COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

BACKGROUND

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that sequence changes in one part of a particular genomic region may not be able to be linked to sequence changes in other parts of the same genome (e.g., the same chromosome) because the sequence changes reside on different fragments. (See Figure 5 and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex sequence process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to scale. Indeed, the

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dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

Figure 1: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find use in aspects of the reflex process described herein.

Figure 2 shows an exemplary embodiment of using binding partner pairs (biotin/streptavidin) to isolate single stranded polynucleotides of interest.

Figure 3 is a schematic diagram illustrating an exemplary embodiment for moving a primer site and a MID to a specific location in a nucleic acid of interest.

Figure 4 shows a schematic diagram illustrating an exemplary use of the reflex process for generating a sample enriched for fragments having a region of interest (e.g., from a population of randomly fragmented and asymmetrically tagged polynucleotides).

Figure 5 shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

Figure 6: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 7: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 8 shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

Figure 9 shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

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Figure 10 shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Figure 11 shows a schematic and results of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate five different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5).

Figure 12 shows a schematic and results of experiments performed to determine the prevalence of intramolecular rearrangement during the reflex process (as desired) vs. intermolecular rearrangement (MID switching).

Figure 13 shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

DEFINITIONS

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Still, certain elements are defined for the sake of clarity and ease of reference.

Terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like.

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons

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may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. patents 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. patent 5,210,015 (real-time PCR with "TAQMAN™" probes); Wittwer et al, U.S. patent 6,174,670; Kacian et al, U.S. patent 5,399,491 ("NASBA"); Lizardi, U.S. patent 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. In one aspect, amplicons of the invention are produced by PCRs. An amplification reaction may be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like.

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

Polynucleotides that are "asymmetrically tagged" have left and right adapter domains that are not identical. This process is referred to generically as attaching adapters asymmetrically or asymmetrically tagging a polynucleotide, e.g., a polynucleotide

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fragment. Production of polynucleotides having asymmetric adapter termini may be achieved in any convenient manner. Exemplary asymmetric adapters are described in: U.S. Patents 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. Patent Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety.

As one example, a user of the subject invention may use an asymmetric adapter to tag polynucleotides. An "asymmetric adapter" is one that, when ligated to both ends of a double stranded nucleic acid fragment, will lead to the production of primer extension or amplification products that have non-identical sequences flanking the genomic insert of interest. The ligation is usually followed by subsequent processing steps so as to generate the non-identical terminal adapter sequences. For example, replication of an asymmetric adapter attached fragment(s) results in polynucleotide products in which there is at least one nucleic acid sequence difference, or nucleotide/nucleoside modification, between the terminal adapter sequences. Attaching adapters asymmetrically to polynucleotides (e.g., polynucleotide fragments) results in polynucleotides that have one or more adapter sequences on one end (e.g., one or more region or domain, e.g., a primer site) that are either not present or have a different nucleic acid sequence as compared to the adapter sequence on the other end. It is noted that an adapter that is termed an "asymmetric adapter" is not necessarily itself structurally asymmetric, nor does the mere act of attaching an asymmetric adapter to a polynucleotide fragment render it immediately asymmetric. Rather, an asymmetric adapter-attached polynucleotide, which has an identical asymmetric adapter at each end, produces replication products (or isolated single stranded polynucleotides) that are asymmetric with respect to the adapter sequences on opposite ends (e.g., after at least one round of amplification/primer extension).

Any convenient asymmetric adapter, or process for attaching adapters asymmetrically, may be employed in practicing the present invention. Exemplary asymmetric adapters are described in: U.S. Patents 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. Patent Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety.

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"Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

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"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. patent 4,883,750; Letsinger et al, U.S. patent 5,476,930; Fung et al, U.S. patent 5,593,826; Kool, U.S. patent 5,426,180; Landegren et al, U.S. patent 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifier" (MID) as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence)

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can be used to differentiate polynucleotides in a sample. In certain embodiments, the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived. For example, a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues or cells, or polynucleotides isolated at different times points), where the polynucleotides from each different source are tagged with a unique MID. As such, a MID provides a correlation between a polynucleotide and its source. In certain embodiments, MIDs are employed to uniquely tag each individual polynucleotide in a sample. Identification of the number of unique MIDs in a sample can provide a readout of how many individual polynucleotides are present in the sample (or from how many original polynucleotides a manipulated polynucleotide sample was derived; see, e.g., U.S. Patent No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more and may include multiple subunits, where each different MID has a distinct identity and/or order of subunits. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Patent 7,544,473, issued on June 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., Tm, length, base composition, etc.), as the methods described herein can accommodate a wide variety of unique MID sets. It is emphasized here that MIDs need only be unique within a given experiment. Thus, the same MID may be used to tag a different sample being processed in a different experiment. In addition, in certain experiments, a user may use the same MID to tag a subset of different samples within the same experiment. For example, all samples derived from individuals having a specific phenotype may be tagged with the same MID, e.g., all samples derived from control (or wildtype) subjects can be tagged with a first MID while subjects having a disease condition can be tagged with a second MID (different than the first MID). As another example, it may be desirable to tag different samples derived from the same source with different MIDs (e.g., samples derived over time or derived from different sites within a tissue). Further, MIDs can be generated in a

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variety of different ways, e.g., by a combinatorial tagging approach in which one MID is attached by ligation and a second MID is attached by primer extension. Thus, MIDs can be designed and implemented in a variety of different ways to track polynucleotide fragments during processing and analysis, and thus no limitation in this regard is intended.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional

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PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90°C, primers annealed at a temperature in the range 50-75°C, and primers extended at a temperature in the range 72-78°C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. patent 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. patent 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. patents 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. patent 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for

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quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Patent 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Patent 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York,

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1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with their use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

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A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Primer site" (e.g., a sequencing primer site, and amplification primer site, etc.) as used herein refers to a domain in a polynucleotide that includes the sequence of a primer (e.g., a sequencing primer) and/or the complementary sequence of a primer. When present in single stranded form (e.g., in a single stranded polynucleotide), a primer site can be either the identical sequence of a primer or the complementary sequence of a primer. When present in double stranded form, a primer site contains the sequence of a primer hybridized to the complementary sequence of the primer. Thus, a primer site is a region of a polynucleotide that is either identical to or complementary to the sequence of a primer (when in a single stranded form) or a double stranded region formed between a primer sequence and its complement. Primer sites may be present in an adapter attached to a polynucleotide. The specific orientation of a primer site can be inferred by those of ordinary skill in the art from the structural features of the relevant polynucleotide and/or context in which it is used.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain intramolecularly from its initial location

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to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

"Solid support", "support", and "solid phase support" are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations. Microarrays usually comprise at least one planar solid phase support, such as a glass microscope slide.

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecule in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of

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duplexes or triplexes among polynucleotides and/or oligonucleotides, biotin-avidin or biotin-streptavidin interactions, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in °C) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H.T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

"Sample" means a quantity of material from a biological, environmental, medical, or patient source in which detection, measurement, or labeling of target nucleic acids is sought. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally

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means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in

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connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry,* 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g.,

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placing functional elements of a polynucleotide (e.g., sequencing primer sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, synthetic DNA, etc. Furthermore, any organism, organic material or nucleic acid-containing substance can be used as a source of nucleic acids to be processed in accordance with the present invention including, but not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), tissue samples, bacteria, fungi (e.g., yeast), phage, viruses, cadaveric tissue, archaeological/ancient samples, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing

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amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, Thermococcus litoralis DNA polymerase, Pyrococcus furiosus DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. Patent Application Serial Number 11/656,746, filed on January 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

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In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in Figure 1A.

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step 1, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form polynucleotide structure 112, with the polynucleotide folding back on itself and hybridizing to form a region of complementarity (i.e., double stranded reflex/reflex' region). In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step 2 producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in polynucleotide 114; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide 114 (e.g., by heat) generates linear single stranded polynucleotide 116. As shown in Figure 1, resultant polynucleotide 116 contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any

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subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In alternative embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide 114 (shown in polynucleotide 118; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure 114 with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated. If the region is removed enzymatically, resultant polynucleotide 118 is used in place of polynucleotide 116 in subsequent steps (e.g., copying to reverse polarity).

In certain embodiments, polynucleotide 116 or 118 is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this copying is not shown (see, e.g., Figure 3). For example, copying to reverse the polarity of polynucleotide 116 results in single-stranded polynucleotide 120 having, in a 5' to 3' orientation, the first domain (122); the reflex sequence (124); the complement of polynucleotide 106 (oriented with the complement of Site A (Site A'; 126) proximal to the reflex sequence); the complement of the reflex sequence (128); and the complement of the first domain (130).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, CT) technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific

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for the polynucleotide region that are positioned in such a way that we can obtain direct sequences of all parts of the insert, as shown in Figure 1B. Note that the polynucleotide shown in Figure 1B (140) has a domain (142) containing a primer site and an MID denoting from which original sample(s) the polynucleotide is derived. Site 142 thus represents an example of a First Domain site such identified as 122 in the Figure 1A. The polynucleotide also includes a reflex site (144), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction).

It is noted here that polynucleotide 140 can be categorized as a precursor to polynucleotide 100 in Figure 1A, as it does not include a 3' reflex sequence complementary to the reflex site (domain 108 in Figure 1A). As detailed below, polynucleotide 140 can be converted to a polynucleotide having the structural configuration of polynucleotide 100, a polynucleotide suitable as a substrate for the reflex process described herein (e.g., by primer extension using a B_n primer and reversal of polarity).

In an exemplary embodiment, each A_n-B_n primer pair defines a nucleic acid region that is approximately 400 bases in length or less. This size range is within the single-sequencing run read length of the current Roche 454 sequencing platform; a different size range for the defined nucleic acid region may be utilized for a different sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in Figure 1B can be used to define regions 1 to 5 shown in Figure 3 (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer site (e.g., for Roche 454 sequencing), an MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S.

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Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in Figure 2. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having streptavidin bound to it. The biotin moiety (i.e., the binding partner of streptavidin) on the extended strands will bind to the solid-phase streptavidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for B_n primer extension reactions), may be eluted from the streptavidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using B_n primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single

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stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the streptavidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR, strand-specific enzymatic degradation, and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in Figure 1B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer site in the first domain' (complement of the first domain 102). The resulting nucleic acid has structure 100 shown in Figure 1A, where the first domain 102 includes the primer site and the MID. Site A (110) in Figure 1 is determined by the specificity of the 5' reflex-B_n primer used.

The reflex process (e.g., as shown in Figure 1) is then performed to produce a product in which the primer site and the MID are now in close proximity to the desired site (or region of interest (ROI)) within the original polynucleotide (i.e., the site defined by the primer used, e.g., B_1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in Figure 1A and 1B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double

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stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer site that can be used in subsequent steps, including sequencing reactions. Providing a primer site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain 102 and the primer site in the A_n primer (not shown in Figure 1B). Because of the position of the primer sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site (or ROI) and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of the ROI as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment obtained by amplification/extension from primer B_0 directly, the polarity of the ROI in the resulting fragment is reversed as compared to the ROI in fragments obtained by primers B_1-B_n. This is because the B_0-generated fragment, unlike the B_1-B_n generated fragments, has not been subjected to a reflex process which reverses the orientation of the ROI sequence with respect to the first domain/reflex sequence (as described above). Therefore, the B_0 primer may have appended to it a primer site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system) rather than a reflex sequence as with primers B_1-B_n. However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer site).

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It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

Figure 3 provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions 1, 2, 3, 4 and 5 in an 11kb region of lambda DNA).

A single parent DNA fragment 202 is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR), cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site), or by attaching adapters to polynucleotide fragments (e.g., fragment made by random fragmentation, by sequence-specific restriction enzyme digestion, or combinations thereof). While only a single fragment with a single MID is shown, the steps in Figure 3 are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). Figure 3 describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions 1, 2, 3, 4 and 5 (shown in polynucleotide 204) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region 4 only (206).

In step 1, the five regions of interest are defined within the parent fragment (labeled 1 to 5 in polynucleotide 204) and corresponding primer pairs are designed for each. The distance of each region of interest from the reflex sequence is shown below polynucleotide 204. The primer pairs are designed as described and shown in Figure 1B (i.e., the A_n-B_n primer pairs). For clarity, only primer sites for region 4 are shown in Figure 3 ("primer sites" surrounding region 4). In step 2, sequence specific primer extensions are performed (only region 4 is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure 208 (i.e., having the reflex sequence on the 5' terminus). As shown, the B_n primer for region 4 will include a sequence specific primer site that primes

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at the 3'-most primer site noted for region 4 (where "3'-most" refers to the template strand, which in Figure 3 is the top strand). This polynucleotide is copied back to produce polynucleotide 210 having reversed polarity (e.g., copied using a primer that hybridizes to the 454A' domain). Polynucleotide 210 has structure similar to polynucleotide 100 shown at the top of Figure 1. Step 4 depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (polynucleotide 212). In the embodiments shown in Figure 3, polynucleotide 212 is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The polynucleotide formed for region 4 is shown as 216 with polynucleotides for the other regions also shown (214).

It is noted here that the formation of each of the polynucleotides 214 may be accomplished either in separate reactions (i.e., structure with region 1 in proximity to the adapter domains is in a first sample, the structure with region 2 in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step 6 the polynucleotides 214 are copied to reverse polarity to form polynucleotides 218. In step 7, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in Figure 1B) each having a second Roche 454 primer site (454B) attached at the 5' end, and extended to form products 220. Steps 6 and 7 may be combined (e.g., in a single PCR or other amplification reaction).

In summary, Figure 3 shows how the reflex process can be employed to produce five daughter fragments 220 of similar length (e.g., ~500 bp) each of which contain DNA sequences that differ in their distance from the reflex sequence in the starting structure 202 while maintaining the original MID.

Figure 4 shows another exemplary use of the reflex process as described herein. In the embodiment shown in Figure 4, a target sequence (i.e., containing region of interest "E") is enriched from a pool of adapter-attached fragments. In certain embodiments, the fragments are randomly sheared, selected for a certain size range (e.g., DNA having a length from 100 to 5000 base pairs), and tagged with adapters (e.g., asymmetric adapters, e.g., as described in US Patent Application Ser. No. 12/432,080, filed on April 29, 2009). The asymmetric adaptor employed in Figure 4 contains a sequencing primer site (454A, as used in the Roche 454 sequencing platform), an MID, an X sequence, and an internal stem region (ISR), which denotes the region of complementarity for the asymmetric adapter that is adjacent to the adapter attachment site (see, e.g., the description in US Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety). The X

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sequence can be any sequence that can serve as a binding site for a polynucleotide containing the complement of the X sequence (similar to a primer site). As described below, the X sequence allows for the annealing of an oligonucleotide having a 5' overhang that can serve as a template for extension of the 3' end of the adaptor oligonucleotide. The sequencing direction of the sequencing primer site (454A primer site in structure 401 of Figure 4) is oriented such that amplification of the adapter ligated fragment using the sequencing primer site proceeds away from the ligated genomic insert. This has the effect of making the initial asymmetric adapter ligated library 'inert' to amplification using this primer, e.g., in a PCR reaction.

To extract a region of interest (the "E" region), the library is mixed with an oligonucleotide (403) containing a 3' X' sequence and a target specific priming sequence (the 1' sequence) under hybridization/annealing conditions. The target specific sequence 1' is designed to flank one side of the region of interest (the 1' sequence adjacent to E in the genomic insert; note that only the E-containing polynucleotide fragment is shown in Figure 4), much like a PCR primer. After annealing primer 403, the hybridized complex is extended, whereby all of the adaptor tagged fragments will obtain the complement of the target specific sequence (i.e., the 1 sequence) on the 3' end (see structure 405; arrows denote the direction of extension).

Extended products 405 are then denatured and the 1/1' regions allowed to hybridize intramolecularly in a reflex process priming event, after which nucleic acid extension is performed to form structure 407 (extension is from the 1 priming site; shown with an arrow). This reflex reaction creates a product (407) that, unlike its parent structure (405), has a sequencing primer site (454A) that is oriented such the extension using this primer sequence proceeds towards the region of interest. Thus, in the absence of a priming and extension reflex reaction, extension with a sequencing primer will not generate a product containing the region of interest (the E region). In other words, only E-region containing target polynucleotides will have a 454A sequence that can amplify genomic material (structure 407).

After completing the reflex process (using 1/1' as the reflex sequences), a PCR amplification reaction is performed to amplify the region of interest (with associated adapter domains). However, before performing the PCR reaction, the fragment sample is "inactivated" from further extension using terminal transferase and ddNTPs. This inactivation prevents non-target adaptor tagged molecules from performing primer extension from the 3' primer 1 site. Once inactivated, a PCR reaction is performed using a sequencing

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primer (i.e., 454A primer 409) and a second primer that primes and extends from the opposite side of the region of interest (i.e., primer 411, which includes a 5' 454B sequencing primer site and a 3' "2" region that primes on the opposite end of E from the 1 region). Only fragments that have undergone the reflex process and contain the E region will be suitable templates for the PCR reaction and produce the desired product (413).

Thus, the process exemplified in Figure 4 allows for the movement of an adapter domain (e.g., containing functional elements and/or MID) into proximity to a desired region of interest.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and /or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Patent 7,537,897, issued on May 26, 2009; U.S. Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in the majority of individual nucleic acid molecules being labeled with a unique ID tag (see, e.g., Brenner et al., Proc. Natl. Acad. Sci. USA. 2000 97(4):1665-70). Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes (or a specific genomic region) or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring

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the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in Figure 5. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the

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subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above).

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as A_n-B_n in Figure 1B). This results in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein function intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are

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linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example 1

Figures 6 and 7 provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

METHODS

Substrate:

The 100 base oligonucleotide substrate (as shown diagrammatically in Figure 6A) was synthesized with internal fluorescein-dT positioned between the REFLEX and REFLEX' sequences. This label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, presence or absence of 1 μM competitor oligonucleotide, 0.5 μl of each DNA polymerase ("DNAP"): Vent (NEB, 2 units/μl), Taq (Qiagen HotStarTaq 5 units/μl) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_20. For Taq titrations 0.5 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95 °C for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55 °C or 50 °C for 30 seconds, and a final incubation at 72 °C for 10 minutes.

T7 exonuclease digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB, 10 units/μl), and made up to 50 μl using NEB Buffer 4 and dH_20. Reactions were incubated at 25 °C for 30 minutes.

Gel Electrophoresis analysis:

An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 μl of extension reactions, and 2 μl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using an Amersham/General Electric Typhoon imager.

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RESULTS

Figure 6A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (the Roche 454 sequencing primer A site; listed as 454A); an MID; a reflex sequence; the insert; and a complement of the reflex sequence is 100 nucleotides in length. After self-annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

Figure 6B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the annealing was carried out is shown at the top of each lane (either 50°C or 55°C). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in figure 6B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, after T7 exonuclease digestion the 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product after T7 exonuclease digestion (see lane 13).

Figure 7 shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in Figure 7A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in only a small

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decrease (~5-10%) of fully extended product. Thus, as expected, the intramolecular reaction is heavily favored. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (~5-10%).

The concentration of the competitor, the concentration of the reflex substrate, and the overall genetic complexity, will all likely affect specific results. The experiments shown in Figures 6 and 7 demonstrate that the core parts of the reflex processes as described herein is functional and can be implemented.

Example II

Figure 8 shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (700) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having three ROIs (2, 3 and 4) at different locations therein. This starting material was subjected to reflex processes (as described in above) specific for ROI 2 as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (702) with or without the T7 exonuclease step.

As shown in the gel on the right of Figure 8, the 488 base pair product was produced in reflex processes with and without the T7 exonuclease step.

Figure 9 shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt SPRI beads to remove primer oligos). One reason for performing such purification steps is to reduce the potential for generating side products in a reaction (e.g., undesirable amplicons). While Figure 9 indicates three purification steps, fewer or additional purification steps may be employed depending on the desires of the user. It is noted that the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation) / second reversing polarity step can be performed without intervening purification steps.

The protocol shown in Figure 9 includes the following steps:

annealing a first primer containing a 5' reflex sequence (or reflex tail, as noted in the figure) specific for the 3' primer site for the R' region to the starting polynucleotide and extending (the primer anneals to the top strand at the primer site at the right of R in

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polynucleotide 902, indicated with a *; this step represents the first denature, anneal and extend process indicated on the right);

after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added;

after purification, adding a second primer specific for the second primer site for the R' region having a 5' 454B tail (this primer anneals to the primer site 3' of the R' region in polynucleotide 904, indicated with a *) and denaturing, annealing and extending resulting in a polynucleotide product having 454A and 454B sites surrounding the MID, the reflex sequence, and R'. Note that the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in Figure 1B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route 1 in Figure 13). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

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Figure 10 shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). A size ladder is included in the left hand lane of each gel to allow estimation of the size of the test material. This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Example IV

Figure 11 shows a schematic of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate 5 different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5). The schematic in Figure 11 shows the starting fragment (11,060 base pairs) and resulting products (each 488 base pairs) generated from each of the different region of interest-specific reflex reactions (reflex reactions are performed as described above). The panel (gel) on the bottom of Figure 11 shows the larger starting fragment (Lane 1) and the resulting daughter products for each region-specific reflex reaction (lanes 2 to 6, with the region of interest noted in each in the box), where the starting and daughter fragments have the expected lengths. Sequencing of the products confirmed the identity of the region of interest in each of the reflex products shown in the gel. These results demonstrate that multiple different reflex products can be generated from a single, asymmetrically tagged parent fragment while maintaining the adapter domains (e.g., the primer sites and MID).

Example V

Figure 12 details experiments performed to determine the prevalence of intramolecular rearrangement (as desired in the reflex process) vs. intermolecular rearrangement. Intermolecular rearrangement is undesirable because it can lead to the transfer of an MID from one fragment to another (also called MID switching). MID switching can occur if a reflex sequence in a first fragment hybridizes to its complement in a second fragment during the reflex process, leading to appending the MID from the second fragment to the first fragment. Thus, intermolecular rearrangement, or MID switching, should be minimized to prevent the transfer of an MID from one fragment in the sample to another, which could lead to a misrepresentation of the source of a fragment.

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To measure the prevalence of MID switching under different reflex conditions, fragments having different sizes were generated that included two different MIDs, as shown in the top panel of Figure 12. The common sequence on these fragments serves as the priming site for the first extension reaction to add the second reflex sequence (see, e.g., step 2 of Figure 3). Three exemplary fragments are shown in Figure 12 for each different fragment size (i.e., 800 base pairs with an MIDB and MIDA combination; 1900 base pairs with MIDC and MIDA combination; and 3000 base pairs with MIDD and MIDA combination). For each MID family (A, B, C and D), there are 10 different members (i.e., MIDA had 10 different members, MIDB has 10 different members, etc.). A set of 10 dual MID fragments for each different size fragment (i.e., 800, 1900 and 3000 base pairs) were generated, where the MID pairs (i.e., MIDA/MIDB, MIDA/MIDC, and MIDA/MIDD) were designated as 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10. All 10 fragments of the same size were then mixed together and a reflex protocol was performed.

Due to the domain structure of the fragments, a successful reflex process results in the two MIDs for each fragment being moved to within close enough proximity to be sequenced in a single read using the Roche 454 sequencing platform (see the reflex products shown in the schematic of Figure 12). The reflex reactions for each fragment size were performed at four different fragment concentrations to determine the effect of this parameter, as well as fragment length, in the prevalence of MID switching. The reflex products from each reaction performed were subjected to 454 sequencing to determine the identity of both MIDs on each fragment, and thereby the proportion of MID switching that occurred.

The panel on the bottom left of Figure 12 shows the rate of MID switching (Y axis, shown in % incorrect (or switched) MID pair) for each different length fragment at each different concentration (X axis; 300, 30, 3 and 0.3 nM). As shown in this panel, the MID switch rate decreases with lower concentrations, as would be expected, because intermolecular, as opposed to intramolecular, binding events are concentration dependent (i.e., lower concentrations lead to reduced intermolecular hybridization/binding). In addition, the MID switch rate decreases slightly with length. This is somewhat unexpected as the ends of longer DNA fragments are effectively at a lower concentration with respect to one another. The reasons for why we do not see this is probably because the production of reflex priming intermediates continues during the final PCR, which means that reflex priming reactions are happening continuously which contributes to MID switching. It is probably the case that the shorter reflex products are able to undergo a higher rate of 'background' reflexing, and therefore increase the overall MID switch rate a little.

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These results demonstrate that MID switching can be minimized (e.g., to below 2%, below 1% or even to nearly undetectable levels) by altering certain parameters of the reaction, e.g., by reducing fragment concentration and/or fragment length.

The panel on the bottom right of Figure 12 shows the frequency of MID switching in the reflex process for the 800 base pair fragments (i.e., MIDA/MIDB containing fragments). In this figure, the area of each circle is proportional to the number of reads containing the corresponding MIDA and MIDB species (e.g., MIDA1/MIDB1; MIDA1/MIDB2; etc.). Thus, a circle representing 200 reads will be 40 times larger in terms of area than a circle representing 5 reads.

As noted above, the MIDA/MIDB combinations having the same number (shown on the X and Y axis, respectively) represent the MIDA/MIDB combinations present in the sample prior to the reflex process being performed (i.e., MIDA/MIDB combinations 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10 were present in the starting sample). All other MIDA/MIDB combinations identified by Roche 454 sequencing were the result of MID switching.

This figure shows that the MID switching that occurs during the reflex process is random, i.e., that MID switching is not skewed based on the identity of the MIDs in the reaction).

Exemplary Reflex Protocols

Figure 13 shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route 3, a pooled and tagged extended library is subjected directly to a reflex process. In Route 2, the pooled library is enriched by target-specific hybridization followed by performing the reflex process. In Route 1 employs enrichment by PCR amplification. As shown in Figure 13, PCR enrichment can be performed directly on the pooled tagged extended library or in a secondary PCR reaction after a hybridization-based enrichment step has been performed (as in Route 2) to generate an amplicon substrate that is suitable for the reflex process. Additional routes for preparing a polynucleotide sample for performing a reflex process can be implemented (e.g., having additional amplification, purification, and/or enrichment steps), which will generally be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes

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and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

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CLAIMS

That which is claimed is:

1. A method of moving a domain in a nucleic acid, the method comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain;

the reflex sequence;

a polynucleotide of interest; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand; and

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region;

whereby a complement of the first domain is present at the 3' end of the extended polynucleotide strand, thereby moving the first domain in the polynucleotide strand.

2. The method of claim 1, wherein the first domain comprises a multiplex identifier (MID).

3. The method of claim 1, wherein the first domain comprises a primer site.

4. The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

5. The method of claim 1, wherein the reflex sequence comprises an RNA polymerase site.

6. The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

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9. The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. The method of claim 13, wherein the length of the second domain is from 30 bases to 30 kilobases.

15. The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

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16. The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

18. The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. A polynucleotide strand comprising the following in a 5' to 3' orientation:

a first domain;

a reflex sequence;

a polynucleotide of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand.

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ABSTRACT OF THE DISCLOSURE

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method". In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

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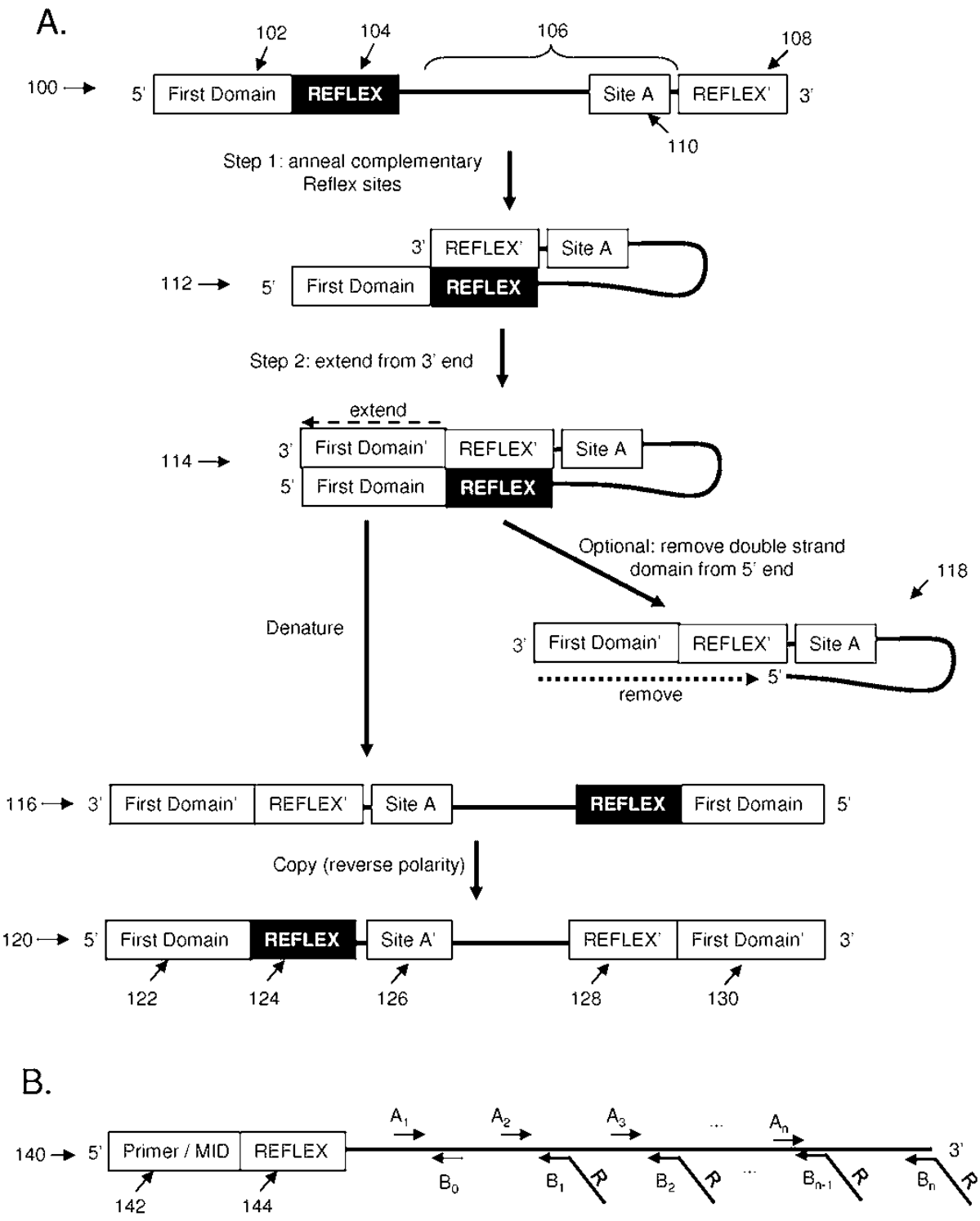

A.
102
104
106
108
100
5'
First Domain
REFLEX
Site A
REFLEX'
3'
110
Step 1: anneal complementary Reflex sites
3'
REFLEX'
Site A
112
5'
First Domain
REFLEX
Step 2: extend from 3' end
extend
114
3'
First Domain'
REFLEX'
Site A
5'
First Domain
REFLEX
Optional: remove double strand domain from 5' end
118
Denature
3'
First Domain'
REFLEX'
Site A
5'
remove
116
3'
First Domain'
REFLEX'
Site A
REFLEX
First Domain
5'
Copy (reverse polarity)
120
5'
First Domain
REFLEX
Site A'
REFLEX'
First Domain'
3'
122
124
126
128
130
B.
140
5'
Primer / MID
REFLEX
142
144
A1
A2
A3
An
B0
B1
B2
Bn-1
Bn
R
3'

Fig. 1

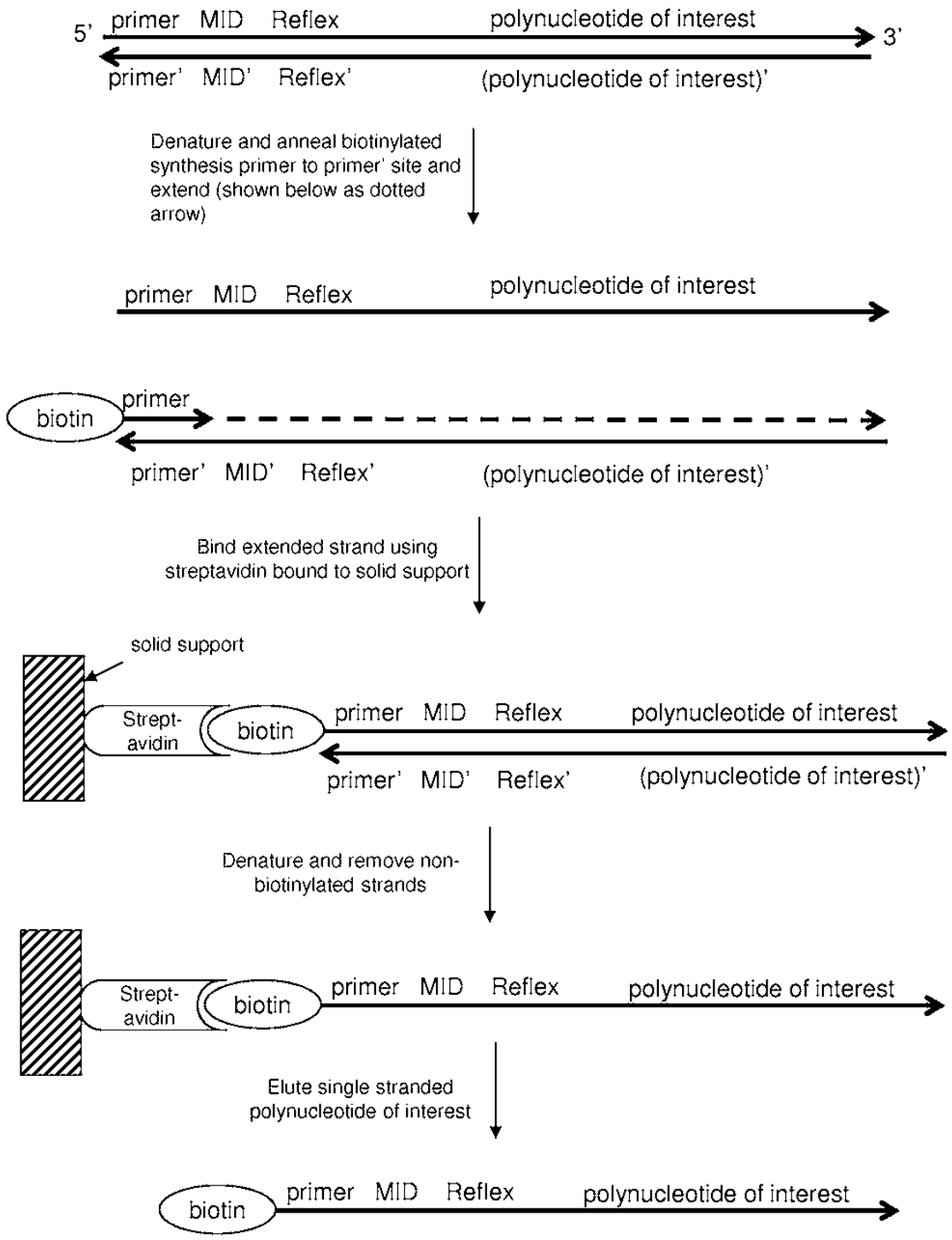
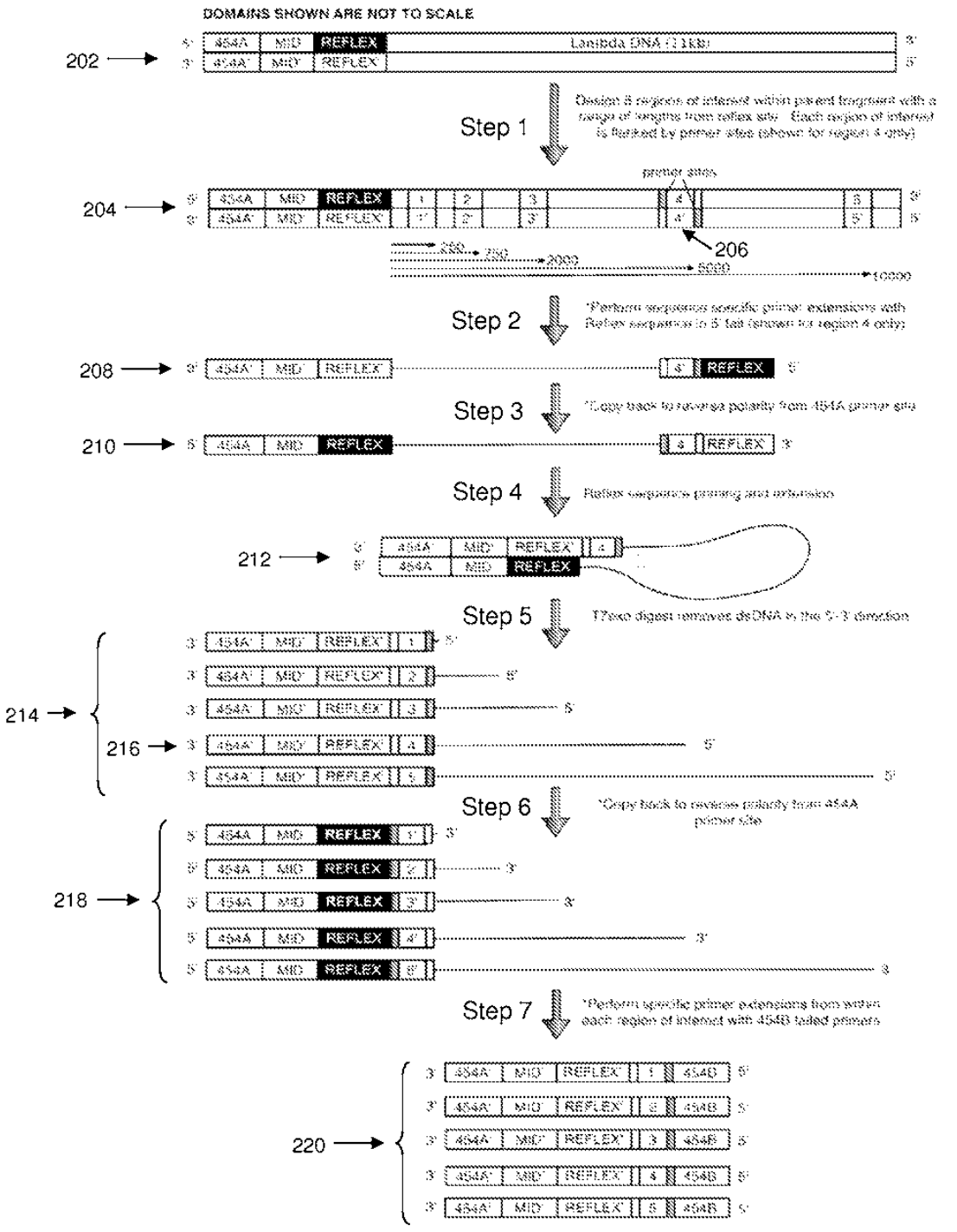

5'
primer MID Reflex
polynucleotide of interest
3'
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and anneal biotinylated synthesis primer to primer' site and extend (shown below as dotted arrow)
primer MID Reflex
polynucleotide of interest
biotin
primer
primer' MID' Reflex'
(polynucleotide of interest)'
Bind extended strand using streptavidin bound to solid support
solid support
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and remove non-biotinylated strands
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
Elute single stranded polynucleotide of interest
biotin
primer MID Reflex
polynucleotide of interest

Fig. 2



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 3

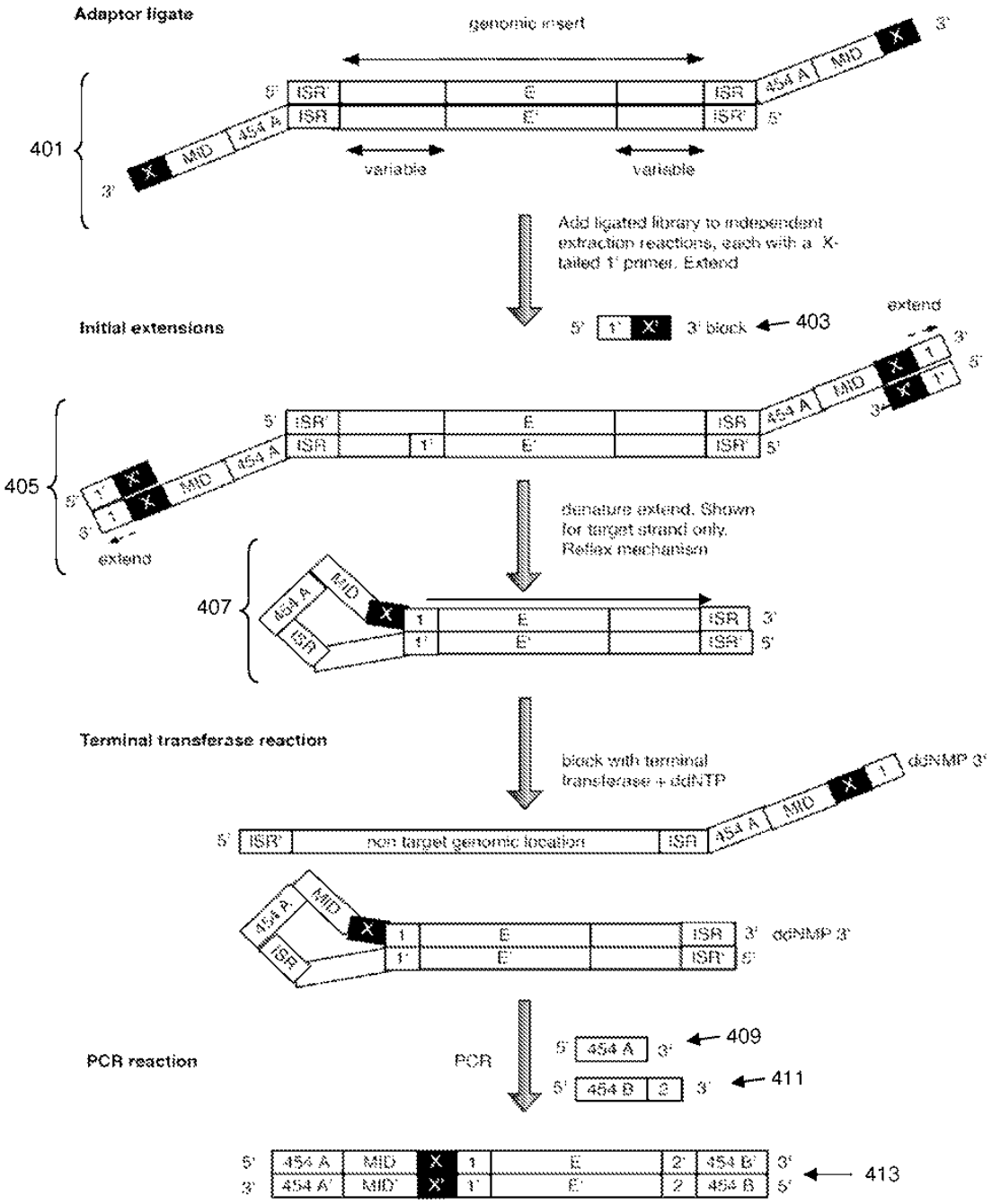

Adaptor ligate
genomic insert
401
454 A
MID
X
ISR
E
variable
Add ligated library to independent extraction reactions, each with a X-tailed 1' primer. Extend
Initial extensions
403
3' block
extend
405
407
Denature extend. Shown for target strand only. Reflex mechanism
Terminal transferase reaction
block with terminal transferase + ddNTP
ddNMP 3'
non target genomic location
PCR reaction
PCR
409
411
413

Fig. 4

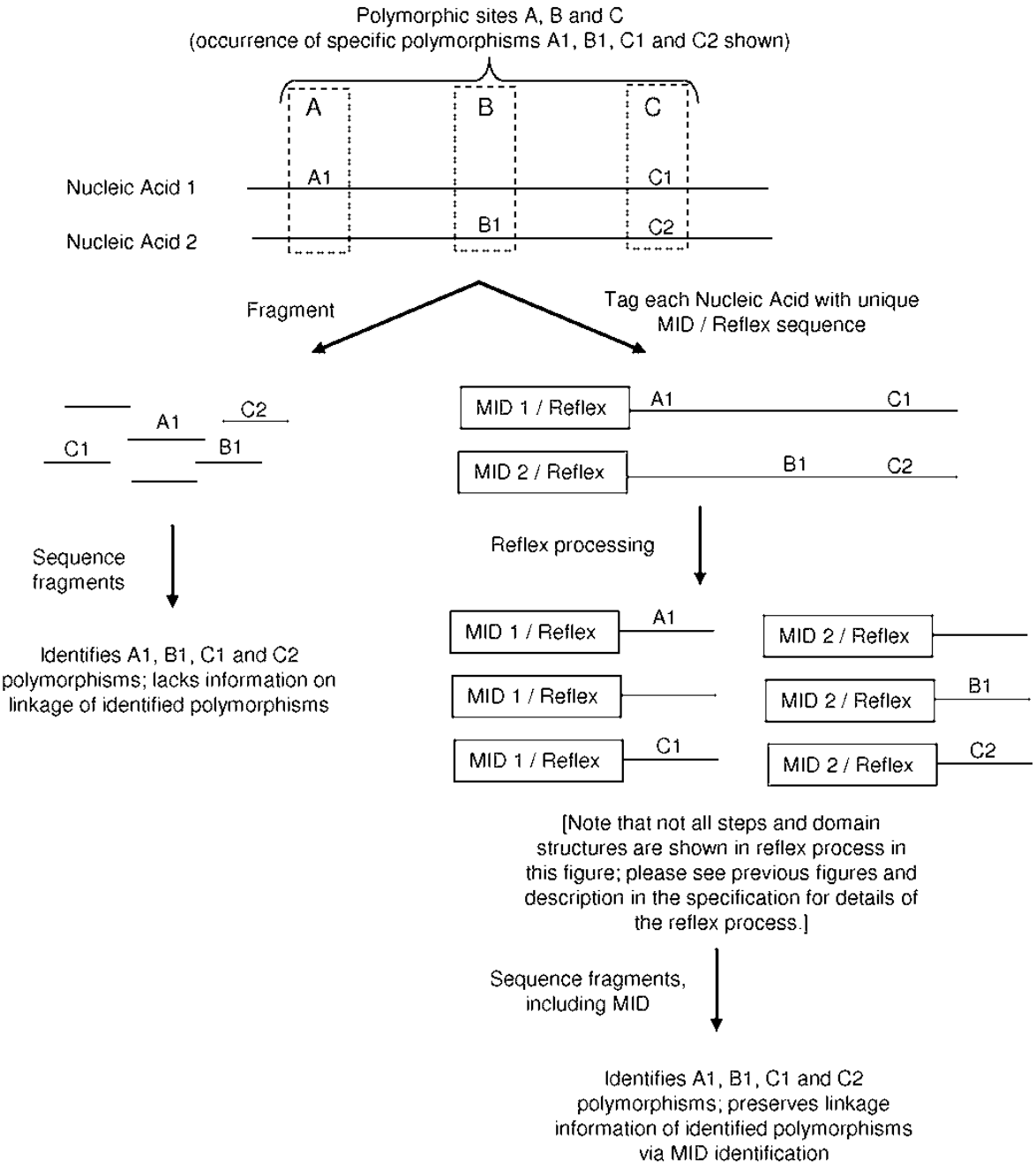
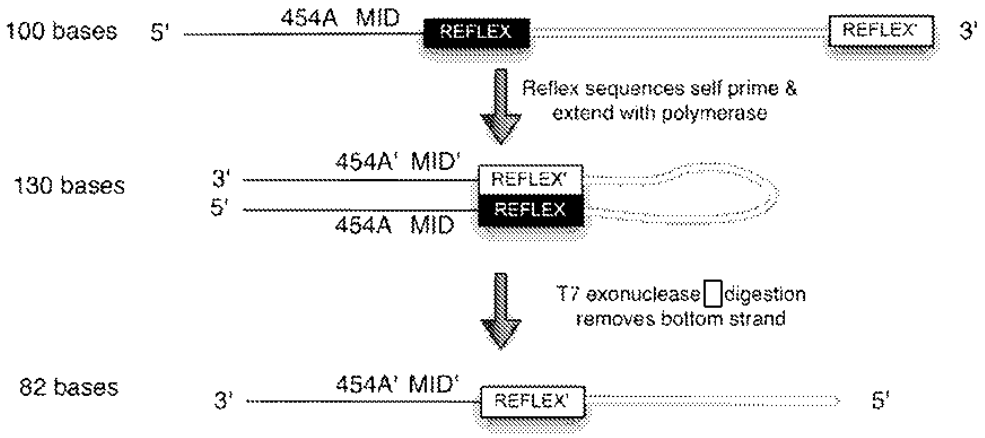
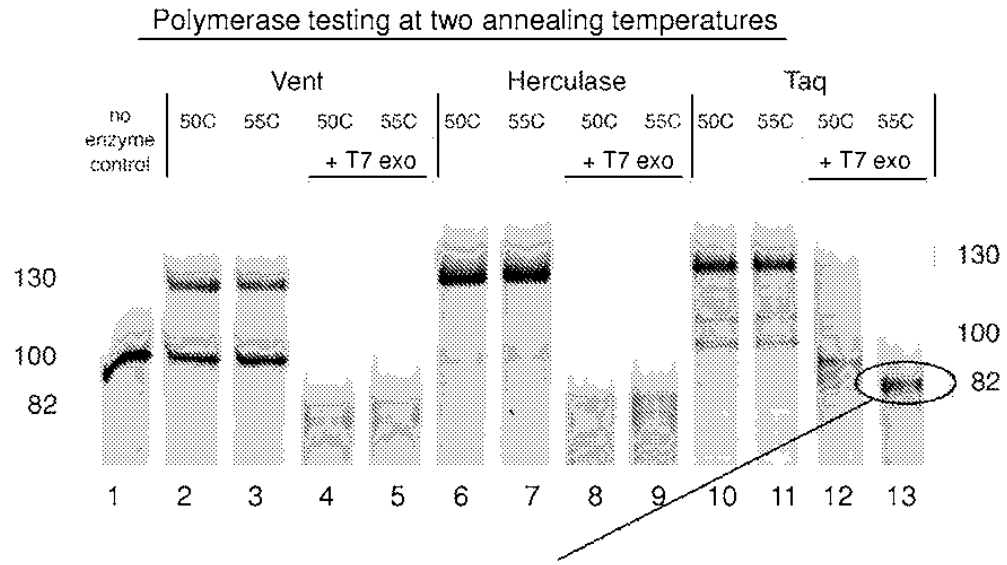

Polymorphic sites A, B and C
(occurrence of specific polymorphisms A1, B1, C1 and C2 shown)
A
B
C
Nucleic Acid 1
A1
C1
Nucleic Acid 2
B1
C2
Fragment
Tag each Nucleic Acid with unique MID / Reflex sequence
A1
C2
C1
B1
MID 1 / Reflex
A1
C1
MID 2 / Reflex
B1
C2
Sequence fragments
Reflex processing
Identifies A1, B1, C1 and C2 polymorphisms; lacks information on linkage of identified polymorphisms
MID 1 / Reflex
A1
MID 2 / Reflex
MID 1 / Reflex
MID 2 / Reflex
B1
MID 1 / Reflex
C1
MID 2 / Reflex
C2
[Note that not all steps and domain structures are shown in reflex process in this figure; please see previous figures and description in the specification for details of the reflex process.]
Sequence fragments, including MID
Identifies A1, B1, C1 and C2 polymorphisms; preserves linkage information of identified polymorphisms via MID identification

Fig. 5

A.



B.



Extension is best with Herculase, but 3'-5' exonuclease activity results in partial digestion of the desired 82 base product. Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product.

Fig. 6

A.


5'
454A MID
REFLEX
REFLEX'
3'
+ Competitor
5'
REFLEX'
3'

B.

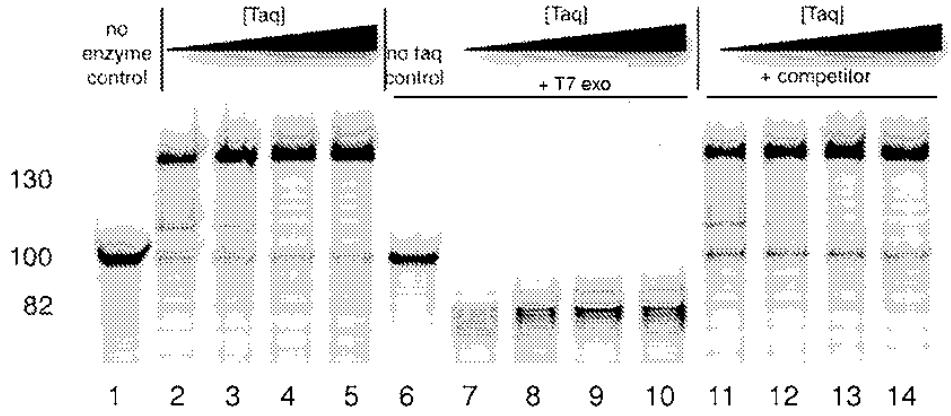
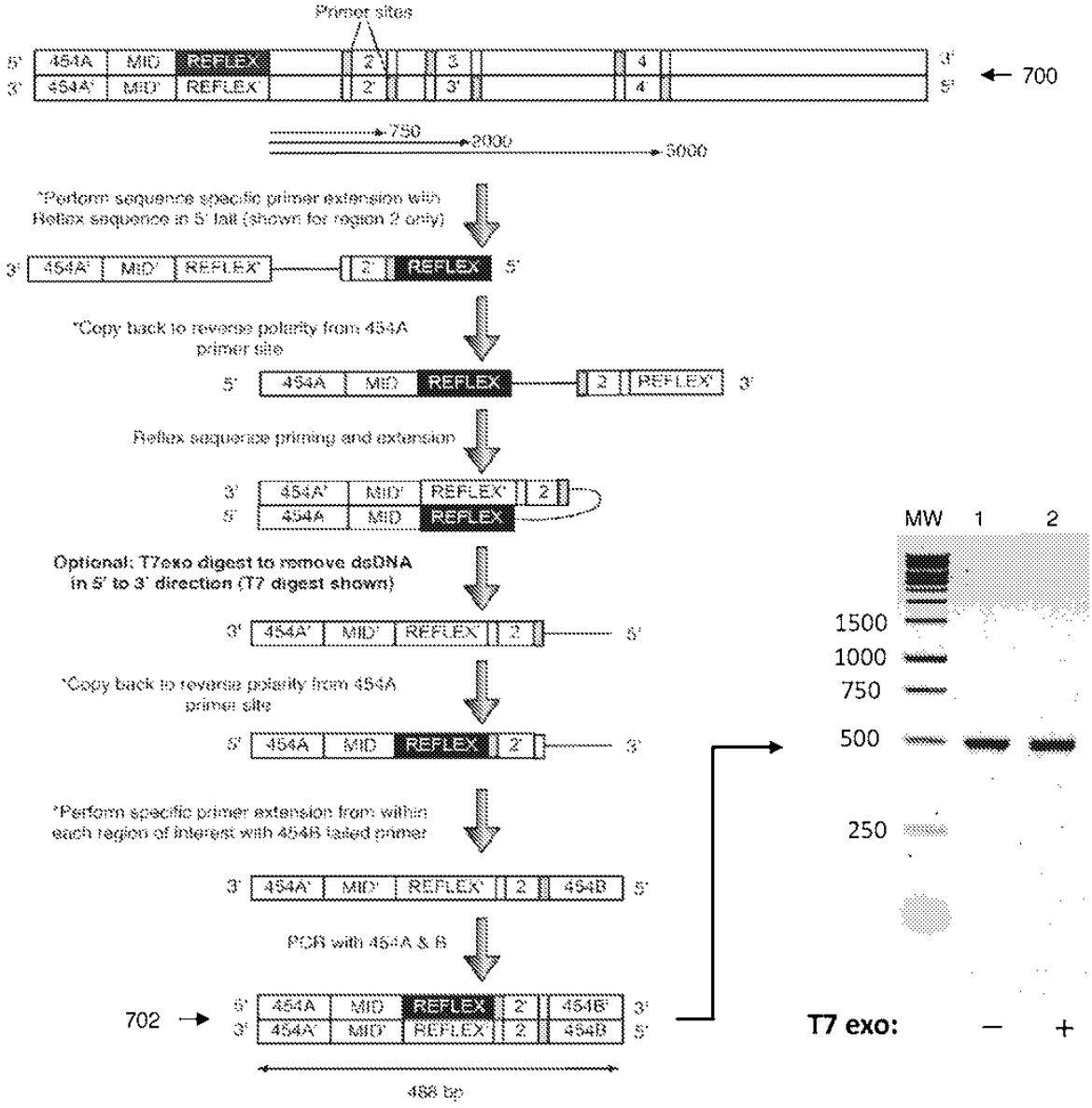

no enzyme control
[Taq]
no taq control
[Taq]
+ T7 exo
[Taq]
+ competitor
130
100
82
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Fig. 7



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 8

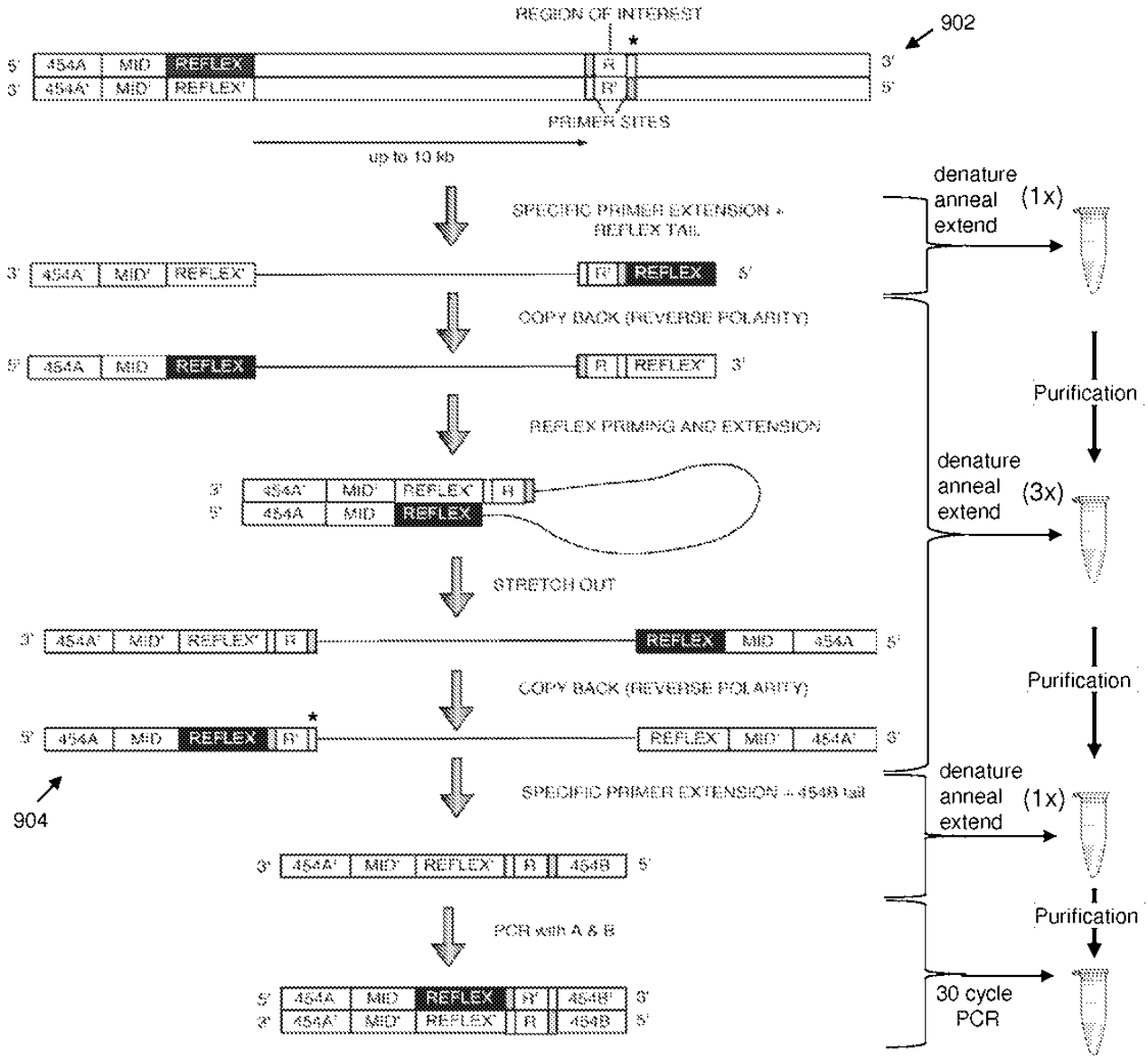

REGION OF INTEREST
902
5' 454A MID REFLEX R 3'
3' 454A' MID' REFLEX' R' 5'
PRIMER SITES
up to 10 kb
SPECIFIC PRIMER EXTENSION + REFLEX TAIL
3' 454A' MID' REFLEX' R' REFLEX 5'
COPY BACK (REVERSE POLARITY)
5' 454A MID REFLEX R REFLEX' 3'
REFLEX PRIMING AND EXTENSION
3' 454A' MID' REFLEX' R
5' 454A MID REFLEX
STRETCH OUT
3' 454A' MID' REFLEX' R REFLEX MID 454A 5'
COPY BACK (REVERSE POLARITY)
5' 454A MID REFLEX R' REFLEX' MID' 454A' 3'
SPECIFIC PRIMER EXTENSION + 454B tail
904
3' 454A' MID' REFLEX' R 454B 5'
PCR with A & B
5' 454A MID REFLEX R' 454B' 3'
3' 454A' MID' REFLEX' R 454B 5'
denature
anneal (1x)
extend
Purification
denature
anneal (3x)
extend
Purification
denature
anneal (1x)
extend
Purification
30 cycle
PCR

Fig. 9

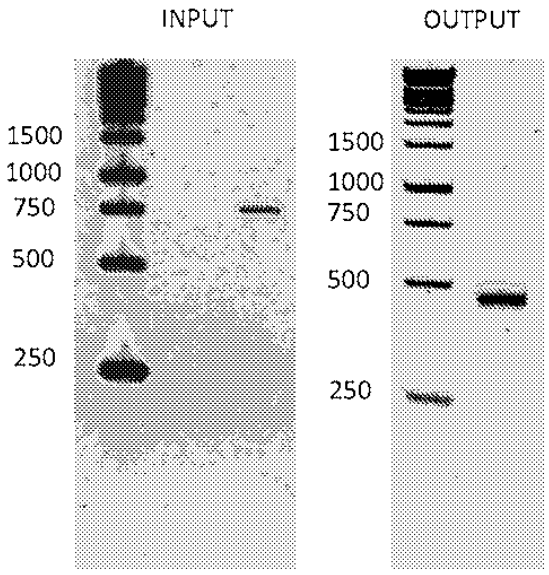
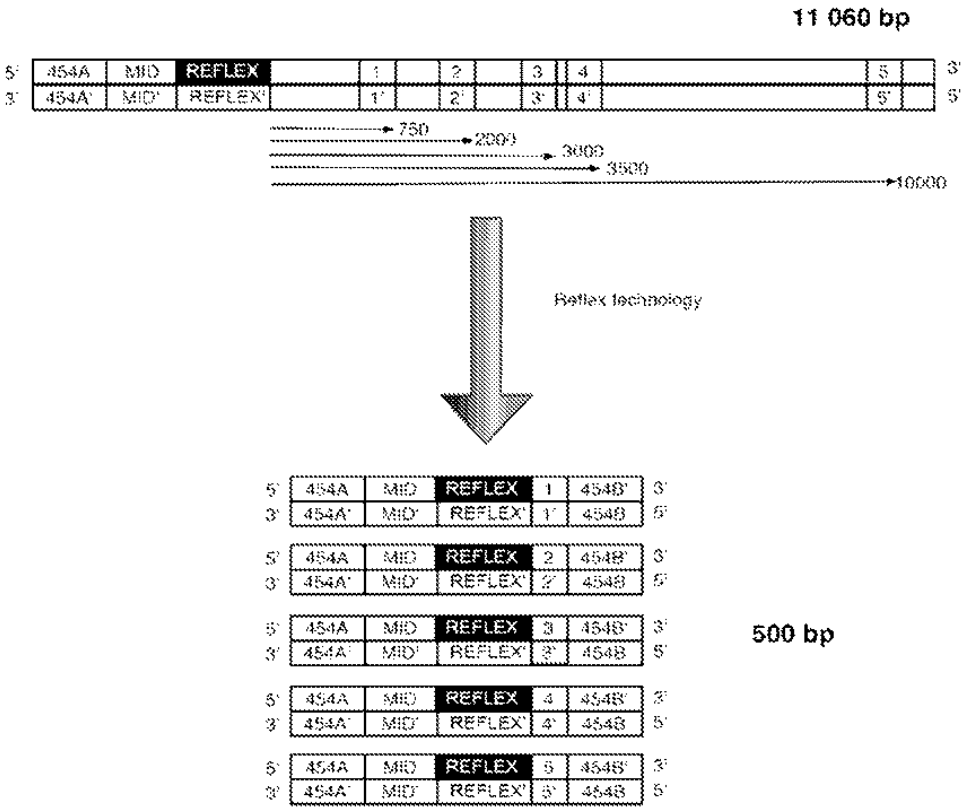


Fig. 10


11 060 bp
454A
MID
REFLEX
454A'
MID'
REFLEX'
750
2000
3000
3500
10000
Reflex technology
454B
454B'
500 bp

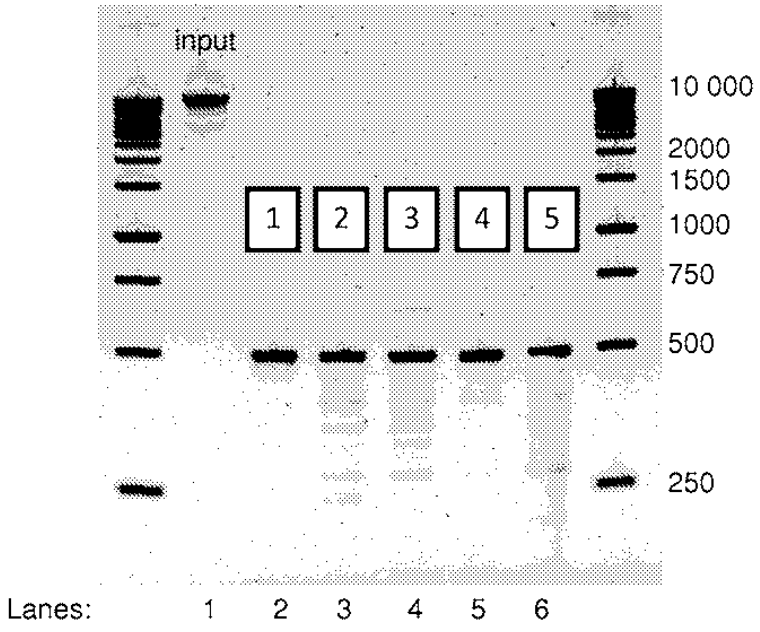
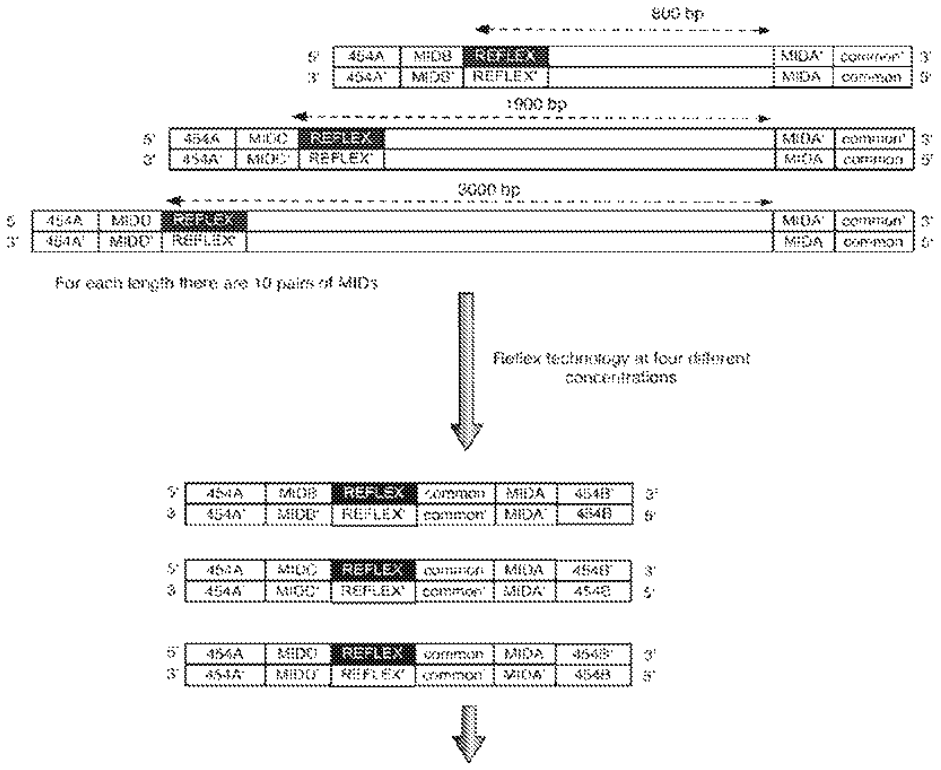
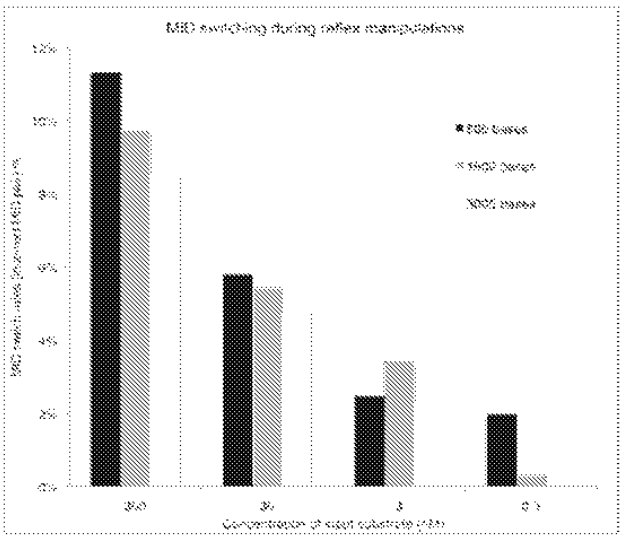

input
10 000
2000
1500
1000
750
500
250
1
2
3
4
5
Lanes: 1 2 3 4 5 6

Fig. 11


800 bp
454A
MID8
REFLEX
MIDA
common
>900 bp
3000 bp
For each length there are 10 pairs of MIDs
Reflex technology at four different concentrations
Roche 454 Sequencing


MID switching during reflex manipulations
Concentration of input substrate (ng/µL)

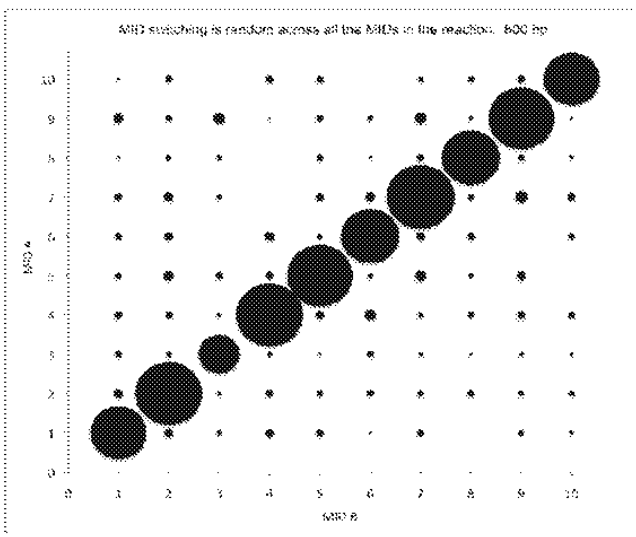

MID switching is random across all the MIDs in the reaction. 800 bp
MID A
MID B

Fig. 12

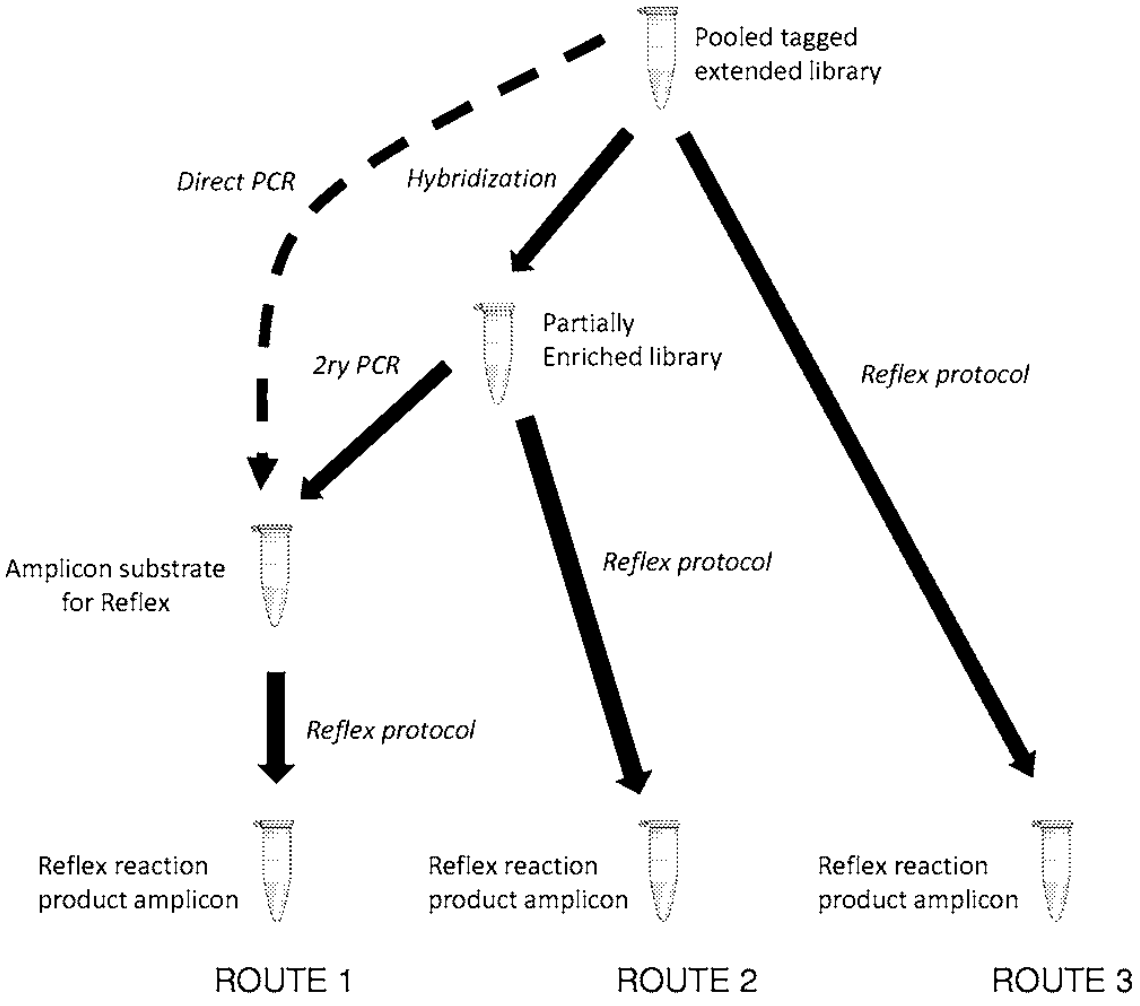

Pooled tagged
extended library
Direct PCR
Hybridization
Partially
Enriched library
2ry PCR
Reflex protocol
Amplicon substrate
for Reflex
Reflex protocol
Reflex protocol
Reflex reaction
product amplicon
Reflex reaction
product amplicon
Reflex reaction
product amplicon
ROUTE 1
ROUTE 2
ROUTE 3

Fig. 13

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

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☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

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All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

Application Information:

Title of the Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement		
Attorney Docket Number	CGLC-020CON5	**Small Entity Status Claimed**	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	13	**Suggested Figure for Publication (if any)**	

Filing By Reference :

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country i

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	93726		

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the application number blank.

Prior Application Status	Pending				Remove
Application Number	Continuity Type		Prior Application Number		Filing Date (YYYY-MM-DD)
	Continuation of		14172694		2015-02-04
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14172694	Continuation of	14021790	2013-09-09	8679756	2014-03-25
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14021790	Continuation of	13859450	2013-04-09	8563274	2013-10-22
Prior Application Status	Abandoned				Remove
Application Number	Continuity Type		Prior Application Number		Filing Date (YYYY-MM-DD)
13859450	Continuation of		13622872		2012-09-19
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13622872	Continuation of	13387343	2012-02-15	8298767	2012-10-30
Prior Application Status	Expired				Remove
Application Number	Continuity Type		Prior Application Number		Filing Date (YYYY-MM-DD)
13387343	a 371 of international		IB2010002243		2010-08-13
Prior Application Status	Expired				Remove
Application Number	Continuity Type		Prior Application Number		Filing Date (YYYY-MM-DD)
IB2010002243	Claims benefit of provisional		61288792		2009-12-21
Prior Application Status	Expired				Remove
Application Number	Continuity Type		Prior Application Number		Filing Date (YYYY-MM-DD)
IB2010002243	Claims benefit of provisional		61235595		2009-08-20

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country	Filing Date (YYYY-MM-DD)	Access Code (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization to Permit Access:

☒ Authorization to Permit Access to the Instant Application by the Participating Offices

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
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If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date o f filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name	Population Genetics Technologies LTD.		
Mailing Address Information For Applicant:			
Address 1			
Address 2	Babraham Research Campus		
City	Cambridge	**State/Province**	
Country [i]	GB	Postal Code	CB22 3AT
Phone Number		Fax Number	

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

Email Address	

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not subsitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Signature	/James S. Keddie, Reg. No. 48,920/			Date (YYYY-MM-DD)	2015-07-06
First Name	James S.	Last Name	Keddie	Registration Number	48920

Additional Signature may be generated within this form by selecting the Add button. Add

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00063

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent C o o p eration Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00065

Electronic Acknowledgement Receipt	
EFS ID:	22828352
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	06-JUL-2015
Filing Date:	
Time Stamp:	16:50:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		CGLC-020-CON5_Appln_fr_parent.pdf	311265 [illegible]	yes	43

	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	39	
	Claims		40	42	
	Abstract		43	43	
Warnings:					
Information:					
2	Drawings-only black and white line drawings	CGLC-020CON5-Figures.pdf	1552139	no	13
Warnings:					
Information:					
3	Application Data Sheet	CGLC-020CON5-ADS.pdf	1820160	no	9
Warnings:					
Information:					
		Total Files Size (in bytes):	3683564		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode - SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 14792094 Document Date: 07/06/2015

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- Drawings – Other than Black and White Line Drawings

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

To access the documents in the SCORE database, refer to instructions below.

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Form Revision Date: September 30, 2013

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 13622872

filed on September 19, 2012.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Sydney Brenner Date (Optional): _____

Signature: Sydney Brenner

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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- ■ United States application or PCT international application number 13622872 filed on September 19, 2012

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

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LEGAL NAME OF INVENTOR

Inventor: Gi Mikawa Date (Optional): 5-Nov-2012

Signature: Gi Mikawa

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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LEGAL NAME OF INVENTOR

Inventor: Robert Osborne Date (Optional): 5/11/12

Signature: Robert Osborne

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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This declaration is directed to:

- ☐ The attached application, or
- ☒ United States application or PCT international application number 13622872 filed on September 19, 2012

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Andrew Slatter Date (Optional): 5th November 2012

Signature: Andrew Slatter

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Patent Application Fee Transmittal

Application Number:	14792094
Filing Date:	
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	James Samuel Keddie/Tiffany Turner
Attorney Docket Number:	CGLC-020CON5

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Late Filing Fee for Oath or Declaration	1051	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	140

Electronic Acknowledgement Receipt	
EFS ID:	22872781
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	09-JUL-2015
Filing Date:	
Time Stamp:	17:54:20
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$140
RAM confirmation Number	4564
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Oath or Declaration filed	CGLC-020CON5_AIA_Dec_fr_parent_Signed.pdf	2461258	no	4
			[illegible]		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30019	no	2
			[illegible]		
Warnings:					
Information:					
		Total Files Size (in bytes):	2491277		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 07/17/2015

SDUONG ADJ #00000015 Mailroom Dt: 07/09/2015
Seq No: 4564 Sales Acctg Dt: 07/10/2015 14792094
01 FC : 1051 -140.00 OP

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 07/17/2015

SDUONG SALE #00000017 Mailroom Dt: 07/09/2015 14792094
01 FC : 2051 70.00 OP

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 07/17/2015

SDUONG RF #30164006 Mailroom Dt: 07/17/2015 14792094

Credit Card Refund Total: $70.00

American Express XXXXXXXXXXXX2003

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/792,094	07/06/2015	Sydney Brenner	CGLC-020CON5

CONFIRMATION NO. 9697

93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

FORMALITIES LETTER

OC000000076389787

Date Mailed: 07/22/2015

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
- The application search fee must be submitted.
- The application examination fee must be submitted.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. Small entity discount is in effect. If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **70** basic filing fee.
- $ **300** search fee.
- $ **360** examination fee.
- $(**0**) previous unapplied payment amount.
- $ **730** TOTAL FEE BALANCE DUE.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00080

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/ygizaw/

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00081

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/792,094

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	70		N/A	
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	300		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*	x 40 =	0.00	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*	x 210 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	730		TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE(S)	SMALL ENTITY ADDITIONAL FEE(S)	OR	OTHER THAN SMALL ENTITY RATE(S)	OTHER THAN SMALL ENTITY ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE(S)	ADDITIONAL FEE(S)	OR	RATE(S)	ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/792,094	07/06/2015	1636	70	CGLC-020CON5	20	2

CONFIRMATION NO. 9697

FILING RECEIPT



93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

Date Mailed: 07/22/2015

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Sydney Brenner, Ely, UNITED KINGDOM;
Gi Mikawa, Great Shelford, UNITED KINGDOM;
Robert Osborne, Great Chesterford, UNITED KINGDOM;
Andrew Slatter, London, UNITED KINGDOM;

Applicant(s)

Population Genetics Technologies LTD., Cambridge, UNITED KINGDOM;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 14/172,694 02/04/2014 *
which is a CON of 14/021,790 09/09/2013 PAT 8679756
which is a CON of 13/859,450 04/09/2013 PAT 8563274
which is a CON of 13/622,872 09/19/2012 ABN
which is a CON of 13/387,343 02/15/2012 PAT 8298767
which is a 371 of PCT/IB2010/002243 08/13/2010
which claims benefit of 61/288,792 12/21/2009
and claims benefit of 61/235,595 08/20/2009
(*)Data provided by applicant is not consistent with PTO records.

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00083

Permission to Access - A proper **Authorization to Permit Access to Application by Participating Offices** (PTO/SB/39 or its equivalent) has been received by the USPTO.

If Required, Foreign Filing License Granted: 07/20/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/792,094**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No
** **SMALL ENTITY** **
Title

Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

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No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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Page 00086

PTO/AIA/80 (07-12)
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POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

☒ Practitioners associated with Customer Number: 93726

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

☒ The address associated with Customer Number: 93726

OR

☐	Firm or Individual Name		
	Address		
	City	State	Zip
	Country		
	Telephone	Email	

Assignee Name and Address: Population Genetics Technologies Ltd
Babraham Research Campus
Cambridge, United Kingdom CB22 3AT

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of the practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record
The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature	Mel Kronick	Date	09/25/2012
Name	Mel Kronick, Ph.D.	Telephone	(650) 269-0445
Title	Director		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt	
EFS ID:	23663952
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	02-OCT-2015
Filing Date:	06-JUL-2015
Time Stamp:	16:41:39
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Assignee showing of ownership per 37 CFR 3.73	CGLC-020CON5_373_C_Statement.pdf	32067 [illegible]	no	2
Warnings:					
Information:					

2	Power of Attorney	CGLC-Population_Genetics_Technologies_Ltd.pdf	1460141 c0fbb0317da8857ee16e11a8fdf90106e3dc3d8	no	1

Warnings:	
Information:	
Total Files Size (in bytes):	1492208

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National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: **Population Genetics Technologies Ltd**

Application No./Patent No.: **14/792,094** Filed/Issue Date: **July 6, 2015**

Titled: **Compositions and Methods for Intramolecular Nucleic Acid Rearrangement**

POPULATION GENETICS TECHNOLOGIES LTD., a **Corporation**
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (chose **one** of options 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), or an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2, or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel **036049**, Frame **0472**, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/AIA/96 (08-12)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

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6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/James S. Keddie, Reg. No. 48,920/	October 1, 2015
Signature	Date
James S. Keddie	48,920
Printed or Typed Name	Title or Registration Number

Electronic Patent Application Fee Transmittal

Application Number:	14792094
Filing Date:	06-Jul-2015
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	James Samuel Keddie
Attorney Docket Number:	CGLC-020CON5

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility filing Fee (Electronic filing)	4011	1	70	70
Utility Search Fee	2111	1	300	300
Utility Examination Fee	2311	1	360	360
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
		Total in USD ($)		**1030**

Electronic Acknowledgement Receipt	
EFS ID:	24016435
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	06-NOV-2015
Filing Date:	06-JUL-2015
Time Stamp:	19:13:57
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$1030
RAM confirmation Number	5359
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Fee Worksheet (SB06)	fee-info.pdf	36954 c9bca7a2ca1f8c0b1f403d40bca2c923ae7d4104fb	no	2

Warnings:	
Information:	
Total Files Size (in bytes):	36954

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc code : PET.OP.AGE
Description : Petition to make special based on Age/Health

PTO/SB/130 (07-09)
Approved for use through 07/31/2012. OMB 0651- 0031
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PETITION TO MAKE SPECIAL BASED ON AGE FOR ADVANCEMENT OF EXAMINATION UNDER 37 CFR 1.102(c)(1)

Application Information

Application Number	14792094	Confirmation Number	9697	Filing Date	2015-07-06
Attorney Docket Number (optional)	CGLC-020CON5	Art Unit	1636	Examiner	
First Named Inventor	SYDNEY BRENNER				
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement				

Attention: Office of Petitions
An application may be made special for advancement of examination upon filing of a petition showing that the applicant is 65 years of age, or more. No fee is required with such a petition. See 37 CFR 1.102(c)(1) and MPEP 708.02 (IV).

APPLICANT HEREBY PETITIONS TO MAKE SPECIAL FOR ADVANCEMENT OF EXAMINATION IN THIS APPLICATION UNDER 37 CFR 1.102(c)(1) and MPEP 708.02 (IV) ON THE BASIS OF THE APPLICANT'S AGE.

A grantable petition requires one of the following items:
(1) Statement by one named inventor in the application that he/she is 65 years of age, or more; or
(2) Certification by a registered attorney/agent having evidence such as a birth certificate, passport, driver's license, etc. showing one named inventor in the application is 65 years of age, or more.

Name of Inventor who is 65 years of age, or older

Given Name	Middle Name	Family Name	Suffix
SYDNEY		BRENNER	

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18.
Please see 37 CFR 1.4(d) for the format of the signature.

Select (1) or (2) :

○ (1) I am an inventor in this application and I am 65 years of age, or more.

◉ (2) I am an attorney or agent registered to practice before the Patent and Trademark Office, and I certify that I am in possession of evidence, and will retain such in the application file record, showing that the inventor listed above is 65 years of age, or more.

Signature	/James S. Keddie, Reg. No. 48920/	Date (YYYY-MM-DD)	2015-11-09
Name	James S. Keddie	Registration Number	48920

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Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Fr eedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about indivi duals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt	
EFS ID:	24024472
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	09-NOV-2015
Filing Date:	06-JUL-2015
Time Stamp:	17:47:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		CGLC-020CON5-Prelim_Amend.pdf	42815 0c589e0b97d68827&b920aa15e4b4508b	yes	7

	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Preliminary Amendment		1	1	
	Specification		2	2	
	Claims		3	5	
	Applicant Arguments/Remarks Made in an Amendment		6	7	
Warnings:					
Information:					
2	Application Data Sheet	CGLC-020CON5-ADS.pdf	1028819	no	9
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
3	Transmittal Letter	CGLC-020CON5-IDS_Transmittal.pdf	52439	no	3
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Form (SB08)	CGLC-020CON5-IDS-1449.pdf	615170	no	8
Warnings:					
Information:					
5	Petition to make special based on Age/ Health	CGLC-020CON5_S-Brenner-PassportCopy.pdf	132890	no	1
Warnings:					
Information:					
6	Petition to make special based on Age/ Health	CGLC-020CON5_Ptn2MakeSpecial.pdf	68949	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1941082		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

FILED ELECTRONICALLY

PRELIMINARY AMENDMENT UNDER CFR § 1.115 Address to: Mail Stop Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket No.	CGLC-020CON5
	Confirmation No.	9697
	First Named Inventor	Brenner, Sydney
	Application Number	14/792,094
	Filing Date	July 6, 2015
	Group Art Unit	1636
	Examiner Name	TBA
	Title: *"Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"*	

Sir:

Prior to the examination of the above-referenced application on the merits, please enter the amendments below.

AMENDMENTS TO THE SPECIFICATION

Please insert the following header and paragraph immediately after the title on page 1:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is continuation of 14/172,694, filed February 4, 2014, which is a continuation of 14/021,790, filed September 9, 2013, which is a continuation of 13/859,450, filed April 9, 2013, which is a continuation of 13/622,872, filed September 19, 2012, which is a continuation of 13/387,343, filed February 15, 2012, which is a §371 National Phase Application of PCT/IB2010/002243, filed August 13, 2010, which claims priority to US Provisional Application No. 61/235,595, filed August 20, 2009 and US Provisional Application No. 61/288,792, filed December 21, 2009; all of which are herein incorporated by reference.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00102

AMENDMENTS TO THE CLAIMS

Please amend the claims as shown below. A complete listing of the claims, including their current status identifiers, is set forth below.

1-20 (Cancelled)

21. (New) A method of analyzing nucleic acids, comprising:

providing a plurality of nucleic acids from a single cell source;

tagging the plurality of nucleic acids from the single cell source with multiplex identifier (MID) sequences; and

determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences.

22. (New) The method of claim 21, wherein the MID sequences uniquely identify the single cell source.

23. (New) The method of claim 21, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source.

24. (New) The method of claim 21, further comprising amplifying the plurality of nucleic acids and attached MID sequences before determining the nucleotide sequence of the plurality of nucleic acids and attached MID sequences.

25. (New) The method of claim 21, wherein the determining step comprises sequencing the plurality of nucleic acids and attached MID sequences.

26. (New) The method of claim 21, wherein the plurality of nucleic acids is selected from DNA and RNA

27. (New) The method of claim 26, wherein the plurality of nucleic acids comprises

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00103

mRNA.

28. (New) The method of claim 23, further comprising identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within a single cell source from a number of unique MID sequences identified.

29. (New) The method of claim 21, wherein the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids.

30. (New) The method of claim 21, wherein the tagging step comprises performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

31. (New) The method of claim 21, wherein:

the providing step comprises providing a plurality of nucleic acids from each of a plurality of single cell sources;

the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and

wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step.

32. (New) The method of claim 21, wherein the MID sequences further comprise a sequencing primer sequence.

33. (New) The method of claim 21, wherein the MID sequences are from 2 to 100 bases in length.

34. (New) The method of claim 31, wherein the plurality of different MID sequences comprises at least 100 different MID sequences.

10x Genomics, Inc. Exhibit 2004
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Page 00104

35. (New) A method of analyzing nucleic acids, comprising:

providing a plurality of nucleic acids from a single cell source;

tagging the plurality of nucleic acids from the single cell source with multiplex identifier (MID) sequences, wherein each of the plurality of molecules is tagged with a unique MID sequence; and

analyzing the plurality of nucleic acids and attached MID sequences.

36. (New) The method of claim 35, wherein the analyzing step comprises sequencing the plurality of nucleic acids and attached MID sequences.

37. (New) A method of analyzing nucleic acids, comprising:

providing a plurality of nucleic acids from a single cell source;

tagging the plurality of nucleic acids from the single cell source with multiplex identifier (MID) sequences, wherein each of the plurality of molecules is tagged with a unique MID sequence;

amplifying the plurality of nucleic acids and associated MID sequences to provide amplified nucleic acids; and

determining a nucleotide sequence of amplified nucleic acids.

38. (New) The method of claim 37, wherein the amplifying step comprises performing a polymerase chain reaction amplification.

39. (New) The method of claim 37, wherein the amplifying step comprises a linear amplification step.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00105

REMARKS

Formal Matters

Claims 1-20 are cancelled without prejudice.

Claims 21-39 are new. Support for new claims 21-39 is found throughout the specification, e.g., at the following exemplary positions: lines 6-7 of page 19 ("the nucleic acid sample being analyzed is derived from a single source (e.g., a ...cell)", page 19, lines 17-19 (i.e., "the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined", page 8, lines 1-3 ("the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived"), and page 25, lines 5-7 ("This sequencing reaction will read through the MID and desired site in the insert"). No new matter has been added.

Applicants respectfully request consideration of the present application.

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number CGLC-020CON5.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: November 9, 2015

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie
Registration No. 48,920

Bozicevic, Field & Francis LLP
1900 University Avenue, Suite 200
East Palo Alto, California 94303
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00107

PTO/AIA/14 (07-14)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	14/792,094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Sydney		Brenner	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Ely	Country of Residence [i]	GB

Mailing Address of Inventor:

Address 1	3 Barton Square		
Address 2			
City	Ely	State/Province	
Postal Code	CB7 4PJ	Country [i]	GB

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Gi		Mikawa	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Great Shelford	Country of Residence [i]	GB

Mailing Address of Inventor:

Address 1	104 High Street		
Address 2			
City	Great Shelford	State/Province	
Postal Code	CB1 6A3	Country [i]	GB

Inventor 3 Remove

Legal Name

PTO/AIA/14 (07-14)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	14/792,094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

Prefix	Given Name	Middle Name	Family Name	Suffix
	Robert		Osborne	

Residence Information (Select One) (•) US Residency ( ) Non US Residency ( ) Active US Military Service

City	Great Chesterford	State/Province		Country of Residence	GB

Mailing Address of Inventor:

Address 1	38 Pilgrim Close		
Address 2			
City	Great Chesterford	State/Province	
Postal Code	CB10 1QG	Country	GB

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Andrew		Slatter	

Residence Information (Select One) (•) US Residency ( ) Non US Residency ( ) Active US Military Service

City	London	State/Province		Country of Residence	GB

Mailing Address of Inventor:

Address 1	65a Huddleston Road		
Address 2			
City	London	State/Province	
Postal Code	N7 0AE	Country	GB

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	93726		
Email Address	docket@bozpat.com	Add Email	Remove Email

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00109

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	14/792,094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

Application Information:

Title of the Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement		
Attorney Docket Number	CGLC-020CON5	**Small Entity Status Claimed**	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	13	**Suggested Figure for Publication (if any)**	

Filing By Reference :

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	93726		

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	14/792,094
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Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the application number blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
14/792,094	Continuation of	14172694	~~2015-02-04~~ 2014-02-04

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14172694	Continuation of	14021790	2013-09-09	8679756	2014-03-25

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14021790	Continuation of	13859450	2013-04-09	8563274	2013-10-22

Prior Application Status	Abandoned		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
13859450	Continuation of	13622872	2012-09-19

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13622872	Continuation of	13387343	2012-02-15	8298767	2012-10-30

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
13387343	a 371 of international	IB2010002243	2010-08-13
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
IB2010002243	Claims benefit of provisional	61288792	2009-12-21
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
IB2010002243	Claims benefit of provisional	61235595	2009-08-20

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

PTO/AIA/14 (07-14)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	14/792,094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization to Permit Access:

☒ Authorization to Permit Access to the Instant Application by the Participating Offices

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	Application Number	14/792,094
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If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date o f filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name	Population Genetics Technologies LTD.

Mailing Address Information For Applicant:

Address 1			
Address 2	Babraham Research Campus		
City	Cambridge	**State/Province**	
Country	GB	Postal Code	CB22 3AT
Phone Number		Fax Number	

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	14/792,094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

Email Address	

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not subsitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Signature	/James S. Keddie, Reg. No. 48,920/			Date (YYYY-MM-DD)	2015-11-09
First Name	James S.	Last Name	Keddie	Registration Number	48920

Additional Signature may be generated within this form by selecting the Add button.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	CGLC-020CON5
	Application Number	14/792,094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent C o o p eration Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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Electronically Filed

INFORMATION DISCLOSURE STATEMENT Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450		
	Attorney Docket	CGLC-020CON5
	First Named	Brenner, Sydney
	Application Number	14/792,094
	Confirmation No.	9697
	Filing Date	July 6, 2015
	Group Art Unit	1636
	Examiner Name	TBA
	Title:	*"Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"*

Sir:

Applicants submit herewith documents which may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 C.F.R. § 1.56. This submission is not intended to constitute an admission that any document referred to therein is "prior art" for this invention unless specifically designated as such. A listing of the documents is shown on enclosed Form PTO/SB/08A.

The Examiner is requested to make the documents listed on the enclosed PTO/SB/08A of record in this application. Applicants would appreciate the Examiner initialing and returning the initialed copy of form PTO/SB/08A, indicating the document cited therein have been considered and made of record herein.

All of the references identified herein were disclosed in parent application serial number 14/172,694, and as such, copies thereof are not included pursuant to the provisions of 37 CFR § 1.98(d).

Statements

☒ **No statement**

☐ **PTA Statement under 37 CFR § 1.704(d)(1):** Each item of information contained in the information disclosure statement filed herewith:

(i) Was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c)

more than thirty days prior to the filing of the information disclosure statement; or

(ii) Is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement.

☐ **IDS Statement under 37 CFR § 1.97(e)(1):** Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement; or

☐ **IDS Statement under 37 CFR § 1.97(e)(2):** No item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of the information disclosure statement.

Fees

☒ No fee is believed to be due.

☐ The appropriate fee set forth in 37 C.F.R. §1.17(p) accompanies this information disclosure statement.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number CGLC-020CON5.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: November 9, 2015

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie
Reg. No. 48,920

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	14792094
Filing Date	2015-07-06
First Named Inventor	BRENNER, Sydney
Art Unit	1636
Examiner Name	
Attorney Docket Number	CGLC-020CON5

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6723513		2004-04-20	LEXOW, Preben	
	2	7544473		2009-06-09	BRENNER, Sydney	
	3	7700325		2010-04-20	CANTOR, et al.	
	4	8101346		2012-01-24	TAKAHAMA, Shinichiro	
	5	8298767		2012-10-30	BRENNER, et al.	
	6	8318460		2012-11-27	CANTOR, et al.	

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	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	
	Attorney Docket Number	CGLC-020CON5

	1	20040224331		2004-11-11	CANTOR, et al.	
	2	20060008799		2006-01-12	CAI, et al.	
	3	20070020640		2007-01-25	MCCLOSKEY, et al.	
	4	20070238113		2007-10-11	KANDA, et al.	
	5	20070259357		2007-11-08	BRENNER, Sydney	
	6	20090155780		2009-06-18	XIAO, et al.	
	7	20100086914		2010-04-08	BENTLEY, et al.	
	8	20100113296		2010-05-06	MYERSON, Joel	
	9	20100120098		2010-05-13	GRUNENWALD, et al.	
	10	20110287435		2011-11-24	GRUNENWALD, et al.	
	11	20120208724		2012-08-16	STEEMERS, et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	
	Attorney Docket Number	CGLC-020CON5

	12	20120316074		2012-12-13	SAXONOV, Serge	
	13	20130059310		2013-03-07	BRENNER, et al.	

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	EP1841879	EP		2007-10-10	COMPASS GENETICS, LLC.		☐
	2	EP1672064	EP		2006-06-21	RIKEN		☐
	3	WO200190418	WO		2001-11-29	THE REGENTS OF THE UNIVERSITY OF CALIFORNIA		☐
	4	WO2004061083	WO		2004-07-22	AMGEN INC		☐
	5	WO2004065617	WO		2004-08-05	THE TRUSTEES OF BOSTON UNIVERSITY		☐
	6	WO2007018601	WO		2007-02-15	RUBICON GENOMICS, INC.		☐
	7	WO2008150432	WO		2008-12-11	454 LIFE SCIENCES CORPORATION		☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	
	Attorney Docket Number	CGLC-020CON5

	8	WO2013036929	WO		2013-03-14	THE BOARD OF TRUSTEES OF THE LELAND STANFORD...		☐
	9							☐

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T5
	1	BOULANGER, et al, "Massively parallel haplotyping on microscopic beads for the high-throughput phase analysis of single molecules", PLoS One, Vol 7:1-10, 2012.	☐
	2	CASBON, et al, "Reflex: intramolecular barcoding of long-range PCR products for sequencing multiple pooled DNAs", Nucleic Acids Res., Pages 1-6, 2013.	☐
	3	FAN, et al, "Whole-genome molecular haplotyping of single cells", Nat Biotechnol., 29: 51-59, 2011.	☐
	4	HIATT, et al, "Parallel, tag-directed assembly of locally derived short sequence reads", Nat Methods., 7:119-122, 2010.	☐
	5	IMBURGIO, et al, "Studies of promoter recognition and start site selection by T7 RNA polymerase using a comprehensive collection of promoter variants", Biochemistry., 39:10419-30, 2000.	☐
	6	KAPER, et al, "Whole-genome haplotyping by dilution, amplification, and sequencing", Proc Natl Acad Sci U. S. A., 110: 5552-7, 2013.	☐
	7	KIRKNESS, et al, "Sequencing of isolated sperm cells for direct haplotyping of a human genome", Genome Res., 23: 826-32, 2013.	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	
	Attorney Docket Number	CGLC-020CON5

	8	KITZMAN, et al, "Haplotype-resolved genome sequencing of a Gujarati Indian individual", Nat Biotechnol., 29:59-63, 2011.	☐
	9	KOZAREWA, et al. "Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of GC-biased genomes", Nat Methods., 6: 291-5, 2009.	☐
	10	KONAREWA, et al, "96-plex molecular barcoding for the Illumina Genome Analyzer", Methods Mol Biol., 733:279-98, 2011.	☐
	11	KWOK, et al, "Single-molecule analysis for molecular haplotyping", Hum Mutat., 23:442-6, 2004.	☐
	12	LAIRD, et al, "Hairpin-bisulfite PCR: Assessing epigenetic methylation patterns on complementary strands of individual DNA molecules", 101:204-9, 2004.	☐
	13	LUNDIN, et al, "Hierarchical molecular tagging to resolve long continuous sequences by massively parallel sequencing", Sci Rep., 3:1186, 2003.	☐
	14	OYOLA, et al, "Optimizing Illumina next-generation sequencing library preparation for extremely AT-biased genomes", BMC Genomics.,13:1, 2012.	☐
	15	PCT/IB2010/002243, International Search Report and Written Opinion, Mailed February 9, 2011, 13 pages.	☐
	16	REISNER, et al, "Single-molecule denaturation mapping of DNA in nanofluidic channels", Proc Natl Acad Sci U.S.A., 107: 13294-9, 2010.	☐
	17	SCHWARTZ, et al, "Capturing native long-range contiguity by in situ library construction and optical sequencing", PNAS, 109:18749-54, 2012.	☐
	18	TURNER, et al, "Assaying chromosomal inversions by single molecule haplotyping", Nat Methods., 3:439-445, 2006.	☐

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	
	Attorney Docket Number	CGLC-020CON5

	19	TURNER, et al, "High-throughput haplotype determination over long distances by haplotype fusion PCR and ligation haplotyping", Nat Protoc., 4:1771-83, 2009.	☐
	20	USHIJIMA, "Detection and interpretation of altered methylation patterns in cancer cells", Nat Rev Cancer., 3:223-31, 2005.	☐
	21	VAN NIEUWERBURGH, et al, "Illumina mate-paired DNA sequencing-library preparation using Cre-Lox recombination", Nucleic Acids Res., 40:1-8, 2012.	☐
	22	WANG, et al, "Self-Formed Adaptor PCR: a Simple and Efficient Method for Chromosome Walking", Appl Environ Microbiol., 15: 5048–51, 2007.	☐
	23	XIAO, et al, "Determination of haplotypes from single DNA molecules: a method for single-molecule barcoding", Hum Mutat., 28:913-21, 2007.	☐
	24	YAN, et al, "Rapid one-step construction of hairpin RNA", Biochem Biophys Res Commun., 4:464-8, 2009.	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	
	Attorney Docket Number	CGLC-020CON5

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/James S. Keddie, Reg. No. 48,920/	Date (YYYY-MM-DD)	2015-11-09
Name/Print	James S. Keddie	Registration Number	48920

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

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3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

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8 Authority/ Awdurdod/ Ùghdarras/ Instanse/ Autorité/ Behörde/ Autorità/ Myndighet/ Údarás/ Εκδούσα Αρχή/ Autoridade/ Autoridad/ Viranomainen/ Myndighet/ Pas vydal/ Väljaandja/ Izdevējiestāde/ Išdavė/ Awtorità/ Organ Wydający/ Organ/ Úrad/ Kiállító hatóság

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10 Holder's signature/ Llofnod y deiliad/ Ainm sgrìobhte an neach-seilbhe/ Handtekening van de houder/ Signature du titulaire/ Unterschrift der Inhaberin (des Inhabers)/ Firma del titolare/ Indehaverens underskrift/ Sínitú an tseilbhóra/ Υπογραφή του κατόχου/ Assinatura do titular/ Firma del titular/ Haltijan nimikirjoitus/ Passinnehavarens namnteckning/ Podpis držitele/ Omaniku allkiri/ Pilsoņa paraksts/ Savininko parašas/ Firma ta' min hariġlu l-passaport/ Podpis posiadacza/ Podpis imetnika/ Podpis držiteľa/ A tulajdonos aláírása

11 Observations page/ Sylwadau tudalen/ Fiosrachadh eile/ Opmerkingen bladzijde/ Page d'observations/ Vermerke seite/ Annotazioni/ Bemærkninger side/ Leathanach tagairtí/ Παρατηρήσεις σελίδα/ Página para observações/ Página de observaciones/ Lisämerkintöjä sivu/ Observationer side/ Poznámky strana/ Märkused/ Īpašas atzīmes/ Pastabos puslapis/ Tagħrif iehor/ Achtanacje/ Stran za opombe/ Pripomienky strana/ Megjegyzések oldal

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10 Holder's signature/ Llofnod y deiliad/ Ainm sgrìobhte an neach-seilbhe/ Handtekening van de houder/ Signature du titulaire/ Unterschrift der Inhaberin (des Inhabers)/ Firma del titolare/ Indehaverens underskrift/ Síniú an tsealbhóra/ Υπογραφή του κατόχου/ Assinatura do titular/ Firma del titular/ Haltijan nimikirjoitus/ Passinnehavarens namnteckning/ Podpis držitele/ Omaniku allkiri/ Pilsoņa paraksts/ Savininko parašas/ Firma ta' min inhariġlu l-passaport/ Podpis posiadacza/ Podpis imetnika/ Podpis držiteľa/ A tulajdonos aláírása

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UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND

PASSPORT
PASSEPORT

Type/Type: P
Code/Code: GBR
Passport No./Passeport No.: [redacted]



Surname/Nom (1): BRENNER
Given names/Prénoms (2): SYDNEY
Nationality/Nationalité (3): BRITISH CITIZEN
Date of birth/Date de naissance (4): 13 JAN /JAN 27
Sex/Sexe (5): M
Place of birth/Lieu de naissance (6): GERMISTON
Date of issue/Date de délivrance (7): 23 AUG /AOUT 06
Authority/Autorité (8): FCO
Date of expiry/Date d'expiration (9): 23 AUG /AOUT 16
Holder's signature/Signature du titulaire (10): Sydney B...

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 = *		X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 = *		X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	11/09/2015	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 19	Minus	** 20	= 0	X $40 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $210 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

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LIE
/PAULA MCCRAY STANLEY/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/792,094

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	70		N/A	
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	300		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	19 minus 20 =	*	x 40 =	0.00	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*	x 210 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	730		TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE(S)	SMALL ENTITY ADDITIONAL FEE(S)	OR	OTHER THAN SMALL ENTITY RATE(S)	OTHER THAN SMALL ENTITY ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x		OR	x	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x		OR	x	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE(S)	ADDITIONAL FEE(S)		RATE(S)	ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x		OR	x	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x		OR	x	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/792,094	07/06/2015	1636	800	CGLC-020CON5	19	3

CONFIRMATION NO. 9697

UPDATED FILING RECEIPT



93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

Date Mailed: 11/19/2015

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Sydney Brenner, Ely, UNITED KINGDOM;
Gi Mikawa, Great Shelford, UNITED KINGDOM;
Robert Osborne, Great Chesterford, UNITED KINGDOM;
Andrew Slatter, London, UNITED KINGDOM;

Applicant(s)

Population Genetics Technologies LTD., Cambridge, UNITED KINGDOM;

Power of Attorney: The patent practitioners associated with Customer Number 93726

Domestic Priority data as claimed by applicant

This application is a CON of 14/172,694 02/04/2014 PAT 9102980 *
which is a CON of 14/021,790 09/09/2013 PAT 8679756
which is a CON of 13/859,450 04/09/2013 PAT 8563274
which is a CON of 13/622,872 09/19/2012 ABN
which is a CON of 13/387,343 02/15/2012 PAT 8298767
which is a 371 of PCT/IB2010/002243 08/13/2010
which claims benefit of 61/288,792 12/21/2009
and claims benefit of 61/235,595 08/20/2009
(*)Data provided by applicant is not consistent with PTO records.

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

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Permission to Access - A proper **Authorization to Permit Access to Application by Participating Offices** (PTO/SB/39 or its equivalent) has been received by the USPTO.

If Required, Foreign Filing License Granted: 07/20/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/792,094**

Projected Publication Date: 02/25/2016

Non-Publication Request: No

Early Publication Request: No
** **SMALL ENTITY** **
Title

Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

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countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/792,094	07/06/2015	Sydney Brenner	CGLC-020CON5

CONFIRMATION NO. 9697

93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

POA ACCEPTANCE LETTER

OC000000078730981

Date Mailed: 11/19/2015

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 10/02/2015.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/dgela/

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MAILED
DEC 22 2015
OFFICE OF PETITIONS

In re Application of	:	
Brenner et al.	:	DECISION ON PETITION
Application No. 14/792,094	:	TO MAKE SPECIAL UNDER
Filed: July 06, 2015	:	37 CFR 1.102(c)(1)
Attorney Docket No. CGLC-020CON5	:	

This is a decision on the petition under 37 CFR 1.102(c)(1), filed November 09, 2015 to make the above-identified application special based on applicant's age as set forth in M.P.E.P. § 708.02, Section II.

The petition is **GRANTED**.

A grantable petition to make an application special under 37 CFR 1.102(c)(1) and MPEP § 708.02, Section II: Applicant's Age must be accompanied by evidence showing that at least one of the applicants is 65 years of age, or more, such as a birth certificate or a statement by applicant. No fee is required

The instant petition includes a statement from the attorney of record declaring that he is in possession of such evidence that proves the applicant, Sydney Brenner, is 65 years of age or older. Accordingly, the above-identified application has been accorded "special" status.

Telephone inquiries concerning this decision should be directed to the undersigned at 571-272-0593. All other inquiries concerning either the examination or status of the application should be directed to the Technology Center.

This application is being referred to Technology Center Art Unit 1634 for action on the merits commensurate with the decision.

/DeTya Flowers/
DeTya Flowers
Petitions Paralegal Specialist
Office of Petitions

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	CGLC-020CON5	9697

93726 7590 01/13/2016
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EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
01/13/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

docket@bozpat.com

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Office Action Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *11/09/2015*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *21-39* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *21-39* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☒ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *7/6/15* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.

4) ☐ Other: _____.

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DETAILED ACTION

1. The present application is being examined under the pre-AIA first to invent provisions. The petition to make the instant application "special" has been granted by the office on December 22, 2015 and thus has been examined on expedited basis. Claims 21-39 pending in this application and are under prosecution. Claims 1-20 are cancelled.

Specification

2. The abstract of the disclosure is objected to because it is not in line with the claimed invention. Also, the abstract contain acronym "MID", the meaning of which may change over time. It is suggested to use the full version of the acronym "MID".

3. The specification is also objected to for missing the header "Brief Description of the Drawings" in page 2 of the specification prior to the description of the figures.

Appropriate corrections are required (See MPEP § 608.01(b)).

Double Patenting

4. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

5. Claims 21-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-21 of U.S. Patent No. 7,544,473 in view of Braverman et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) for the following reasons.

Regarding instant independent claims 21, 35 and 37, claims 1 and 4 of '473 patent is drawn to a method of tagging a plurality of populations of polynucleotides each with a unique sequence token tag, comprising: generating a plurality of unique sequence token tags; dividing the polynucleotides of each of said populations of polynucleotides into at least two non-overlapping nucleic acid segments; and ligating a unique sequence token tag to the at least two non-overlapping nucleic acid segments of each of said populations of polynucleotides to provide a plurality of populations of polynucleotides each tagged with a unique sequence token tag, wherein each unique sequence token tag is used to tag only one of said populations of polynucleotides.

Claim 4 of '473 patent is drawn to a single subject, which is a single cell as defined in the instant specification (pg. 19, paragraph 2). One having ordinary skill in the art would recognize that the single subject of '473 patent meets the limitation of nucleic acids from the single cell source. Regarding instant claims 21, 35 and 37, claims of '473 patent does not specifically drawn to determining the nucleic acid sequence of the plurality of nucleic acids.

Regarding claim 37, although claims '473 patent are drawn to the step of a plurality of populations of polynucleotides, said claims are not specifically drawn to the step of amplifying the plurality of nucleic acids associated with the MID sequences.

However, the steps of amplifying the plurality of nucleic acids associated with the MID sequences and determining the sequences attached to the mid sequences are routine, well established and conventional in the art and therefore obvious improvements over '473 patent. The steps missing in the instant claims are taught by Braverman (Figs. 1 and 2 and paragraphs 0077-0084) for enriching the concentration of nucleic acids from the single cell and to determine the error rate in the sequencing run (paragraph 0082).

With regard to the limitations of dependent claims 22-34, 36, 38 and 39 claims 1-21 of '473 patent in view of Braverman are drawn to the limitations of said dependent claims and therefore are not patentably significant.

6. Claims 21-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,481,292 in view of Braverman

et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) for the following reasons.

Regarding instant independent claims 21, 35 and 37 claim 1 of 292 patent is drawn to a method for enhancing a determination of the presence of alleles in a genomic sample, said method comprising: a) attaching a set of oligonucleotides that comprises a degenerate base region (DBR) comprising at least one nucleotide base selected from: R, Y, S, W, K, M, B, D, H, V, N and modified versions thereof to the nucleic acid molecules of a genomic sample that contains a polymorphic target region, thereby producing a population of adapter-attached polynucleotides in which each of said adaptor-attached polynucleotides that comprises said polymorphic target region is attached to a DBR sequence of an oligonucleotide of said set of oligonucleotides, wherein said oligonucleotides further comprise a unique multiplex identifier (MID) sequence that identifies a source for each of the nucleic acid molecules to which it is attached; b) amplifying the adapter-attached polynucleotides, thereby producing amplified adapter-attached polynucleotides that contain the polymorphic target region; c) sequencing some of the amplified adapter-attached polynucleotides that contain the polymorphic target region, thereby producing a plurality of sequences, wherein the sequencing step provides, for each of the amplified adaptor-attached polynucleotides that contain the polymorphic target region and are sequenced: (i) the nucleotide sequence of at least a portion of said polymorphic target region and (ii) the DBR sequence of the oligonucleotide to which said polymorphic target region is attached; d) determining, for one allele of the polymorphic target region, the number of different DBR

sequences in the oligonucleotides that are associated with said allele; and e) repeating step d) for additional alleles of said polymorphic target region, thereby enhancing said determination of the presence of said alleles in said genomic sample based on the numbers of different DBR sequences in the oligonucleotides that are associated with said allele and said additional alleles (steps in common with instant claims are emphasized by the examiner with underlining).

Although claims of '292 patent is not drawn to a single cell source, it is noted that single cell source is an obvious variant of a genomic sample. Also the step of providing nucleic acid from a single cell source is routine well established and conventional in the art as taught by Braverman (paragraph 0077).

With regard to the limitations of dependent claims 22-34, 36, 38 and 39 claims 1-20 of '292 patent in view of Braverman are drawn to the limitations of said dependent claims and therefore are not patentably significant.

7. Claims 21-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 8,685,678 in view of Braverman et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) for the following reasons.

The subject matter of claims of '678 patent (e.g., claims 1 and 11) is similar to that of '292 patent and are not patentably distinct as discussed in section 6.

8. Claims 21-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,741,606 in view of Braverman

et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) for the following reasons.

The subject matter of claims of '606 patent (e.g., claims 1 and 9) is similar to that of '292 patent and are not patentably distinct as discussed in section 6.

9. Claims 21-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 8,715,967 in view of Braverman et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) for the following reasons.

The subject matter of claims of '967 patent (e.g., claims 1 and 15) is similar to that of '292 patent and are not patentably distinct as discussed in section 6.

10. Claims 21-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-25 of U.S. Patent No. 8,722,368 in view of Braverman et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) for the following reasons.

The subject matter of claims of '368 patent (e.g., claim 1) is similar to that of '292 patent and are not patentably distinct as discussed in section 6.

11. Claims 21-39 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 25-45 of copending Application No.

14/273,197 in view of Braverman et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) for the following reasons.

Regarding instant independent claims 21, 35 and 37 claim 25 of '197 copending application is drawn to a method for assessing the presence of a genetic variation of a virus in a sample, comprising: (a) amplifying a population of initial target DNA molecules from a tagged viral sample to produce a population of amplified target DNA molecules, wherein at least some of the initial target DNA molecules are tagged with sequences tags that discriminate between the different initial target DNA molecules and each of the amplified target DNA molecules comprise an associated sequence tag; and (b) sequencing a plurality of the amplified target DNA molecules to produce a plurality of sequence reads, wherein the sequencing step provides, for those amplified target DNA molecules that are sequenced: (i) the nucleotide sequence of a target DNA molecule and (ii) the nucleotide sequence of an associated tag; (c) examining the sequence reads of b) to identify a potential genetic variation; (d) assigning a confidence, using a computer, to the existence of the genetic variation in said tagged viral sample, based on: (i) a determination of the number of said different sequence tags that are associated with said genetic variation; and (ii) a determination of the number of said sequence reads that comprise each of the different sequence tag that are associated with said genetic variation. Claim 34 of '197 copending application is drawn to the initial target DNA molecules are further tagged with a unique multiplex identifier (MID) sequence that identifies the source of a nucleic acid molecule to which it is tagged (common steps

between instant independent claims and claims of '197 copending application are underlined by the examiner).

Although claims of '197 copending application are not drawn to a single cell source, it is noted that single cell source is an obvious variant of a genomic sample. Also the step of providing nucleic acid from a single cell source is routine well established and conventional in the art as taught by Braverman (paragraph 0077).

With regard to the limitations of dependent claims 22-34, 36, 38 and 39 claims 25-45 of '197 copending application in view of Braverman are drawn to the limitations of said dependent claims and therefore are not patentably significant.

This is a provisional nonstatutory double patenting rejection.

Claim Rejections - 35 USC § 102

12. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

13. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

14. Claims 21-25 and 28-39 are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Brenner (US 2007/0259357 published Nov. 8, 2007, cited in the IDS of 11/7/2015).

Regarding claims 21-25, 28, 31 and 35-39, Brenner teaches a method of tagging a plurality of genomic DNA samples from a plurality of subjects each with a unique sequence token by generating a plurality of unique sequence token tags; dividing each genomic DNA sample from each subjects into at least two non-overlapping nucleic acid segments; and ligating a unique sequence token tag to each non-overlapping nucleic acid segment of each genomic DNA sample to provide a plurality of genomic DNA samples from a plurality of subjects each tagged with a unique sequence token tag, wherein each unique sequence token tag is used to tag only the genomic DNA from one subject (paragraphs 0008 and 0047). Brenner also teaches the genomic DNA is from a cell line (paragraph 0055), which meets the limitation of nucleic acids is from single cell source. The unique sequence token is the multiplex identifier because it identifies only one sample out of the plurality of samples.

Brenner further teaches the amplification step to enrich tagged fragments before sequencing (paragraph 0096) and determining the nucleotide sequence of the plurality of nucleic acid sequence attached to the unique sequence token (paragraph 0005).

Regarding claim 29, Brenner teaches tagging step comprise ligating token to the DNA (paragraph 0054).

Regarding claim 31, Brenner teaches token comprise primer sequence for sequencing (paragraph 0086).

Regarding claims 33 and 34, Brenner teaches that the token sequences are triplet or 9 bases and comprise at least 4480 different token sequences (Constructing of a Set of Mutually Discriminatory Sequence Tokens section).

15. Claims 21-25 and 28-39 are rejected under pre-AIA 35 U.S.C. 102(a) and 102(e) as being anticipated by Braverman et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007).

Regarding claims 21-23 and 25, Braverman method of analyzing nucleic acids, comprising providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify

each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088).

Regarding claim 24, Braverman teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claim 28, Braverman teaches identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within a single cell source from a number of unique MID sequences identified (Fig. 1 and paragraphs 78-83).

Regarding claim 29, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claim 30, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claims 31 and 32, Braverman teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Fig. 2 and paragraphs 0085-0088).

Regarding claims 33 and 34, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claim 35-37, Braverman teaches a method of analyzing nucleic acids, comprising: providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with a unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003). Braverman further teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088).

Regarding claim 38, Braverman teaches that the amplifying step comprises performing a polymerase chain reaction amplification (paragraph 0086).

Regarding claim 39, Braverman teaches that the amplifying step comprises a linear amplification (i.e., isothermal amplification) step (paragraph 0007).

Claim Rejections - 35 USC § 103

16. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

17. The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under pre-AIA 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

18. This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

19. Claims 21-39 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Braverman et al (US 2009/0105959 published Apr. 23, 2009, effective filing date Jun. 1, 2007) in view of Bosnes (WO 03/062462 published Jul. 31, 2003).

Braverman and Bosnes teach a method for analyzing nucleic acid from a sample and therefore are analogous arts. The teachings of Bosnes are specifically applied for the limitations of claims 26 and 27 of nucleic acid is DNA and mRNA for correlating information relating to mRNA to genomic information.

Regarding claims 21-23 and 25, Braverman method of analyzing nucleic acids comprising providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088).

Regarding claim 24, Braverman teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claims 26 and 27, Braverman teaches nucleic acids (0018). However, Braverman does not specifically teach that the nucleic acid is DNA and mRNA.

Regarding claim 28, Braverman teaches identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within a single cell source from a number of unique MID sequences identified (Fig. 1 and paragraphs 78-83).

Regarding claim 29, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claim 30, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claims 31 and 32, Braverman teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Fig. 2 and paragraphs 0085-0088).

Regarding claims 33 and 34, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claim 35-37, Braverman teaches a method of analyzing nucleic acids, comprising: providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with a unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003). Braverman also teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic

acid sequence (paragraph 0088) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088).

Regarding claim 38, Braverman teaches that the amplifying step comprises performing a polymerase chain reaction amplification (paragraph 0086).

Regarding claim 39, Braverman teaches that the amplifying step comprises a linear amplification (i.e., isothermal amplification) step (paragraph 0007).

As discussed above regarding claims 26 and 27, Braverman does not specifically teach that the nucleic acid is DNA and mRNA. However, the DNA and mRNA from the same sample were known in the art at the time the claimed invention was made as taught by Bosnes, who teaches a method for isolating DNA and mRNA from the same sample and further teaches having both DNA and mRNA from the same sample allows for correlating information relating to mRNA to genomic information (Abstract).

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the DNA and mRNA from the single cell source of Bosnes in the method of Braverman with a reasonable expectation of success with the expected benefit of correlating information relating to mRNA to genomic information thus understanding the interrelationship of genotype and phenotype as taught by Bosnes (Abstract). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the mRNA from

the single cell source because isolation of DNA, mRNA and protein are is routinely practiced in the art as exemplified by Bosnes.

It is further noted that in KSR, the Supreme Court particularly emphasized "the need for caution in granting a patent based on the combination of elements found in the prior art," (USPQ2d at 1395), and reaffirmed principles based on its precedent that "[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."

In the instant case, the steps of claims 21-39 are a combination of known steps which are well understood, routine, conventional steps already engaged in by the scientific community for improving the sensitivity, specificity of the nucleic acid analysis and were known in the art at the time the claimed invention was made. As discussed above, Braverman in view of Bosnes teaches the claimed steps and combined as suggested above would arrive at the claimed invention. Also, one having ordinary skill in the art would recognize that choosing both DNA and mRNA template would be a mere design choice because steps of analyzing mRNA templates would not be different as the steps for converting mRNA to cDNA are routine, conventional and well established in the art. Thus the steps of pending claims when viewed as whole, add nothing significant beyond the sum of the steps taken from Braverman and Bosnes. Therefore the steps of claims 21-39 do not amount to "significantly more" and thus obvious improvement over Braverman.

Conclusion

20. No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 7 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

Notice of References Cited	Application/Control No. 14/792,094	Applicant(s)/Patent Under Reexamination BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	Page 1 of 1

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*	A	US-2009/0105959 A1	04-2009	Braverman; Michael S.	C12Q1/68	702/19
	B	US-				
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N	WO 03/062462	07-2003	GB	Bosnes	
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	28662	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or population.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:41
L2	232	1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:45
L3	12	2 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:47
L4	0	3 and (single or one) near3 (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:53
L5	10	3 and (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:53
L6	65	1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:00
L7	55	6 and (single or one) same(source or cell) same (nucleic or dna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:01
L8	8	1 and "reflex sequence".clm.	US-PGPUB;	OR	ON	2016/01/05

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			USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			16:08
L9	174	("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:11
L10	25	9 and (single or one) same (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:11
L11	69	("20040224331" | "20060008799" | "20070020640" | "20070238113" | "20070259357" | "20090155780" | "20100086914" | "20100113296" | "20100120098" | "20110287435" | "20120208724" | "20120316074" | "20130059310" | "6723513" | "7544473" | "7700325" | "8101346" | "8298767" | "8318460").PN.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:30
L12	8	11 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:31
L13	138	"7393665"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:36
L14	2	"7393665".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:36
L15	599	("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:37
L16	147	15 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (determin$3 or obtain$3) near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2016/01/05 16:38

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			JPO; DERWENT; IBM_TDB			
L17	697052	435/$.ccls. or 506/$.ccls. or 422/$.ccls. or 536/$.ccls. or (c12q1/68 or c07h21/02 or c07h21/04 or c12p19/34 or c12m1/34 or g01n33/52 or c40b30/04 or c40b40/04 or c40b50/04 or g01n31/22).cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:50
L18	481	17 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:51
L19	131	18 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (determin$3 or obtain$3) near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:51
L20	45	19 and (single or one) near5 (source or cell) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:52
L21	17	1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:55
L22	3	21 and (single or one or indivudual) same (source or cell) same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:56
L23	3	21 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (determin$3 or obtain$3) same sequence.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:58

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PubMed barcode sequencing

Summary 200 per page Sort by Publication Date

Search results

Items: 113

Filters activated: Publication date from 1950/01/01 to 2009/08/19. Clear all to show 493 items.

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(54) Title: METHOD FOR ISOLATING NUCLEIC ACIDS AND PROTEIN FROM A SINGLE SAMPLE

(57) Abstract: The present invention comprises a method of isolating nucleic acid and protein from the same sample with solid supports, wherein nucleic acid and protein components contained in the sample become bound to distinct solid supports. The invention also allows for kits for isolating nucleic acid and protein from the same sample and for use of the method of isolating nucleic acid and protein for the analysis and/or comparison of mRNA and/or protein expression and/or their correlation to genomic information.

Method for isolating nucleic acids and protein from a single sample

The present invention relates to a method of analysing a single sample for DNA, mRNA and protein expression. This is advantageous in correlating information relating to both mRNA and protein expression to genomic information.

The human genome is now more or less mapped and the gene number has been found to be much lower than first estimated. This shows that human complexity does not lie in the number of genes, but in how the genes are utilized and gene products combined and modified. Thus, the focus on studying the gene products and their interaction has become more significant.

It is known that only a fraction of the genes are transcribed in a single cell or cell type, and that one gene can give rise to many different proteins. This is partly due to alternative reading frames, alternative translational start/stop codons and alternative splicing of mRNA. It is also known that the relative quantitative levels of mRNA and protein do not always- or seldom correspond. One mRNA species can also give rise to different proteins because of post-translational modifications.

Thus an individual's genome (the collection of all genes), can be regarded as representing the genetic "possibilities" of an individual, while the transcriptome (the collection of mRNAs transcribed from the genome) represents what might possibly happen. The proteome, however (i.e. the total collection of all proteins translated from the transcriptome and modified post-translationally) represents reality, i.e. what really happened.

Accordingly, all three levels, DNA, RNA and

protein, give information which is valuable for different reasons. The DNA or genotype gives important information about genetic pre-dispositions and acquired mutations/local rearrangements. Both mRNA and protein profiles generate "molecular portraits" of a biological state/stage or disease, and may also be used for staging and monitoring of the disease development and treatment. As opposed to the DNA, both mRNA and protein profiles represent "snap shots" of the cell's biology, since they are continuously changing in response to the surrounding environment. Due to regulatory mechanisms acting both at the transcriptional, translational and post-translational levels, mRNA and protein levels do not always correlate. It is therefore crucial to study both mRNA and protein from the same sample.

Thus, as mentioned above, there is however not necessarily a 1:1 correlation between mRNA and protein levels. If protein levels and corresponding mRNA levels are compared, then for every protein for which the ratio of mRNA and protein is not 1:1 then this protein is subject to some form of interesting post transcriptional and/or post translational regulation. mRNA/protein ratios for specific genes are often shifted during disease conditions. To be able to study regulatory mechanisms and to unravel the reasons behind such a shift in mRNA/protein ratios, it is crucial to isolate mRNA and protein from the same sample. A novel and advantageous method for carrying out such isolation is presented herein.

Variation in protein or mRNA levels between individuals, for example in an individual with a disease state compared with a "normal" patient, are often due to single nucleotide polymorphisms (SNPs) or other forms of mutation or genetic predisposition in their genes (i.e. their DNA) or their regulatory regions. For this reason it is advantageous to be able to isolate DNA and protein, preferably DNA, RNA and protein, and especially

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DNA, mRNA and protein from the same sample to enable direct comparison. Such methods are also provided by the present invention.

In particular, it has now been found that nucleic acid and protein, and in particular DNA, RNA and protein can be isolated from a single sample in a form suitable for downstream manipulation and analysis, by a simple and easy to perform procedure. This isolation method involves binding nucleic acid and protein or preferably DNA, RNA and protein from the same sample on to distinct solid supports or areas of solid supports so that the isolated components can be analysed separately. Advantageously these methods can be combined with a further initial step involving the specific isolation of one or more particular cell types or populations (e.g. B-cells, T-cells, monocytes) from the sample before the isolation of nucleic acid and protein is carried out. In particular, it has been shown that DNA (e.g. genomic DNA), RNA (e.g. mRNA) and proteins may be isolated from a single sample with a high, documented purity. Different cell populations can also be isolated sequentially from the same sample and then each of these can be subjected to DNA, RNA and protein isolation.

In one aspect, the present invention thus provides a method of isolating nucleic acid and protein from the same sample, said method comprising contacting said sample with solid supports, whereby the nucleic acid and protein components in said sample become bound to distinct solid supports.

The nucleic acid to be isolated may be DNA, RNA or any naturally occurring modification thereof, and combinations thereof. Thus, the entire nucleic acid based component of a sample can be isolated in the method of the invention. In a preferred embodiment of the invention however DNA and/or RNA are separately isolated from the same sample onto distinct solid supports.

Thus, in preferred embodiments the present invention provides a method of isolating RNA and protein or DNA and protein from the same sample. In especially preferred embodiments the invention provides a method of isolating DNA, RNA and protein from the same sample.

In embodiments and steps of the methods of the invention, where the nucleic acid to be isolated from the sample is DNA, preferably the DNA is genomic DNA (gDNA) and may be in a single or double stranded or in any other form. In certain embodiments a specific DNA molecule, e.g comprising a gene encoding a specific protein may be isolated.

In embodiments and steps of the methods of the invention, where the nucleic acid to be isolated from the sample is RNA, total RNA may be isolated or a particular form or subset of RNA may be isolated. In preferred embodiments of the invention mRNA is isolated from the sample. In certain embodiments a specific RNA molecule, e.g. a specific mRNA encoding a specific protein may be isolated.

In the methods of the present invention described herein the steps of isolating nucleic acid and protein onto distinct solid phases involving contacting the sample with said solid phases can be carried out sequentially or simultaneously. Similarly, in embodiments where it is desired to isolate protein, DNA and RNA as separate components then these isolations can be carried out in the same step or in separate sequential steps. As will be described further below, in certain situations it may be desirable to carry out protein and nucleic acid (e.g. DNA and/or RNA) isolations on separate aliquots (or fractions) of the same sample (i.e. on a divided or split sample). In such cases the isolations may be carried out in parallel steps, e.g. simultaneously in parallel.

Preferably the required isolations are carried out in separate sequential steps which can, in turn, be

carried out in any order. Thus, although in the preferred embodiments of the invention DNA is isolated in a first step, followed by RNA in a second step and protein in a third step the different components can be isolated in any order.

The term "distinct solid phases" as used herein includes the use of different solid supports (i.e. supports with different binding and surface properties) in different steps of the method of the invention (for example in embodiments where sequential steps are used to bind the desired nucleic acid components and proteins), different supports added in same step (for example in embodiments where the isolation of the desired nucleic acid components and protein are carried out in the same step) and the use of supports with the same surface properties added in different steps, where binding of a particular nucleic acid or protein component is enabled by virtue of different incubation conditions. Furthermore, in some situations a particular solid support may bind different components (e.g. protein and nucleic acid, or cells and protein or nucleic acid etc.) when placed under different conditions e.g. different incubation conditions, reagents, environment etc. Such differences may also be exploited to bind different components to the same solid support, but in distinct or different steps, e.g. at different stages of the procedure, such that the desired components may be separately isolated. This term also includes different areas of a single solid support, which may for example be designed to have different surface properties such that particular nucleic acid and protein components bind to different areas or different ligands of the same support.

As mentioned above, it is an important aspect of the invention that nucleic acid and protein are analysed from the same sample.

"Same sample" as used herein refers to the

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isolation of the appropriate nucleic acid and protein components from a single, i.e. undivided sample. In this way the method of the invention is distinguished from prior art methods which may involve obtaining multiple samples or equivalent procedures, for example obtaining one initial sample, and straightaway at the outset dividing it into aliquots e.g. obtaining a sample and dividing it into aliquots on which a separate analysis of DNA, RNA and protein (one for each aliquot) is carried out and the results compared. Thus, an "undivided sample" may be viewed as a sample which is not divided straightaway e.g. immediately or initially after sampling, or at the outset before any isolation procedures are carried out. Thus, subsequent later division of the sample is not precluded, but it is not divided initially, or as a first step in the sample treatment process. Conveniently, as will be described further below, prior to carrying out nucleic acid and/or protein isolation procedures, it may be advantageous, or desirable first to isolate a particular desired population or sub-population of cells, or indeed to isolate all (or substantially all) the cells or any desired fraction thereof from the sample, and/or to lyse the cells in the sample or in any isolated population or fraction thereof. In such situations it may be desired or convenient to divide the single (i.e. same) sample after the cell isolation or cell lysis steps have been carried out.

Thus, division of the sample after an initial, or first, isolation step, or after two or more isolation steps is contemplated. This may be desirable if, for example, if it is desired to change conditions (e.g. ionic strength, salt concentration etc), e.g. to isolate different components. Thus, for example, protein and nucleic acid components (i.e. DNA and/or RNA) may be isolated from different aliquots of the sample after such a sample splitting or division step. However, in

one advantageous embodiment of the invention, the method is carried out using a single sample which is not divided at any stage or at any time. The fact that the multiple analysis of DNA, RNA and protein can be carried out on a single, undivided sample using the methods of the present invention is clearly advantageous and will allow for a more direct and accurate comparison between the various nucleic acid and protein components of the sample.

The samples suitable for use in the methods of the present invention may be any material containing nucleic acid and protein, including for example foods and allied products, clinical and environmental samples. However, the sample will generally be a biological sample, which may contain any viral or cellular material, including all prokaryotic or eukaryotic cells, viruses, bacteriophages, mycoplasmas, protoplasts and organelles. Such biological material may thus comprise all types of mammalian and non-mammalian animal cells, plant cells, algae including blue-green algae, fungi, bacteria, protozoa etc. Representative samples thus include whole blood and blood-derived products such as plasma, serum and buffy coat, bone marrow and samples obtained from other haematopoetic tissues, urine, faeces, cerebrospinal fluid or any other body fluids, tissues (for example solid tissues), cell cultures, cell suspensions etc. Preferably the sample is a live (e.g. a non-fixed sample), i.e. comprises viable cells, or at least cells which have not been treated in any way.

The sample may be freshly obtained, or stored or treated in any desired or convenient way, for example by dilution, or adding buffer, or other solutions or solvents, enzyme-containing solutions etc.), as long as the integrity of the nucleic acids and proteins, or the cells or other entities containing such nucleic acids and proteins within the original sample are maintained, i.e. are not substantially degraded. Nucleic acids and

protein may be denatured but not degraded.

It is further advantageous that a crude sample can be taken directly from its source, e.g. directly from a patient or from the environment (e.g. a direct clinical or environmental sample) and its protein and nucleic acid analysed using the methods of the invention.

The nucleic acid- and protein-containing sample may, generally speaking, simply be contacted with the appropriate solid supports under conditions whereby the nucleic acids or proteins as appropriate will become bound to the solid support. If necessary, e.g. if the nucleic acid and protein to be isolated is not available for binding within the initial sample e.g. is contained within a biological particle as described above e.g. a viral coat or a cell membrane or wall, the initial binding step may be preceded by one or more separate steps to free the nucleic acid and protein components, e.g. by disrupting structural components such as cell walls or to achieve lysis. Procedures for achieving this are well known in the art. Thus, for example, although some cells e.g. blood cells, may be lysed by reagents such as detergent alone, other cells, e.g. plant or fungal cells or solid animal tissues may require more vigorous treatment such as, for example, grinding in liquid nitrogen, heating in the presence of detergent, alkaline lysis in the presence of detergent. The main requirement is that procedures for freeing the nucleic acid and protein are chosen such that the particular nucleic acid and protein species which are to be isolated in the methods of the invention remain sufficiently intact, e.g. are not substantially degraded.

Preferably samples are 10 μl to 100 ml in size, preferably from 200 μl to 10 ml. The method of the invention may be used for small samples, e.g. less than 1 ml or for larger samples e.g. at least 2 ml, e.g. more than 5 ml or 10 ml or 50 ml. A major advantage of the

present invention is that small sample volumes may be used, and in particular that protein may be isolated from such small sample volumes following, or concurrently with, DNA and/or RNA isolation. This feature makes the method of the invention particularly suitable for automation. Advantageously the sample volume as subjected to the nucleic acid/protein isolation procedures is 1 ml or less, e.g. 10 to 800 μl, e.g. 20 to 500 μl, or 50 to 200 μl. For example, the method may be performed on a sample containing 1 x 10^4 to 1 x 10^6 cells, preferably 1-10 x 10^5 cells. The method described is highly scalable and can be used to detect DNA/RNA/protein in 1,5,10,20,200 or 2000 or more cells.

As mentioned above an optional and preferred initial step once the sample has been obtained is to enrich the sample for particular populations, e.g. particular populations of cells or organelles for which the nucleic acid and protein is to be analysed.

Methods of enrichment are well known and documented in the art. Preferred methods involve the use of a solid phase, i.e. the binding of the population(s) in question to a solid phase and exemplary methods will be discussed in more detail below. In such methods, one or more steps of positive and/or negative solid phase selection can be used, as appropriate, depending on the desired purity of sub-population required. Such techniques are well known and described in the art. Briefly, in a negative selection step, the cells/populations which bind to the solid phases are removed from the sample to be analysed, whereas in a positive selection step the cells/populations which bind to the solid phases are retained in the sample to be analysed.

Exemplary and preferred antibodies for use in the isolation of specific cell populations are CD15 and CD45 antibodies (specific for leukocytes), CD14 antibodies (specific for monocytes), CD2, CD3, CD4 and CD8 antibodies (specific for T-cells), CD19 antibodies

(specific for B-cells) and Anti Ber EP4 antibodies (specific for epithelial cells and in particular circulating carcinoma cells). Other antigens may also be used as the basis for detection by binding to specific antibodies (or antibody fragments, derivatives etc.), as indeed may any other cell surface molecule which may bind specifically to a binding partner or ligand.

If desired, prior to cell lysis, cell surface proteins may be subjected to an *in vitro* modification procedure for example chemical modification, e.g. biotinylation or radiolabelling, for example to introduce a label or reporter or marker group etc., prior to nucleic acid and protein isolation steps. Such modified surface proteins may then be isolated by a specific or adapted isolation procedure. Thus, for example, a biotinylated surface (e.g. membrane) protein may be isolated by binding to a solid support carrying streptavidin or avidin.

Other methods of isolating a desired cell population may also be used, as desired, for example Laser Capture Microdissection.

Where the method of the invention involves such a preliminary cell/organelle etc. isolation step, it will be appreciated that larger sample volumes may be used, e.g. 1-50 ml, e.g. 2 to 20 ml or 5-10 ml, for example of a blood or other clinical sample.

Sample preparation is one of the bottlenecks in proteomics in that due to the variation in abundancy and lack of specific amplification technology for proteins, it is advantageous to reduce sample complexity in order to increase resolution. Thus the optional step of the present invention by which a particular sample can be treated to specifically enrich for a particular specific population or populations of cells, organelles, etc., and thereby proteins is extremely advantageous. Thus, it can be seen that the methods of the invention allow

the enrichment of a particular population of cells and the analysis of DNA, RNA and protein therefrom using the same single sample. The fact that this sample can be taken directly from a patient is a further advantage.

The feature of specifically or selectively isolating a desired population may be applied advantageously to comparing multiple (e.g. 2 or more, e.g. 2 to 10 or 2 to 6) populations from the same sample. Thus, for example, by the use of different appropriate binding partners specific for particular desired populations and coupled to different solid supports, different populations may be isolated, or separated from the same sample.

Thus, a preferred embodiment of the invention provides a method of isolating DNA, RNA, and protein from the same sample, said method comprising the following steps:

a) isolating from said sample the populations from which the nucleic acid and protein is to be isolated and preferably isolating one or more specific populations;

b) lysis of the populations, e.g. cell populations, to obtain a sample containing nucleic acid and protein in a free form available for binding to a solid support;

c) contacting sample with an appropriate solid phase under conditions whereby DNA, preferably genomic DNA, becomes bound to the solid support;

d) separation of this solid phase from the remainder of the sample;

e) contacting said remainder of the sample with an appropriate solid phase under conditions whereby RNA, preferably mRNA, becomes bound to the solid support;

f) separation of this solid phase from the remainder of the sample;

g) contacting said remainder of the sample with an appropriate solid phase under conditions whereby a

specific protein or population of proteins becomes bound to the solid support;

h) separation of this solid phase from the remainder of the sample.

The method outlined above is a preferred embodiment of the invention and clearly these specific steps can be altered depending on the overall analysis it is wished to achieve to form further embodiments of the invention. In particular, some of the above discussed steps are optional and need not be included. For example, steps a) and b) are optional and whether or not they are carried out depends on the nature of the sample which is initially obtained. If this sample does not contain the nucleic acid and protein which it is desired to analyse in a free and released form, for example if the nucleic acid and protein is contained within cells or tissues or other biological packages, then generally steps a) and b) will be carried out.

In addition, the above described methods describes the sequential isolation of DNA, RNA and protein in separate steps. The isolation of the whole nucleic acid component as one fraction and protein, and the isolation of DNA and protein, and RNA and protein, are also within the scope of the invention and the steps of the method can be adapted as appropriate to fulfil this purpose. For example, if it is desired to isolate the total nucleic acid and protein, step c) can be replaced by a step which involves contacting the sample with an appropriate solid phase under conditions whereby the total nucleic acid component, e.g. DNA and RNA, becomes bound to the solid support. Step e) would then be omitted. If it is desired to isolate DNA and protein then the RNA steps e) and f) can be omitted. Vice versa, if it is desired to isolate RNA and protein then the DNA steps c) and d) can be omitted.

In addition, the nucleic acid and protein isolation steps of the above described methods can be carried out

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in any order. Thus, although in a preferred embodiment the order is DNA followed by RNA followed by protein (if all three components are to be analysed), the steps may be carried in any order, e.g. the RNA or the protein may be isolated first. In methods where nucleic acid and protein are to be isolated, or DNA and protein, or RNA and protein are to be isolated, although in preferred embodiments the nucleic acid component is isolated first, the protein component may be isolated first.

In addition, in a further embodiment of the invention the desired nucleic acid and protein components can be isolated simultaneously from the same sample in the same step. This may be achieved in any appropriate manner but will generally be carried out by contacting the sample simultaneously with distinct solid supports which have different surface properties and are able to bind specifically to DNA (e.g. genomic DNA), RNA (e.g. mRNA), all nucleic acid species, or protein, as appropriate. Appropriate solid supports which have specificity for the various nucleic acid and protein components which it is desired to isolate from a particular sample are described herein and any of these may be used. These distinct solid supports with different surface properties may be provided on different areas of the same solid support, or may be provided as separate solid supports which are brought into contact with the sample in the same step. These separate solid supports may conveniently be provided for example as separate dipsticks with different appropriate surface properties. Once the desired nucleic acid and protein components have been isolated in such a single step then they can be used and analysed in the same way as if isolated in separate steps.

Once the desired nucleic acid and protein components have been separated onto solid phases these can be subjected to further analysis, e.g. by conventional methods. Again appropriate methods of

further analysis will be discussed in more detail below.

The binding of nucleic acid to a solid support is generally independent of its sequence and the specificity of binding is controlled by varying the properties of the solid support and the conditions under which binding of the nucleic acid is induced. As described above the whole nucleic acid component of a sample can be induced to bind to a solid phase or the appropriate choice of binding conditions and/or nature of the solid support can be effected so that DNA or RNA can be selectively bound to a solid phase, thereby enabling a selective DNA or RNA isolation procedure, which may be carried out simultaneously or preferably sequentially. Appropriate methods and conditions whereby all nucleic acid may be bound or DNA and RNA are selectively bound to a solid phase are well known and documented in the art and any of these methods can be used in the methods of the present invention. Some appropriate and preferred methods are discussed briefly here and in more detail below where alternative methods are also discussed.

Conveniently, where DNA and RNA are isolated in two separate steps the sequential separation may take place using two different solid phases, for example solid supports which can differentiate between DNA and RNA. Thus, such embodiments may comprise carrying out a first separation step to isolate DNA. A further solid support can then be added to the sample to capture the RNA remaining in the sample, either by using a solid support that can bind the RNA, e.g. a silica based support (described in more detail below), or a solid support that can capture specific RNA molecules (e.g. by carrying a complementary nucleic acid probe), or a solid support which can capture a subset of RNA molecules e.g. polyadenylated RNA, using for example a poly-dT or poly-dU based oligonucleotide capture probe. In this way it is possible rapidly to isolate and separate DNA and RNA

or subsets of both from the same sample.

In a representative procedure, the sample is lysed in the presence of detergent and the DNA is allowed to bind to a solid support, whereupon the DNA may readily be separated from the sample by removal of the support. Such procedures are described in more detail in WO96/18731 (and in particular Example 12) the disclosure of which is incorporated herein by reference. The procedure of specific DNA capture described in WO96/18731 is also known as the DNA DIRECT method and can be made selective for genomic DNA. Appropriate conditions and methods for carrying out such separations are also described in the Dynabeads DNA DIRECT kit (which is commercially available from Dynal Biotech AS, Oslo, Norway). In methods of the invention where it is desired to isolate DNA and in particular genomic DNA, the DNA DIRECT method of separation is one preferred embodiment. Alternatively, other solid supports having surface chemistries or surface properties supporting DNA binding may be used, for example a support (preferably magnetic beads) carrying surface carboxyl groups.

The RNA may then be isolated from the remaining sample. This can be by a solid phase based system as described above wherein a different solid phase is added to the sample to which RNA becomes specifically bound. Alternatively the same solid phase may be used and the RNA made to bind thereto by inducing an appropriate change in the sample conditions.

Preferably in embodiments where the RNA is isolated by binding to a solid phase, and in the representative procedure as described here, the RNA is mRNA and the surface of the solid phase used has been engineered to carry a capture probe specific for mRNA, e.g. the support has attached to its surface dT oligonucleotides or dU oligonucleotides, e.g. Dynabeads oligo (dT), Dynabeads cDNA release (available from Dynal Biotech ASA) or Dynabeads T_7-oligo(dT) (a modified construct) in

which a T7 promoter is included following the principles set out in Eberwine *et. al.*, Biotechniques (1996), 20(4): 584-91 and US-A-5,514,575). The oligonucleotide attached to the "T7 beads" is a combined oligo dT and T7 promoter sequence of the structure 5'AAAAAA-T7sequence(39nt)-dT(20-25)'. VN is added to the 3'end of the oligonucleotide for Dynabeads T_7-oligo(dT).

Appropriate conditions and methods for carrying out such RNA separations are also described in the product inserts provided with the Dynabeads commercial product. In particular Dynabeads oligo (dT)(which are commercially available from Dynal Biotech AS, Oslo, Norway) are sold with an mRNA DIRECT kit protocol which describes how mRNA separation may be carried out. In methods of the invention where it is desired to isolate RNA and in particular mRNA, the mRNA DIRECT method of separation is preferred.

The choice of oligo dT or dU beads to be used depends on the downstream applications of the isolated mRNA (see further discussion below).

Support bound oligo dU may be used for isolation of mRNA as discussed in WO 00/58329 of Dynal Biotech ASA. This is analogous to the use of oligo dT, but with the added advantage that the "U's" provide a site for cleavage by a glycosylase enzyme.

In preferred embodiments, the oligo dT/dU (including the T7 modification) constructs additionally include a further nucleotide sequence (designated "VN", wherein "V" may be any nucleotide other than T (e.g. A, G or C) and "N" may be any nucleotide, including T. This additional "VN" sequence serves the advantageous purpose of assisting in the targeting or positioning of binding of the oligo dU/dT probe sequence to the nucleic acid; the positioning of probe binding is advantageously at the border between the poly A tail and the coding sequence of the mRNA.

In the step of the method where protein is isolated, this is conveniently carried out by any appropriate method using a solid phase with appropriate surface properties. Appropriate methods will depend on the type of proteins it is desired to analyse. In one embodiment specific proteins may be isolated using a solid support, which has an appropriate binding partner/ligand attached to its surface, e.g. an antibody to the particular protein which it is desired to isolate. A particularly preferred antibody to be used in this regard is anti-CK19 (a support, e.g. beads can be coated with anti-CK19 and can be used to isolate specific problems. Anti-CK19 antibodies are commercially available). This thus applies well known principles of affinity separation of proteins, as widely described in the prior art. Any such standard and well known methods may be used or adapted for the present invention.

Alternatively a solid phase with more general surface binding properties can be selected, e.g. a solid phase which has surface chemistry which effects classical chromatography interactions such as ion exchange (including both anion exchange and cation exchange), reverse phase interactions or hydrophobic interactions. Such surfaces are conveniently provided on magnetic beads. The use of these more general surfaces conveniently allows the fractionation of proteins in the sample into subsets depending on the structure and properties (charge, hydrophobicity) etc. of the proteins present.

Such general solid phase surfaces can be prepared using conventional techniques which are standard and well documented in the art of column chromatography. Furthermore, supports (e.g. particles or beads) have been widely described in the art for fluidised bed separation technology, and have properties e.g. ion exchange, designed for protein separation (e.g. US-A-5,084,169, US-A-5,079,155 and US-A-473,231). Magnetic particulate solid supports having such surface chemistry

are not known in the art and thus form a further embodiment of the invention. A solid support suitable for protein isolation comprises on its surface positively charged amine groups, which are believed to bind negatively charged proteins. The bound proteins may be eluted under conditions of high salt and low pH. Amine beads are available from Dynal Biotech AS as Dynabeads M-270 amine.

In embodiments of the invention where the protein isolation step is carried out after the various nucleic acid isolation steps, the remaining sample may be subjected to any convenient treatment before the protein is isolated using the appropriate solid support. For example, if it is desired to fractionate the proteins present in the sample, for example using the solid supports with more general surface binding properties as described above, this step can be preceded by a step whereby the protein sample is subdivided into different fractions, e.g. subdivided into one or more of membrane fraction, cytosolic fraction and nuclear fraction. Methods for carrying out such subdivision are standard and well documented in the art of protein analysis.

In all the embodiments of the invention described herein, whilst any solid support formats may be used, as well known in the art and widely described in the literature, preferred solid supports are particles and more preferably magnetic particles. Magnetic particles (beads) are well known in the art and widely commercially available. Different sizes of beads are available, or may be prepared, and different applications (e.g. different DNA, mRNA, protein etc. separations) may be optimised on beads of different sizes, e.g. 4.5 μm, 2.8 μm, 2.7 μm, 1.0 μm in diameter.

The methods of the invention are advantageously amenable to automation, particularly if particles, and especially, magnetic particles are used as the support. In a particularly favoured embodiment of the invention,

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the nucleic acid and protein isolation method is performed using an automated system for handling of the solid support during the cell lysis, nucleic acid binding, protein binding and, optionally, washing steps. Thus the isolated support- bound cells may be transferred to such an apparatus, washed if desired, and lysed; the nucleic acid (i.e. whole nucleic acid component, DNA or RNA) or protein, as appropriate depending on the particular step of the method and the particular support and conditions used may bind to the support, and the bound nucleic acid or protein may readily be washed, using such an apparatus. Furthermore, such an apparatus may also be used to handle the support during the cell/population isolation stage. Thus, it can be seen that the option is there to automate all of the steps of the method if desired.

Particular mention may be made in this regard of the Bead Retriever™, available from Dynal Biotech ASA, Norway. The apparatus has a system for ready and efficient transfer of the support (carrying cells or nucleic acid or protein) from one well to another. Other automated robotic workstations which may be used include the BioMek 2000 with the Dynal MPC-auto96 magnet station and the TECAN GENESIS RSP with its own built in magnet station. Both the BioMek and the TECAN GENESIS RSP are liquid handling robots.

The present invention provides the first description of a technology which can isolate specific cell populations, DNA, mRNA and proteins from the same sample in an automated way, e.g. by carrying out all the steps using a solid phase and in particular a magnetic solid phase. Other methods such as laser microdissection are much slower than the methods described herein, are not automatable and require fixed, sectioned material.

The various reactants and components required to perform the methods of the invention may conveniently be

supplied in kit form. Such kits represent a further aspect of the invention.

At its simplest, this aspect of the invention provides a kit for isolating nucleic acid and protein from the same sample comprising:

(a) a solid support suitable for binding nucleic acid components; and

(b) a solid support suitable for binding proteins.

In preferred embodiments the supports of (a) comprise supports which are selective for binding to DNA, or supports which are selective for binding to RNA, or both types of support.

Further optional components of the kit include (c) solid supports suitable for isolation of specific cell populations, (d) means for lysing said cells.

The various components (a), (b), (c) and (d) may be as described and discussed above in relation to the methods of the invention.

A further optional component is (e), means for detecting the nucleic acid and/or protein. As discussed above, for detecting nucleic acids such means may include appropriate probe or primer oligonucleotide sequences for use in hybridisation and/or amplification-based detection techniques. For detecting proteins such means include appropriate antibodies.

Optionally further included in such a kit may be buffers, salts, polymers, enzymes etc.

Once one or more nucleic acid and protein components have been isolated onto a solid phase, these can then be analysed or used in any appropriate way. For example, the methods of the invention can be used in any application which involves analysis or comparison of the genome (DNA), transcriptome (RNA) and proteome (expressed protein), or in any analysis where it is desired to compare a particular DNA and/or RNA and the expressed protein, or more preferably a particular DNA, RNA and protein. Such methods are very useful for gene

expression studies as they allow analysis and comparison of mRNA levels with protein levels. In addition, the ability to analyse and compare mRNA and protein levels and profiles of samples (e.g. from different cells and tissues) gives a snapshot of the cell's biology. Furthermore, being able to study the mRNA/protein ratio gives an indication of which genes are subject to post-transcriptional regulation. Such ratios are often shifted during disease states, thereby allowing an indication as to which genes may be involved in disease and allowing a study of the possible regulatory mechanisms behind such a shift in mRNA/protein ratio.

The methods of the invention are also useful for example in determining the molecular changes and mechanisms involved in disease states, e.g. determining which genes are mutated, or which proteins are over or under expressed or incorrectly processed. Studying the DNA composition of a sample can provide information on genetic predispositions and acquired mutations, whereas the mRNA and protein profiles generate "molecular portraits" of a cell's biological state or the stage of disease and may also be used for the staging and monitoring of the disease development and treatment. As mentioned above, the methods described are particularly advantageous for such analysis, as they allow the comparison of DNA, RNA and protein from the same sample which is important to allow a more accurate and direct comparison.

The method of the invention also has use as a method for isolating nucleic acid and protein fractions from a single sample.

More specifically, in the case of the DNA, once isolated this can be used in nucleic acid based detection procedures, e.g. in genotyping, SNP analysis, sequencing reactions etc.

Advantageously the bound nucleic acid need not be eluted or removed from the support prior to carrying out

the detection step, although this may be performed if desired. Whether or not the nucleic acid is eluted may also depend on the particular method which was used in the nucleic acid binding step. Thus certain nucleic acid-binding procedures will bind the nucleic acid more tightly than others. In the case of DNA-binding using detergents (e.g. by DNA Direct) for example, the nucleic acid will elute from the solid support when an elution buffer or other appropriate medium is introduced. Nucleic acid bound by means of a precipitant such as alcohol or a chaotrope will remain more tightly bound and may not elute when placed in a buffer medium, and may require heating to be eluted. A preferred use of the DNA is in solid phase SNP analysis, which may optionally be fully automated. Such SNP analysis can be carried out using any appropriate technique. For example, PCR can be carried out using one biotinylated primer, followed by single stranded DNA template generation on streptavidin beads, followed by probe annealing, ddN incorporation and a labelling reaction.

Thus, the support-bound nucleic acid may be used directly in a nucleic acid based detection procedure, especially if the support is particulate, simply by resuspending the support in, or adding to the support, a medium appropriate for the detection step. Either the nucleic acid may elute into the medium, or as mentioned above, it is not necessary for it to elute.

A number of different techniques for detecting nucleic acids are known and described in the literature and any of these may be used. Conveniently, nucleic acid may be detected by optical methods, for example by measuring or determining optical density (OD). Alternatively, the nucleic acid may be detected by hybridisation to a probe (which may be a labelled probe for detection) and very many such hybridisation protocols have been described (see e.g. Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd Ed.

Cold Spring Harbor Press, Cold Spring Harbor, NY). Most commonly, the detection will involve an *in situ* hybridisation step, and/or an in *vitro* amplification step using any of the methods described in the literature for this, for example probe annealing/hybridization followed by ddN incorporation (which may be labelled for detection). Thus, techniques such as LAR, 3SR and the Q-beta-replicase system may be used. However, PCR and its various modifications e.g. the use of nested primers, or the use of one or more biotinylated primers will generally be the method of choice (see e.g. Abramson and Myers, 1993, Current Opinion in Biotechnology, 4: 41-47 for a review of nucleic acid amplification technologies).

Other detection methods may be based on a sequencing approach, for example, the minisequencing approach as described by Syvänen and Söderlund, 1990, Genomics, 8: 684-692.

In amplification techniques such as PCR, the heating required in the first step to melt the DNA duplex may release the bound DNA from the support. Thus, in the case of a subsequent detection step, such as PCR, the support bound nucleic acid may be added directly to the reaction mix, and the nucleic acid will elute in the first step of the detection process. The entire isolated support bound nucleic acid sample obtained according to the invention may be used in the detection step, or an aliquot.

The results of the PCR or other detection step may be detected or visualised by many means, which are described in the art. For example the PCR or other amplification products may be run on an electrophoresis gel e.g. an ethidium bromide stained agarose gel using known techniques.

The amplified nucleic acid may also be detected, or the result confirmed, by sequencing, using any of the many different sequencing technologies which are now

available, e.g. standard sequencing, solid phase sequencing, cyclic sequencing, automatic sequencing and minisequencing.

If mRNA is isolated, for example using oligo(dT)beads, or oligo(dU) beads (e.g. using Dynabeads oligo(dT), Dynabeads cDNA release or Dynabeads T7 oligo(dT) as described herein) this mRNA can be used or analysed in any appropriate way. The mRNA can for example be analysed using appropriate conventional techniques, e.g. by Northern blotting, or can be used to generate cDNA which may then be subjected to any appropriate DNA based analysis such as those described above.

It can be seen that mRNA and cDNA libraries from specific samples, e.g. specific populations or sub-populations of cells, viruses, etc can be produced using the methods of the invention. This application can either be carried out while the mRNA is still on the solid phase, i.e. resulting in solid phase cDNA libraries, or can be carried out after the mRNA has been eluted. The mRNA may also be used as a substrate for RT-PCR. Solid phase cDNA libraries can be used in any appropriate application and can be re-used, for example as template in different PCRs (or other amplifications), thereby enabling PCR (or other) analysis of multiple transcripts, e.g. from single isolated cells. For example, it can be seen from Figure 2 that a solid phase c-DNA library has been re-used in multiple PCR experiments to detect different transcripts in cultured carcinoma cells.

If Dynabeads cDNA release beads (or commercial or generic equivalents) are used to isolate the mRNA, as described above, these beads have oligo(dU)VN coupled to their surface. These oligo(dU)VN molecules bind to the mRNA and can be used to prime one strand of solid phase cDNA synthesis. The solid phase cDNA can then be released enzymatically by UNG treatment and used, e.g.

for probe generation for gene expression profiling.

If Dynabeads T7 oligo(dT) beads (or commercial or generic equivalents) are used to isolate the mRNA, as described above, these beads have coupled to their surface a T7 promoter 5-prime to the oligo(dT)VN. After double stranded solid phase cDNA synthesis (first strand synthesis carried out using the oligo (dT) as a primer and second strand synthesis initiated with random hexamers), the T7 promoter (which is a 39 nucleotide sequence specific for the enzyme T7 polymerase, which recognises this sequence in double stranded DNA) can be used to in vitro transcribe antisense RNA and thereby amplify mRNA from small samples for further analysis, or e.g. for probe generation for gene expression profiling, e.g. for microarray analysis. Such probes are conveniently generated by carrying out reverse transcription of the antisense RNA (with random hexamers) to give cDNA which can be labelled and used to probe microarrays. These cDNAs can also be used in a second round of amplification using T7 beads.

Although microarrays are a powerful high throughput tool, they are not very sensitive - at least 1-2 μg of mRNA is required per array. This is often impossible to achieve, e.g. by single cell analysis. Thus, in vitro transcription methods are required to amplify the RNA. For this reason the ability to use the Dynabeads T7 oligo(dT) beads to amplify RNA is advantageous in microarray applications and any other applications where amplification of mRNA is advantageous.

Once isolated by binding to a solid phase using the methods of the invention, subsets of proteins (e.g. those isolated using the supports with more generalised chromatography type surfaces) or individual proteins (e.g. those isolated using supports carrying specific ligands) can be subjected to direct analysis whilst still on the bead (e.g. Bead ELISA) or can be subjected to elution from the solid support followed by analysis

by any appropriate conventional technique such as SDS-PAGE/Western blotting, immunoprecipitation, etc.

As discussed extensively above, one of the main advantages of the method of the invention is that one or more of DNA, RNA and protein can be isolated from the same sample ready for analysis. Thus, results obtained by analysing the DNA, RNA and protein from the same sample using any of the individual techniques described above can be compared as appropriate depending on the aim of the analysis.

If the initial sample contains cells or other biological particles which contain the nucleic acid and protein which is to be analysed, then generally the cells/particles to be analysed will need to be separated from the remainder of the sample. Such separation can be carried out by any of the methods which are standard and conventional in the art, such as sedimentation or centrifugation or cell sorting procedures. Alternatively affinity based separation systems may be used and these are described in more detail below. Such affinity based systems are especially preferred if it is desired to analyse the nucleic acid and protein from a sub-population of the cells or particles contained within the initial sample. Where such affinity-based systems are used, the separation is generally carried out by binding the cells or particles to a solid support carrying an appropriate binding partner.

Affinity-based separation or isolation systems for desired target cells are well known in the art, and rely on the specificity of a binding partner, specific or selective for the target cell, to achieve selective isolation of the cell. Such a system is employed as an optional step according to the present invention in order to achieve selective isolation of one or more particular populations of cells or other biological "particles" from the sample before it is treated in order to free the nucleic acid and protein and provide

the nucleic acid and protein containing sample. Thus, a suitable binding partner for use in this regard may be one or more moieties having a binding affinity for the desired cell or other population(s).

The binding partner may be any molecule or moiety capable of binding to the required populations of cells or other particles, but conveniently will be a protein, polypeptide or peptide. Other moieties or molecules of a different chemical nature, for example carbohydrates or small organic molecules may however also be used. Nucleic acid binding partners e.g. aptamers may also be used.

The binding partner may bind to molecules or structures present on the surface of the cells or other particles, for example to cell surface antigens which are expressed (e.g. specifically) on the surface (e.g. cell specific Dynabeads such as Dynabeads Epithelial Enrich, anti CD14, anti CD3, anti CD4, anti CD8, anti CD19). Alternatively, the binding partner may be any moiety binding to a cell surface expressed protein e.g. a cell surface receptor.

The binding partner may, for example, conveniently be an antibody specific for a particular surface antigen. Antibody fragments and derivatives may also be used, according to techniques well known in the art. Methods for preparing such fragments or derivatives are well known in the art and widely described in the literature.

Thus, using knowledge of the molecules or other entities present on the surface of the desired population(s), a binding partner, or combination or mixture of binding partners, may be selected to achieve a desired separation or isolation of cell or other populations from the sample. Advantageously, all or substantially all (i.e. close to all) of the desired cells or other particles present in the sample may be separated. The separation achievable may be dependent

not only on the binding partner(s) selected, but also on the nature of the sample, binding conditions etc. Also, biological systems are by their nature variable, and 100% separation may not always be achieved, and, indeed, is not necessary according to the present invention; as in any biological system, some tolerance must be allowed for. However, in preferred embodiments of the invention at least 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the desired populations present in the sample may be separated.

Where more than one different type of binding partner is used they may be attached to the same or different solid supports. Such a system using different solid supports, is applicable particularly in the case of a particulate support such as beads. Thus, different binding partners may be attached to different beads.

In embodiments where more than one different type of binding partner is used, appropriate amounts or ratios at which the different types of binding partner may be used will be readily determined by a person skilled in the art.

As mentioned above, cell separation techniques based on solid phase affinity binding (e.g. immunomagnetic separation (IMS)) are well known in the art and conditions to achieve this may readily be determined by the skilled worker in this field. IMS is a preferred and advantageous cell separation procedure to use, since it enables the isolation of specific cell populations with high purity, for example more than 99% purity from whole blood, as analysed by flow cytometry and molecular techniques. Thus, in the embodiments where a separate affinity isolation step is used, for example a solid support carrying appropriate binding partner(s) may be brought into contact with the sample. A particulate solid support may, for example, be added to the sample contained (e.g. suspended) in an appropriate medium (e.g. a buffer). The support may

then be left in contact with the sample (e.g. incubated) for a length of time to enable binding to the cells or other particles to occur. Time/temperature conditions during the step are not critical, and the sample-support mixture may be incubated at e.g. 4 to 20°C for 10 minutes to 2 hours e.g. 20-45 minutes. As regards other conditions, these may be determined appropriately having regard to the cells under investigation and the isolation methods used, according to principles and procedures known in the art. The cell isolation procedure however focuses on minimizing the probability of affecting the physiological state of the cells and thus enabling downstream molecular characterisation and profiling. Laser capture microdissection can also be used to isolate cell populations for the downstream applications described.

Following cell binding, or any other steps carried out to separate a desired population of cells from which nucleic acid and protein are to be analysed, the isolated or support-bound cells are lysed to release their nucleic acid and protein. Methods of cell lysis are well known in the art and widely described in the literature and any of the known methods may be used. Any of the following methods could, for example, be used: detergent lysis using e.g. SDS, LiDS, Triton-X-100, urea or sarkosyl in appropriate buffers; the use of chaotropes such as Guanidium hydrochloride (GHCl), Guanidium thiocyanate (GTC), sodium iodide (NaI), perchlorate etc; mechanical disruption, such as by a French press, sonication, grinding with glass beads, alumina or in liquid nitrogen; enzymatic lysis, for example using lysozyme, pronases or cellulases or any of the other lysis enzymes commercially available; lysis of cells by bacteriophage or virus infection; freeze drying; osmotic shock; microwave treatment; temperature treatment; e.g. by heating or boiling, or freezing, e.g. in dry ice or liquid nitrogen, and thawing; alkaline

lysis. As mentioned above, all such methods are standard lysis techniques and are well known in the art, and any such method or combination of methods may be used. The only requirement is that an appropriate method is chosen such that the species of nucleic acid and protein to be analysed in the subsequent procedure remain substantially intact, i.e. substantially non-degraded.

The use of agents such as solvents, alcohols and chaotropes in isolation methods is sometimes disadvantageous. The present invention affords the advantage that the use of such agents may be avoided. Thus, whilst lysis methods such as those mentioned above using such agents may be employed, in advantageous embodiments of the invention the use of such agents is avoided.

Conveniently, lysis may be achieved according to the present invention by using detergents. An exemplary suitable lysis agent thus includes a detergent such as SDS or another alkali metal alkylsulphate salt, e.g. LiDS, or Sarkosyl or combinations thereof. The lysis agents may be supplied in simple aqueous solution, or they may be included in a buffer solution, to form a so-called "lysis buffer". Any suitable buffer may be used, including for example Tris, Bicine, Tricine and phosphate buffers. Alternatively the lysis agents may be added separately. Salts, for example LiCl and NaCl, may also be included in or added to the lysis buffers. In particular, LiCl is preferred when LiDS is used and NaCl is preferred when SDS is used.

Suitable concentrations and amounts of lysis agents will vary according to the precise system etc. and may be appropriately determined, but concentrations of e.g. 2M to 7M chaotropes such as GTC GHCl, NaI or perchlorate may be used, 0.1M to 1M alkaline agents such as NaOH, and 0.1 to 50% (w/v) e.g. 0.5 to 15% detergent.

To carry out the method of the invention, the

support-bound or otherwise isolated cells, may conveniently be removed or separated from the remainder of the sample, thereby concentrating or enriching the cells. To facilitate subsequent steps, it may be desirable, prior to the lysis step, to dilute the isolated cells, e.g. in an appropriate buffer or other medium. If desired the cells may further be treated, e.g. by heating or mixing (e.g. vortexing). A dilution step may be advantageous to prevent agglomeration/aggregation of a particulate support such as beads. Lysis then may conveniently be achieved by adding an appropriate lysis buffer containing the desired lysis agents or by subjecting the isolated cells to the desired lysis conditions. For example, in the case of simply adding a lysis buffer containing appropriate lysis agents, the isolated cells may simply be incubated in the presence of the lysis buffer for a suitable interval to allow lysis to take place. Different incubation conditions may be appropriate for different lysis systems, and are known in the art. For example for a detergent containing lysis buffer, incubation may take place at room temperature or at higher temperatures e.g. 37°C, 50°C or 65°C. Likewise, time of incubation may be varied from a few minutes e.g. 5 or 10 minutes to hours, e.g. 20 to 40 minutes or 1 to 2 hours. For enzymatic lysis, longer treatment times may be required, e.g. overnight.

Following the obtaining of a sample containing free or released nucleic acid and protein (e.g. following lysis, if required), the steps to be taken will depend on the particular type of analysis it is desired to carry out. For example, in the methods of the invention, solid phases can be used to isolate nucleic acid and protein, DNA and protein, RNA and protein or most preferably DNA, RNA and protein from the same sample. Which entity is isolated depends on the nature of the solid support added and/or the conditions to

which the sample and nucleic acid is exposed.

Appropriate conditions and solid supports whereby nucleic acids become bound to the solid support are well known and documented in the art and any of these methods may be used in the embodiments and steps of the invention wherein it is desired that all the released nucleic acids (i.e. DNA and RNA) in a sample are bound to a solid support. Conveniently, the nucleic acid is bound non-specifically to the support i.e. independently of sequence. Thus, for example the released nucleic acid may be precipitated onto the support using any of the known precipitants for nucleic acid, e.g. alcohols, alcohol/salt combinations, polyethylene glycols (PEGs) etc. Precipitation of nucleic acids onto beads in this manner is described for example in WO 91/12079. Thus, salt may be added to the support and released nucleic acid in solution, followed by addition of alcohol which will cause the nucleic acid to precipitate. Alternatively, the salt and alcohol may be added together, or the salt may be omitted. As described above in relation to the cell binding step, any suitable alcohol or salt may be used, and appropriate amounts or concentrations may readily be determined. However, as mentioned above, it is preferred to avoid the use of solvents, alcohols and similar agents. Thus alternative techniques, avoiding the use of such agents are preferred.

In preferred methods of the invention the DNA and RNA are bound to different solid supports in different steps. Again appropriate conditions and solid supports which allow the selective binding of DNA or RNA are well known in the art and any of these techniques can be used. For example, such preferred techniques whereby DNA and RNA can be separated sequentially from the same sample is described in WO96/18731, the teaching of which is incorporated herein by reference.

For example in this regard, by selecting

appropriate "nucleic acid binding" conditions (e.g. appropriate buffer or lysis/binding medium compositions), it may be selected whether to bind DNA released from the cells, or RNA released from the cells to the solid support. Thus, "binding medium" compositions may be selected favouring DNA binding (or more particularly genomic DNA binding) to the solid support. Such binding medium compositions include those mentioned above, those described in the Examples below, and the compositions of WO96/18731. For example, a representative DNA binding medium may include GuHCl and optionally EDTA.

Briefly, DNA is bound to appropriate non-specific solid supports in the presence of various detergents as described in WO 96/18731 of Dynal AS (the so-called "DNA Direct" procedure). Various detergent-based systems for binding nucleic acids to a solid support are described in this publication and may be used according to the present invention. The solid support is then separated from the rest of the sample, e.g. by the use of a magnetic field.

To bind RNA, appropriate medium compositions or conditions are known in the art, or may readily be determined from RNA isolation procedures known in the art, and may include, for example, the buffers and procedures described in EP-A-0389063 and US-A-5,234,809 of Akzo Nobel NV.

Representative RNA-binding compositions may thus include guanidine thiocyanate (GTC) with EDTA.

For RNA binding, the nature of the solid support may be of importance and in particular a "silica" (i.e. comprising silica itself or being based on silica or a silica derivative) solid surface should be used (see further below)

Advantageously, when the method of the invention is used to isolate DNA, it may be combined with a further step separately to isolate the RNA from the sample.

Thus, following the procedure discussed above, and selecting DNA-binding conditions in the nucleic acid binding step (e.g. a lysis/binding or binding medium favouring DNA binding), DNA released from the support-bound cells may be bound to the support, and removed from the sample. RNA, most notably mRNA, released from the leucocytes, remains in the sample (more precisely in the supernatant). This RNA may readily be isolated from the sample using standard procedures, for example by binding to a capture probe, conveniently immobilised (e.g. by binding to a solid support), consisting of oligo dT.

A further solid support can then be added to the sample under conditions such that the RNA in the sample becomes captured. This can be done by for example suing a solid support that can bind the RNA or a solid support that can capture specific RNA molecules (e.g. by carrying a complementary nucleic acid probe), or a subset if RNA molecules, e.g. by carrying a capture probe for polyadenylated RNA, i.e. specific for mRNA.

Conveniently, the nucleic acid or DNA binding step may take place simultaneously or concomitantly with the cell lysis step. This may conveniently be achieved using the detergent-based methods of WO96/18731. Thus, for example, an agent or agents for lysis and nucleic acid binding may conveniently be contained in an appropriate medium (e.g. a buffer or other aqueous solution) and added to the support-bound cells. The cells may then be maintained in contact with the medium e.g. incubated (e.g. as described above) to allow lysis and nucleic acid binding to take place. Such a medium may be referred to as a "lysis/binding" medium. A detergent may function as both lysis agent and to assist in the binding of the nucleic acid to the support.

The detergent may be any detergent, and a vast range are known and described in the literature. Thus, the detergent may be ionic, including anionic and

cationic, non-ionic or zwitterionic. The term "ionic detergent" as used herein includes any detergent which is partly or wholly in ionic form when dissolved in water. Anionic detergents have been shown to work particularly well and are preferred. Suitable anionic detergents include for example sodium dodecyl sulphate (SDS) or other alkali metal alkylsulphate salts or similar detergents, sarkosyl, or combinations thereof.

Conveniently, the detergent may be used in a concentration of 0.2 to 30% (w/v), e.g. 0.5 to 30%, preferably 0.5 to 15%, more preferably 1 to 10%. For anionic detergents concentrations of 1.0 to 5% e.g. 0.5 to 5% have been shown to work well.

The detergent may be supplied in simple aqueous solution, which may be alkaline or acidic, or more preferably in a buffer. Any suitable buffer may be used, including for example Tris, Bicine, Tricine, and phosphate buffers. Conveniently, a source of monovalent cations, e.g. a salt, may be included to enhance nucleic acid capture, although this is not necessary. Suitable salts include chloride salts, e.g. sodium chloride, lithium chloride etc. at concentrations of 0.1 to 1M, e.g. 250 to 500 mM. As mentioned above, other components such as enzymes, may also be included.

Other optional components in the detergent composition include chelating agents e.g. EDTA, EGTA and other polyamino carboxylic acids conveniently at concentrations of 1 to 50 mM etc., reducing agents such as dithiotreitol (DTT) or β-mercaptoethanol, at concentrations of for example 1 to 10 mM.

Preferred detergent compositions may for example comprise:

100 mM Tris-HCl pH 7.5
10 mM EDTA
2% SDS

or:

100mM TrisCl pH 7.5
10mM EDTA
5% SDS
10mM NaCl
or:
100mM TrisCl pH 7.5
500mM LiCl
10mM EDTA
1% LiDS

Reference is made to WO96/18731 for further details, exemplary reaction conditions etc.

Alternative nucleic acid binding techniques may also be used in order to achieve the step of binding released nucleic acid to the solid support. For example, one such method may take advantage of the well known principle of nucleic acid binding to a silica surface.

Thus, in such an embodiment, the solid support may comprise or consist of a silica or silica-based or derived material. Many such materials are known and described in the art, and the literature is replete with references describing the isolation of nucleic acids by binding to silica surfaces (see e.g. EP-A-0389063 of AKZO N.V., US-A-5,342,931, US-A-5,503,816 and US-A-5,625,054 of Becton Dickinson, US-A-5,155,018 of Hahnemann University, US-A-6,027,945 of Promega Corp. and US-A-5,945,525 of Toyo Boseki KK).

Ionic binding of the nucleic acid to the support may be achieved by using a solid support having a charged surface, for example a support coated with polyamines.

The support which is used in the method of the invention may also carry functional groups which assist in the specific or non-specific binding of nucleic acids, for example DNA binding proteins e.g. leucine zippers or histones or intercalating dyes (e.g. ethidium

bromide or Hoechst 42945) which may be coated onto the support.

Likewise, the support may be provided with binding partners to assist in the selective capture of nucleic acids. For example, complementary DNA or RNA sequences, or DNA binding proteins may be used. The attachment of such proteins to the solid support may be achieved using techniques well known in the art. Conveniently, such nucleic acid-binding partners may be intermixed on the solid support with the anti-leucocyte binding partners.

The solid supports used for any of the steps of the methods described herein may be any of the well known supports or matrices which are currently widely used or proposed for immobilisation, separation etc. These may take the form of particles, sheets, gels, filters, membranes (e.g. nylon membranes), fibres, capillaries, needles or microtitre strips, tubes, plates or wells, etc, combs, pipette tips, micro arrays, chips, or indeed any solid surface material.

Conveniently the support may be made of glass, silica, latex, plastic or any polymeric material. Generally speaking, for isolation of nucleic acids, the nature of the support is not critical and a variety of surface materials may be used. The surface of the solid support may be hydrophobic or hydrophilic. Preferred are materials presenting a high surface area for binding of the cells, and subsequently, of the nucleic acid. Such supports will generally have an irregular surface and may be for example be porous or particulate e.g. particles, fibres, webs, sinters or sieves. Particulate materials e.g. beads are generally preferred due to their greater binding capacity, particularly polymeric beads/particles.

Conveniently, a particulate solid support used according to the invention will comprise spherical beads. The size of the beads is not critical, but they may for example be of the order of diameter of at least

1 and preferably at least 2 μm, and have a maximum diameter of preferably not more than 10 and more preferably not more than 6 μm. For example, beads of diameter 2.8 μm, 2.7 μm and 4.5 μm have been shown to work well.

Monodisperse particles, that is those which are substantially uniform in size (e.g. size having a diameter standard deviation of less than 5%) have the advantage that they provide very uniform reproducibility of reaction. Monodisperse polymer particles produced by the technique described in US-A-4336173 are especially suitable.

Non-magnetic polymer beads suitable for use in the method of the invention are available from Dynal Biotech AS as well as from Qiagen, Amersham Pharmacia Biotech, Serotec, Seradyne, Merck, Nippon Paint, Chemagen, Promega, Prolabo, Polysciences, Agowa, Bangs Laboratories and Dyno Particles or Dyno Speciality Polymers.

However, to aid manipulation and separation, magnetic beads are preferred. The term "magnetic" as used herein means that the support is capable of having a magnetic moment imparted to it when placed in a magnetic field, and thus is displaceable under the action of that field. In other words, a support comprising magnetic particles may readily be removed by magnetic aggregation, which provides a quick, simple and efficient way of separating the particles following any cell, nucleic acid and protein binding steps, and is a far less rigorous method than traditional techniques such as centrifugation which generate shear forces which may disrupt cells or degrade proteins or nucleic acids.

Thus, using the method of the invention, the magnetic particles with cells, nucleic acids, or proteins attached may be removed onto a suitable surface by application of a magnetic field e.g. using a permanent magnet. It is usually sufficient to apply a

magnet to the side of the vessel containing the sample mixture to aggregate the particles to the wall of the vessel and to remove the remainder of the sample for further steps.

Especially preferred are superparamagnetic particles for example those described by Sintef in EP-A-106873, as magnetic aggregation and clumping of the particles during reaction can be avoided, thus ensuring uniform cell, nucleic acid and protein extraction. The well-known magnetic particles sold by Dynal Biotech ASA (Oslo, Norway, previously Dynal AS) as DYNABEADS, are particularly suited to use in the present invention. Beads are also automation friendly and allow binding at varying salt concentrations and/or pH. Beads also allow the possibility to manipulate binding and elution conditions of single samples.

Functionalised coated particles for use in the present invention may be prepared by modification of the beads according to US patents 4,336,173, 4,459,378 and 4,654,267. Thus, beads, or other supports, may be prepared having different types of functionalised surface, for example positively or negatively charged, hydrophilic or hydrophobic.

In embodiments where an affinity-based separation of cells or other particles is used, the binding partner(s) may be attached to the solid support in any convenient way, before or after binding to the cells/particles, according to techniques well known in the art and described in the literature. Thus the binding partner may be attached directly or indirectly to the solid support.

In a convenient embodiment, the binding partner may be attached to the support, prior to contact with the sample. Such attachment may readily be achieved by methods (e.g. coupling chemistries) well known in the art, and conveniently the binding partner is bound directly to the solid support, for example by coating.

However it may also be attached via spacer or linker moieties. The binding partner may be covalently or reversibly attached according to choice.

Alternatively, as mentioned above, the binding partner may first be brought into contact with the sample, to bind to the cells/particles before being attached to the solid support. In this case, the solid support may conveniently carry or be provided with a binding moiety capable of binding to the particular specific binding partner used. Again, such binding systems are well known in the art. For example, the solid surface may carry a (secondary) antibody capable of binding to the particular binding partner (e.g. a polyclonal anti-species antibody).

In an advantageous embodiment of the invention the lysis step of the method (and indeed the other steps of the method involving the use of a solid support) may include a further step involving the addition of a further or extra amount of solid support (also referred to herein as a "second" solid support) to the reaction mixture.

The use of a second solid support has been found to offer advantages in sample collection for example by improving pellet formation and hence isolation of the first solid support. The improved pellet formation may also reduce non-specific binding of substances or entities in the pellet, or in other words reduces contamination of the pellet. Whilst not wishing to be bound by theory it is believed that when only a first solid support is used the isolated nucleic acid binds to the first solid support as a loose, non-compact mesh, thereby resulting in a relatively loose non-compact pellet. However, where a second support is used and particularly when this second solid support comprises particles which are smaller or larger than the first solid support, the second solid support fills in the gaps (or vice versa) in the loose mesh, thereby making

the pellet tighter and more compact thus reducing the tendency to trap contaminating material.

Further details of the nature and characteristics of appropriate second solid supports for use in the methods of the present invention are described in WO 01/94572, the disclosure of which is incorporated herein by reference.

Briefly, the second solid support may be of comparable size and density to the first solid support. Preferably however, the second solid support is of a smaller size than the first solid support. For example, where the supports are particulate the second solid support comprises particles of a smaller diameter (e.g. approximately half the diameter), than those comprising the first solid support. In especially preferred embodiments the first solid support comprises particles of 4.5 μm diameter (e.g. the M450 beads described herein) whereas the second solid support comprises particles of 2.8 μm diameter (e.g. the M280 and M270 beads described herein). Alternatively, the first support may be smaller than the second support and the dimensions described above may be reversed.

Especially preferably the second solid support may take a more active role in the isolation of the nucleic acid and in such cases the second solid phase is capable of binding to nucleic acid, i.e. has nucleic acid binding properties. Preferably therefore the second solid support may be made of glass, silica, latex, plastic or any polymeric material (i.e. an uncoated surface) capable of binding nucleic acid and such a solid support may optionally be functionalised, for example to aid or improve nucleic acid binding. Particularly preferred in this regard are functionalised solid supports which have a surface charge, preferably a negative surface charge. Most preferred are solid supports coated with carboxylic acid functional groups. Such solid supports are commercially available, for

example the M-270 carboxylic acid beads or M-280 Hydroxyl beads manufactured by Dynal Biotech ASA. Preferably the second solid supports are particulate, e.g. beads, and especially preferably are magnetic.

The provision of a further or extra amount of solid support after the cell isolation step results in an improved yield of nucleic acid and also makes the elution of nucleic acid from the solid support easier, particularly where solid supports with a negatively charged surface are used. Whilst not wishing to be bound by theory, as described above it is believed that the addition of an extra amount of a "second" solid support improves the compactness of the bead and nucleic acid pellet and particularly where DNA is able to bind to the second solid support, more effective and complete binding of nucleic acid molecules, rather than the nucleic acid molecules being attached to the beads only at one end or being attached to the beads loosely is achieved.

The terms "additional" or "extra" or "further" amount when used herein in connection with the addition of a second solid phase, is used to indicate the addition of any amount (by weight) of second solid phase such that the isolation of nucleic acid is improved, for example the yield of isolated nucleic acid is improved. For example the amount of second solid phase added might be the same amount as the amount of first solid phase used or may be up to approximately 3 to 5 times the amount of first solid phase used. Alternatively, the amount of second solid phase added may be less than the amount of first solid phase providing that the isolation of nucleic acid is improved. Preferably the amount of second solid phase used is 0.5 to 3 times the amount of first solid phase.

As the "amount" of solid phase refers to the weight of the solid phase, in the preferred embodiments of the invention where the first and second solid phases are

particulate and the particles making up the second solid phase are smaller (or larger) than the particles making up the first solid phase, the number of particles used for the first and second solid phases will generally be different.

The invention will now be described in more detail in the following non-limiting Example, with reference to the drawings in which:

Figure 1a represents a schematic diagram showing a summary of a model system according to the present invention; leukocytes are isolated by positive selection, binding to beads carrying anti-CD45. DNA is isolated from the captured leukocytes and bound onto DNA-binding beads, from which it may be eluted in purified form, e.g. for subsequent analysis. As an alternative, the isolated leukocyte population may be further fractionated by selection for circulating carcinoma cells (SW480 cells; Ber EP4$^+$ cells) using beads (Dynabeads® Epthelial Enrich) carrying a specific binding partner for such cells. This fractionated population may also be subjected to genomic DNA isolation as previously, allowing the general (e.g. leukocyte) and sub-fractionated (e.g. SW_480) cell populations to be compared. The isolated cell populations may be subjected to mRNA and protein (specific or general fraction) isolation;

Figure 1b represents a schematic diagram showing a summary of a model system according to the present invention; carcinoma cells (SW480) that have been spiked into blood sample, are isolated by positive selection, binding to beads carrying anti-Ber EP4 (Dynabeads Epithelial enrich). DNA is isolated from the captured carcinoma cells by lysing the cells and adding DNA binding Dynabeads, from which it may be eluted in purified form, e.g. for subsequent analysis. Different blood cell population (e.g. B-cells, T-cells, monocytes) may be isolated from the remaining blood sample

subsequently, and subjected to DNA isolation as described. The isolated cell populations may be subjected to mRNA and protein isolation.

Figure 2 is a photograph of a gel showing the results of sequential PCR experiments carried out as described in Example 1 on solid phase cDNA obtained from isolated mRNA (isolated from cultured carcinoma cells spiked into blood samples), in order to detect different transcripts: Lane 1 - ESX (1st round of PCR); lane 2 - Mucin 1; Lane 3 - CK19 (third round of PCR); Lane 4 - CK19 (first (nested) round of PCR); Lane 4 - ESX (second (nested) round of PCR);

Figure 3 is a photograph of a gel showing the results of PCR and RT-PCR experiments carried out as described in Example 1, showing isolation of genomic DNA (gDNA) and mRNA encoding CK19 sequentially from the same sample: Lane 1 - 100 bp DNA ladder 1 μg; Lane 2 - cDNA CK19 (mRNA isolation)a; Lane 3 - gDNA CK19 (g-DNA isolation)a; Lane 4 - cDNA CK19 (mRNA isolation)b; Lane 5 - gDNA CK19 (gDNA isolation)b; Lane 6 - Positive control; Lane 7 - Negative control;

Figure 4 shows the results of protein fractionation of a cultured carcinoma cell sample as described in Example 1; Figure 4A shows the results of Bead-DELFIA following specific protein isolation using anti-CK19; Figure 4B shows the results of SDS-PAGE comparing the cell lysate against a fractionated cell lysate(fractionated by binding to Dynabeads® M270-amine).

Figure 5a shows the results of an IMS separation of B cells by CD19-IMS and T cells by CD3-IMS, CD8-IMS and CD4-IMS, where mRNA from each cell population isolated after gDNA removal is subjected to an RT-PCR for CD19 as described in Example 2. The gels compare the amount of CD19 (and hence B cells) present in each sample to investigate unspecific binding in IMS;

Figure 5b shows the results of an IMS separation of

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monocytes by Dynabeads CD14, as described in Example 2. This figure shows that RT PCR for CD14 gives a positive result, but RT PCR for CD19 or CD4 result in only low levels being detected and hence demonstrates the low levels of unspecific binding for IMS;

Figure 6 shows the results of an IMS separation of carcinoma cells from whole blood (into which they had been spiked), where RT PCR for CK19 gives a positive result indicating the isolation of the carcinoma cells. RT PCRs for CD4 and CD14 are not detected and CD19 barely detected in the negative controls (unspiked blood samples, lanes 3 and 4) and this again demonstrates that only low levels of unspecific binding occur for IMS;

Figure 7 is a schematic representation of the DNA isolation protocol in Example 3;

Figure 8 shows that DNA obtained from IMS isolated cells is not degraded;

Figure 9a shows the results for protein isolated using ion exchanger beads from a cell lysate comparing Dynabeads ion exchanger prototypes with AP-Biotechs 15 Q/S ion exchange resins;

Figure 9b shows the results for protein isolated from different numbers of carcinoma cells using the cation exchanger prototype beads;

Figure 10a shows PCR results for CK19, CD14, CD4 and CD19 for IMS isolated carcinoma cells, where 2000, 200 or 20 cells were spiked into whole blood. CK19 was detected in samples which only represented mRNA from 20, 2 or 0.2 cells;

Figure 10b shows PCR results for CK19 for IMS isolated carcinoma cells where 1, 5 or 10 cells were spiked into whole blood. CK19 was detected in one of two parallels for one cell;

Figure 11 shows the re-use of the solid phase cDNA library generated from down to one cell in PCR, detecting a second transcript from the same sample, demonstrating the flexibility and scalability of the

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methods.

Example 1

Combined isolation of nucleic acid and proteins from single samples

Experimental procedures:
Cell pellets containing $2x10^5$ cultured carcinoma cells (SW480) were lysed in 200 µl lysis-binding buffer (100 mM Tris-HCl pH 7.5, 500 mM LiCl, 10 mM EDTA pH 8.0, 5 mM DTT, 1% LiDS) containing Dynabeads Epithelial Enrich (200 µg) which were present to improve pellet formation and Dynabeads M270 carboxylic Acid (300 µg), which bind the DNA. DNA is isolated by incubating the mix on a roller at room temperature for 5 minutes. The bead-DNA complex is separated from the remaining lysate, and washed 3x500 µl in 1xWashing buffer (10 mM Tris-HCl pH 7.5, 150 mM LiCl, 1.0 mM EDTA), followed by elution in 100 µl elution buffer (10 mM Tris-HCl pH 8.0, 0.01% Tween 20) at 80°C for 5-10 minutes. The DNA is ready for e.g. PCR (1 µl is more than enough). Reference is made to Fig. 3 which shows gel analysis of PCR products obtained from *inter alia*, isolated genomic DNA.

To the remaining lysate, 20 µl oligo(dT) beads (Dynabeads® Oligo (dT) 25 can be used) were added and mRNA annealed for 5 minutes at room temperature on roller. Bead-mRNA complex is separated from the remaining lysate, washed (as standard Dynal procedure ie. as indicated by the manufacturer) and resuspended in 100 µl ice cold 10 mM Tris-HCl, and ready for RT-PCR. The results are shown in Figs. 2 and 3.

The remaining lysate is diluted (200 µl lysate + 800 µl 10 mM Tris-HCl pH 8.0) and added to 1.5 mg Dynabeads M270-amine, which have been pre-washed in 10 mM Tris-HCl

pH 8.0. The suspension is incubated on a roller for 1 hour at room temperature. The bead-protein complex is then washed 3x100 µl in 10 mM Tris-HCl pH 8.0, and eluted by boiling all the beads for 2-5 minutes in 10 µl SDS-PAGE sample buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 2.5% SDS, 5% b-mercapto-ethanol, 0.01% BPB) and 1 µl is applied on 10-15% SDS-PAGE Phast gel. The results are shown in Fig. 4 (specifically in Figure 4B).

For specific protein isolation, the lysate was diluted: 5 µl, 10 µl and 20 µl lysate in 100 µl PBS with 0.1% BSA, prior to bead-DELFIA (ie separation of specific CK19 protein using anti-CK19 beads). The results are shown in Figure 4A.

Isolated LMW in Figure 4B refers to experiments where we dissolved Low Molecular Weight standard (MW protein from Amersham Pharmacia) in lysis/Binding buffer to a conc. of 1 µg/µl and mixed 20 µl of this with 80 µl 10 mM Tris-HCl, added 1.5 mg amine beads and incubated and washed, eluted and analysed as described for lysate. This shows that the beads bind proteins.

Example 2

Isolation of cell populations by IMS (immunomagnetic separation)

The presence/co-isolation of B cells in different populations of T cells isolated by IMS were investigated. Cells positive for CD19, CD3, CD8 and CD4 were selected by IMS, gDNA was removed and mRNA was then extracted from the isolated cell populations and cDNA was synthesised. PCR was then carried out using CD19 primers on a dilution series for each sample, representing from 40 000 to 75 cells. The results for

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this experiment can be seen in Figure 5a, where the sensitivity of the CD19 RT-PCR is indicated in the first gel panel. The remaining gels demonstrate the low level of unspecific binding in IMS, since CD19 PCR for the T cell populations demonstrated weak bands and hence only a very low level of unspecific B cell binding had occurred.

A similar experiment was carried out, demonstrating a low level of unspecific B cell binding or T_H cell (T helper cells) binding (<0.2% unspecific) when IMS was used with CD14 to isolate monocytes (Figure 5b).

Example 3

Spiking of carcinoma cells into a blood sample and the isolation of the carcinoma cells by IMS

Cultured colon carcinoma cells (SW480) were resuspended in culture medium, diluted and counted in a Coulter counter. Different numbers of carcinoma cells (from $1x10^6$ to 1 cell) were spiked into diluted blood samples (1 ml whole blood + 1 ml DPBS) or used directly as positive controls. Un-spiked blood samples were treated identically and served as negative controls. Samples were kept at 4°C.

Pre-washed Dynabeads Epithelial Enrich ($4x10^7$ beads) were added to each spiked and un-spiked blood sample, and the samples were incubated at 4°C for 15 minutes on a roller.

The cell-bead complex was captured by applying a magnet, washed once in cold DPBS/0.1% BSA and transferred to a new tube. The cell-bead complex was washed 3 times in 1 ml cold DPBS. In the last wash, the sample was split by transferring 500 µl resuspended bead-cell complex to a

new tube (sample a/b).

Sample a ($5x10^5$ cells):

DNA isolation
Washing buffer was removed, and the rosetted cells were lysed by adding lysis-binding buffer, containing DNA binding Dynabeads. The cell lysate was incubated at room temperature, on a roller for 5 minutes.

The DNA-bead complex was captured by applying a magnet. The supernatant, containing mRNA and proteins was transferred to a new tube. The DNA-bead complex was washed 3x in 1xWashing buffer (10 mM Tris-HCl pH 7.5, 150 mM LiCl, 1.0 mM EDTA) by turning the tube upside down 3-5 times. After the third wash, the DNA-bead complex was resuspended in Elution buffer (10 mM Tris-HCl pH 8.0, 0.01% Tween 20) and the tube incubated at 80°C for 10 minutes. The eluted DNA was transferred to a new tube and was ready for e.g. PCR (1 μl is more than enough). As can be seen from Figure 8, there was no degradation of the DNA.

Subsequent mRNA isolation
The remaining lysate was subjected to mRNA isolation by adding pre-washed Dynabeads oligo(dT)25 and incubated at room temperature on a roller for 5 minutes.

The mRNA-bead complex was captured by applying a magnet and washed 2x in washing buffer with LiDS, 2x in washing buffer without LiDS and 1x in 10 mM Tris HCl pH 7.5 (as standard Dynal Procedure...) and was then ready for cDNA synthesis or RT-PCR. cDNA synthesis was performed as follows:

Solid-phase cDNA was synthesised by using the oligo(dT)-sequences on the beads as primer in 1st strand cDNA

synthesis. cDNA was synthesised using Thermoscript, following the manufacturers protocol except for the incubation step. Samples were incubated for 30 minutes at 50°C followed by 30 minutes at 65°C with constant mixing. The solid-phase cDNA was used as template in PCR.

PCR was then carried out using CK19 primers, which amplify a transcript not present in blood. The isolation of carcinoma cells could be seen from the results of the CK19 PCR. CD14, CD4 and CD19 PCRs were then carried out to show the specificity of the IMS process for isolating carcinoma cells in this experiment rather than blood cells (Figure 6a).

Example 4

Protein isolation with anion and cation exchanger beads

Protein fractionation from carcinoma cells was carried out using 50 µl (1.5 mg) ion exchanger beads for each sample. The cation exchanger beads were pre-treated in 100 µl 1 M NaCl, 20 mM Citric Acid, pH 3.5 for 15 minutes. The beads were then washed 2x in 100 µl washing buffer (50 mM NaCl, 20 mM Citric Acid, 0.5% Tween 20, pH 3.5). The anion exchanger beads were pre-treated in 100 µl 1M NaCl, 20 mM Tris-HCl pH 10.0 for 15 minutes. The beads were then washed 2x in 100 µl washing buffer (50 mM NaCl, 20 mM Tris-HCl, 0.5% Tween 20, pH 3.5).

Cell lysis and ion exchange
Anion/Cation exchange (Figure 9a):

Cell pellets were lysed in 500 µl lysis buffer (20 mM Tris, 1% Triton-x-100, 150 mM NaCl, 5 mM EDTA, pH 8) and incubated on a roller for 10 min at 4°C. The lysate was

transferred to tubes containing pre-treated ion exchanger beads and incubated on a roller for 15 minutes at 4°C. The bead-protein complex was washed 3x in 20 mM Tris-HCl, 50 mM NaCl, pH 8.0. The proteins were eluted from the anion exchanger beads by adding 1M NaCl, 20 mM Citrate, pH 3.5, and incubating on a roller for 15 minutes. The proteins were eluted from the cation exchanger beads by adding 1 M NaCl, 20 mM Tris HCl pH 10.0 and incubating on a roller for 15 minutes. Analysed by SDS-PAGE.

Sample b (from Example 3, i.e. $5x10^5$ cells): Removed washing buffer from cells. The rosetted cells were lysed in 300 µl lysis buffer (20 mM Citric Acid, 50 mM NaCl, 20 mM Tris, 1% Triton-x-100, 5 mM EDTA, pH 3.5). Incubated on a roller at 4°C for 15 minutes. Transferred the lysate to a new tube, containing pre-treated cation exchanger beads. Incubated on a roller at 4°C for 15 minutes. Washed the beads 3x in 300 µl washing buffer (20 mM Citric Acid, 50 mM NaCl, 0.5% Tween 20, pH 3.5).

Resuspended beads in 100 µl elution buffer (20 mM Tris HCl, 1 M NaCl, pH 10.0) and incubated on a roller for 15 minutes. Eluted proteins were transferred to a new tube and analysed by SDS-PAGE.

The ion exchanger Dynabeads gave similar results for protein isolation as Amersham Pharmacia Biotech, ion exchangers (Figure 9a). Protein fractionation was also carried out using cation exchanger beads from spiked carcinoma cells and compared to the cell lysate directly. Figure 9b shows the results from this, where the negative control shows the unspecific background from unspiked blood.

Example 5

Scaling down the techniques to isolate reduced numbers of cells

2000, 200 and 20 carcinoma cells were spiked into 1 ml of whole blood. The cell isolation was carried out as described in Example 3 and mRNA was obtained (using 500 μg oligo (dT) beads). cDNA was synthesised on the oligo (dT) beads using the same amount of beads as in previous experiments. PCR was carried out for these samples using CK19, CD14, CD4 and CD19 primers. Analysis was carried out on samples representing mRNA from 20, 2 and 0.2 cells. (Figure 10a). It can be seen that all samples gave positive results for CK19, indicating the presence of carcinoma cells. The results for CD14 and CD4 were negative and low levels of CD19 were detected indicating again the very low levels of unspecific binding for IMS. Therefore 20 cells could easily be isolated and detected by this method.

The experiment was then repeated by spiking whole blood with 1, 5 and 10 carcinoma cells, where single cells had been picked with a micromanipulator. The cells were isolated as previously described, mRNA was isolated using less oligo (dT) beads (100 μg) and a solid phase cDNA library was generated as before. PCR was then carried out using the whole library from each isolation in one reaction with CK19 primers.

Figure 10b shows the results of these experiments, where the first 7 lanes show the MW-marker and then the results for unspiked cells (positive controls). The remaining lanes show the results for the spiked cells which were isolated. It can be seen that in one of two parallels, a single cell could be recovered from a spiked blood sample and detected using CK19 PCR.

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This shows that the method described here is highly scalable. Figure 11 shows the re-use of the cDNA libraries from Figure 10b in a second PCR, detecting another transcript. The technology is also flexible and allows multiple mRNA analysis of single small samples.

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Claims

1. A method of isolating nucleic acid and protein from the same sample, said method comprising contacting said sample with solid supports, wherein nucleic acid and protein components contained in said sample become bound to distinct solid supports.

2. The method of claim 1, wherein both DNA and RNA are bound to the same solid support.

3. The method of claim 1, wherein DNA and RNA are bound to distinct solid supports.

4. The method of claim 3, wherein DNA and RNA are bound to different solid supports in separate steps.

5. The method of any one of claims 1 to 4, wherein RNA and protein, or DNA and protein, or DNA, RNA and protein are isolated from the same sample.

6. The method of claim 5, wherein said RNA is mRNA.

7. The method of any one of claims 1 to 6, wherein said sample is a food or allied product, or is a clinical, environmental or biological sample.

8. The method of any one of claims 1 to 7, wherein prior to contacting said sample with said solid supports, the sample is subjected to a preliminary treatment step to free the nucleic acid and/or protein components from structures or entities in which they may be contained.

9. The method of any one of claims 1 to 8, wherein prior to contacting said sample with said solid supports, the sample is subjected to a cell isolation

procedure.

10. The method of claim 9, wherein one or more particular cell populations are specifically isolated.

11. The method of any one of claims 1 to 10, wherein the sample, or a cell population isolated therefrom, is subjected to a cell lysis step prior to contacting said sample with said solid supports.

12. The method of claim 11, wherein cell surface proteins of cells within or isolated from said sample are subjected to an *in vitro* modification procedure prior to the cell lysis step.

13. The method of any one of claims 1 to 12, wherein the sample is not divided at any stage of the method.

14. The method of any one of claims 8 to 12, wherein the sample is divided after cell isolation and/or lysis or after said preliminary treatment step.

15. The method of any one of claims 1 to 14, wherein said sample is contacted with said solid supports sequentially or simultaneously or in parallel.

16. The method of claim 15, wherein in a first step DNA is isolated from said sample, in a second step RNA is isolated from said sample and in a third step, protein is isolated from said sample, and wherein said steps may be performed in any order.

17. The method of any one of claims 1 to 16, wherein DNA is isolated on a support carrying surface carboxyl groups.

18. The method of any one of claims 1 to 17, wherein DNA is isolated by binding to a solid support, in the presence of a detergent.

19. The method of any one of claims 9 to 18, wherein cell lysis and nucleic acid or DNA binding to a solid support occur simultaneously or concomitantly.

20. The method of any one of claims 1 to 19, wherein RNA is isolated using an RNA-specific capture-probe carried by or attached to, or capable of binding to said solid support.

21. The method of claim 20, wherein said capture probe is or comprises a dT oligonucleotide or dU oligonucleotide.

22. The method of any one of claims 1 to 21, wherein protein is isolated using an appropriate binding partner/ligand carried by or attached to or capable of binding to said solid support.

23. The method of any one of claims 1 to 21 wherein protein is isolated using a solid support having a surface capable of effecting a chromatographic interaction.

24. The method of any one of claims 1 to 23, wherein said solid supports comprise particles.

25. The method of claim 24, wherein said particles are magnetic particles.

26. A kit for isolating nucleic acid and protein from the same sample comprising:

(a) a solid support suitable for binding nucleic acid components;

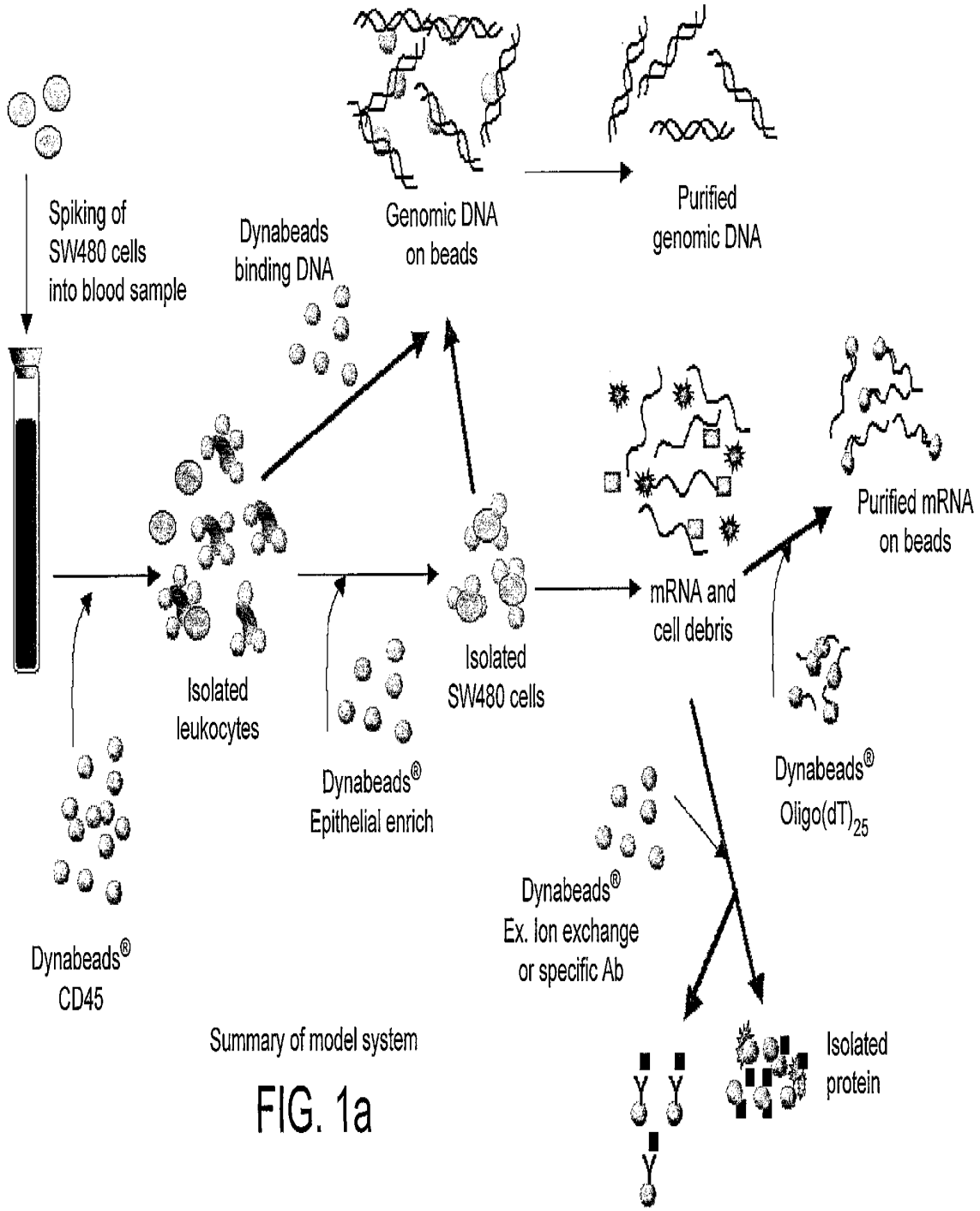
(b) a solid support suitable for binding proteins.

27. The kit of claim 26, wherein the solid support of (a) comprises a support which is selective for binding DNA or RNA or both types of support.

28. The kit of claim 26 or 27, wherein the kit also comprises (c) a solid support suitable for isolation of a specific cell population and/or (d) means for lysing said cells, and/or (e) a means for detecting the nucleic acid and/or protein.

29. Use of the method of any one of claims 1 to 28 for the analysis and/or comparison of mRNA and/or protein expression and/or the correlation thereof to genomic information.

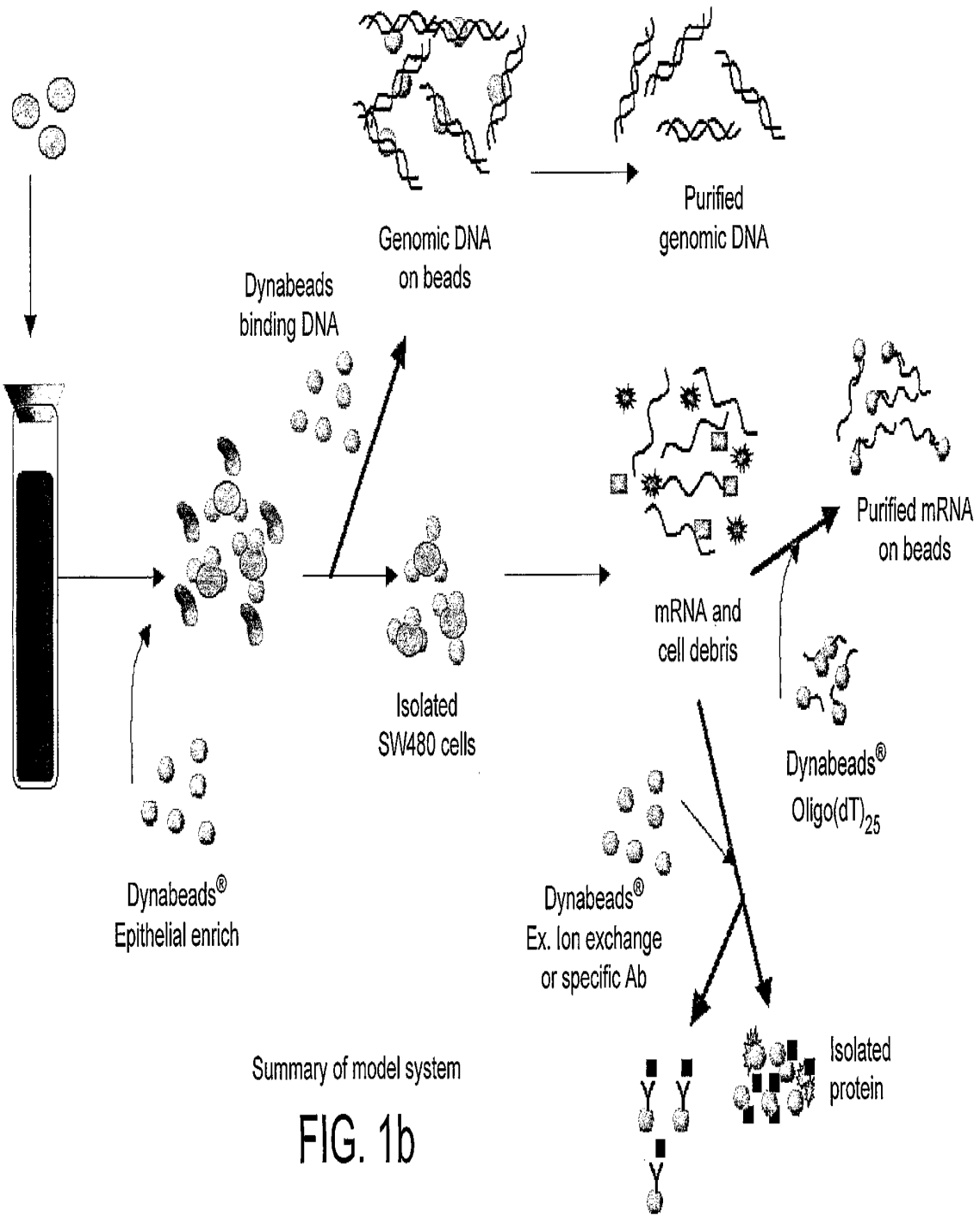
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Summary of model system

FIG. 1a

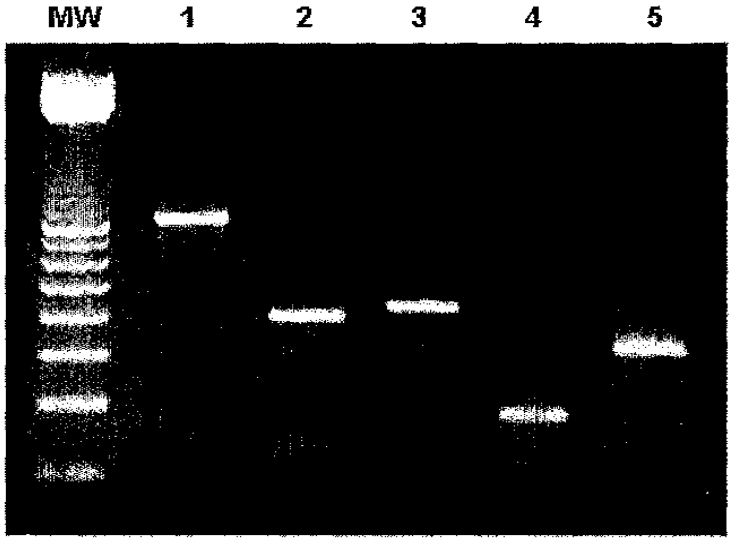
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Summary of model system

FIG. 1b

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Re-use of Solid-phase cDNA library in multiple PCR experiments, detecting different transcripts

Cultured Carcinoma Cells

Lane	No. of cells	Amplicon	Round of PCR
1	10000	ESX	first (45 cycles)
2	10000	Mucin1	second (45 cycles)
3	10000	CK19	third (30 cycles)
4	10	CK19	first (nested)
5	10	ESX	second (nested)

FIG. 2

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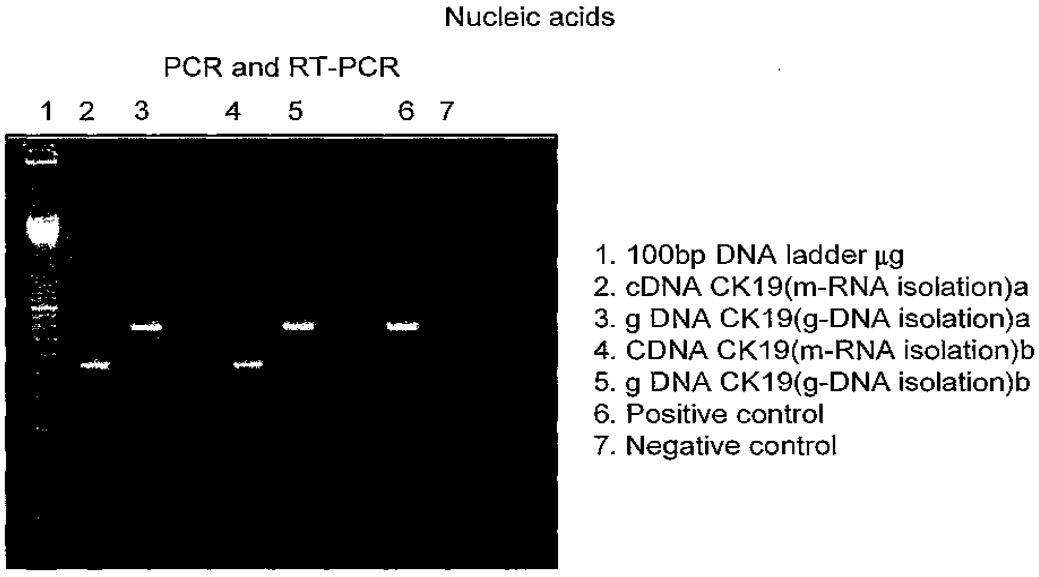


FIG. 3

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FIG. 4

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FIG. 5a

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FIG. 5b

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10x Genomics, Inc. Exhibit 2004
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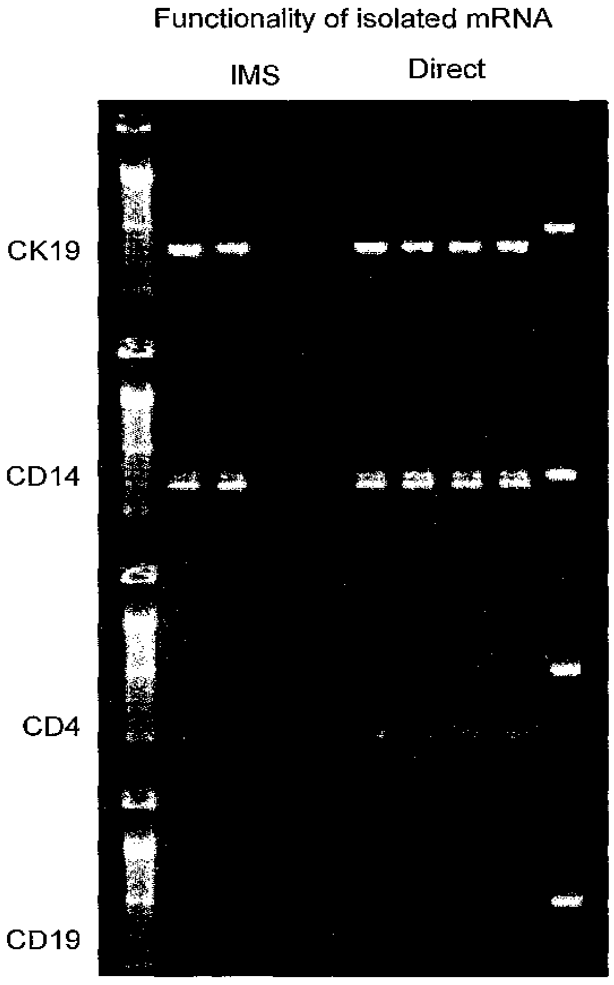


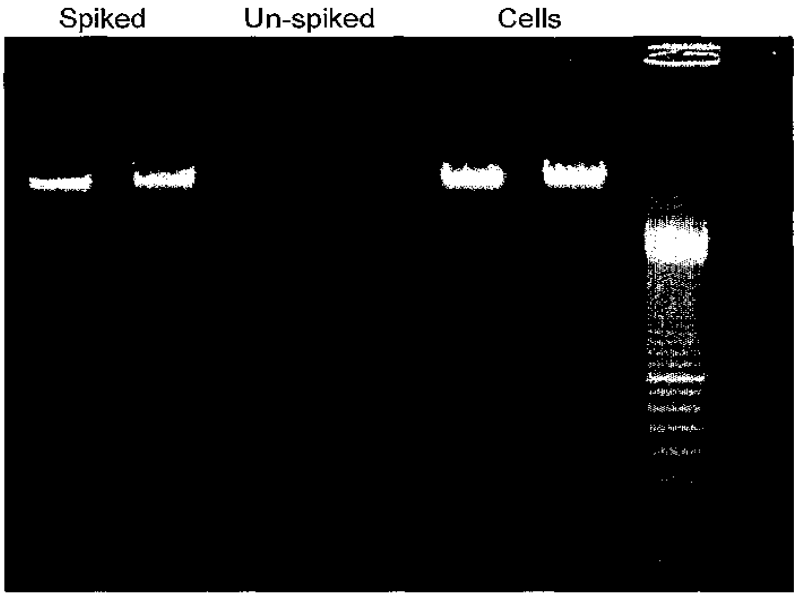
FIG. 6

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FIG. 7

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Integrity of isolated DNA:
No degradation

FIG. 8

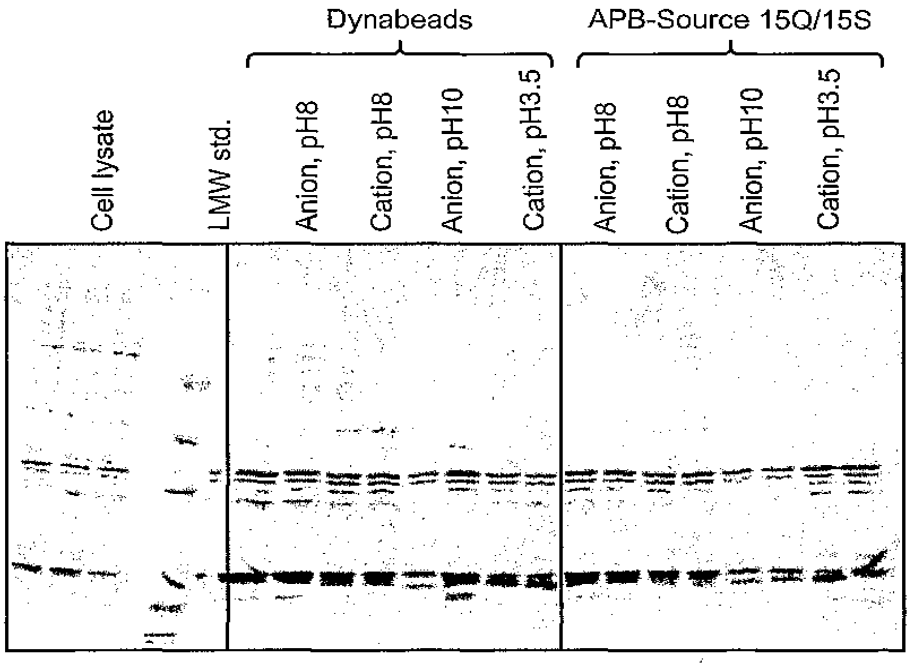


FIG. 9a

10x Genomics, Inc. Exhibit 2004
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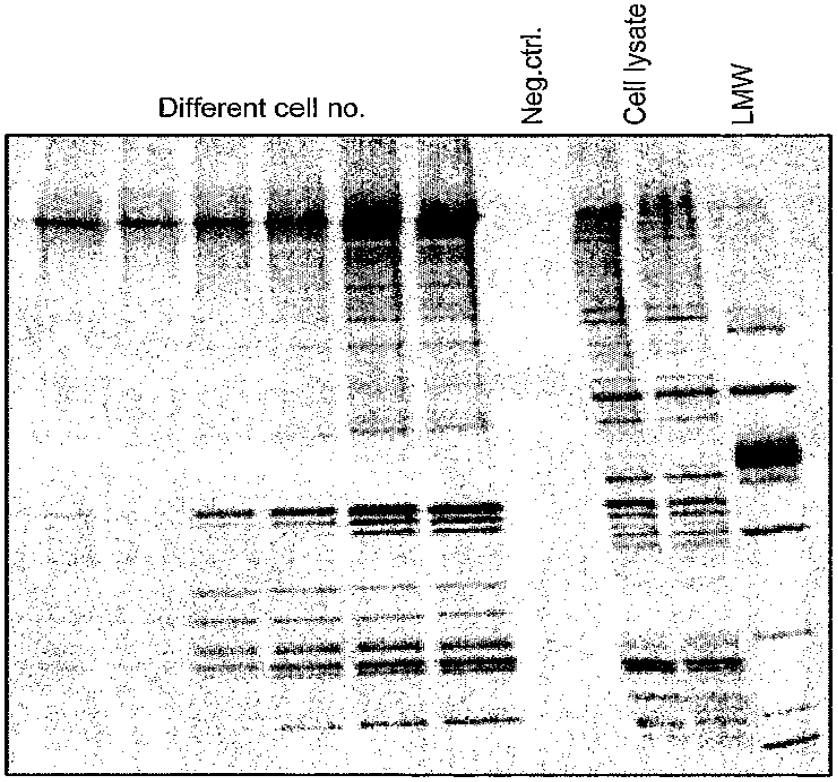
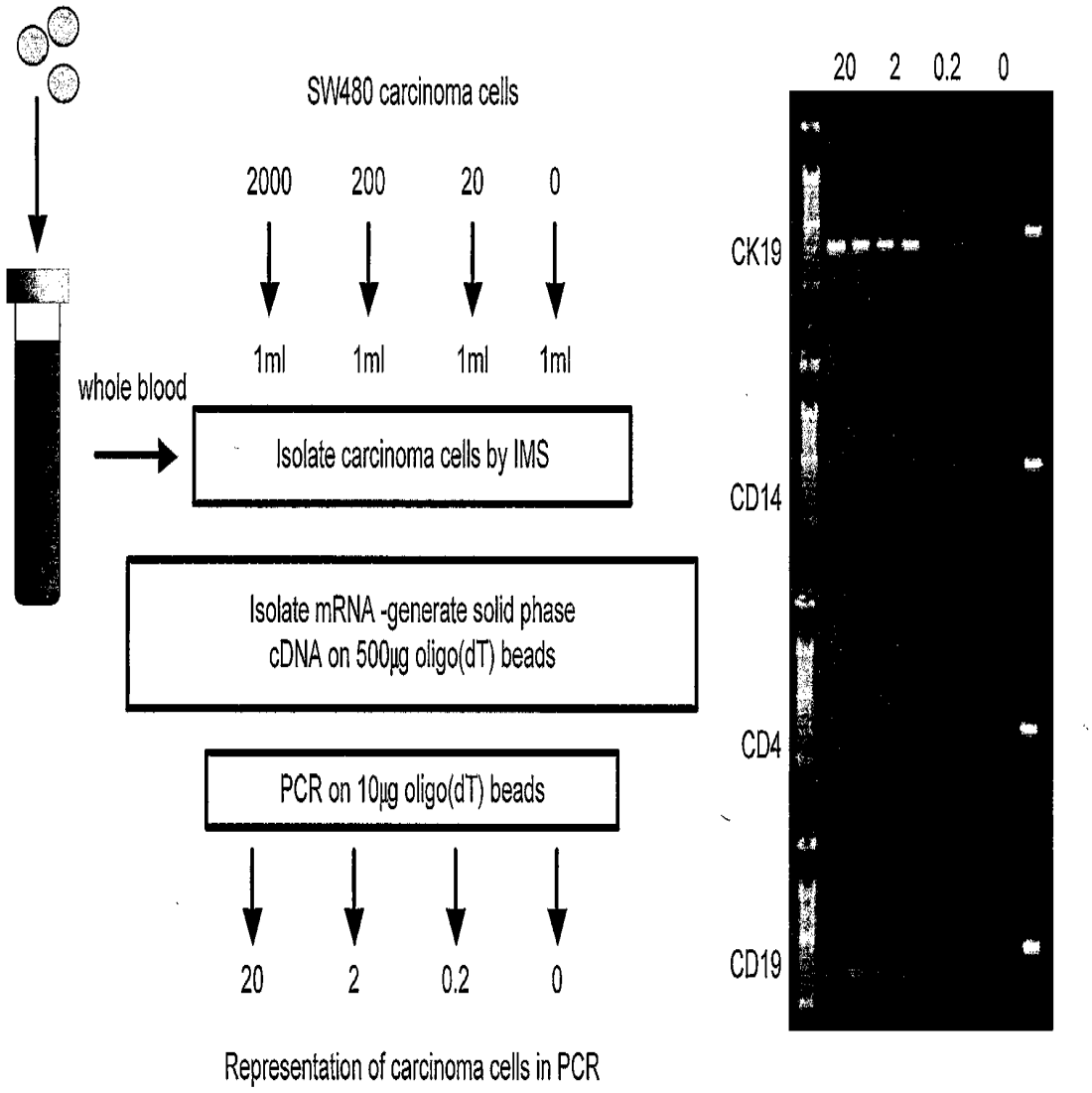


FIG. 9b

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Representation of carcinoma cells in PCR

FIG. 10a

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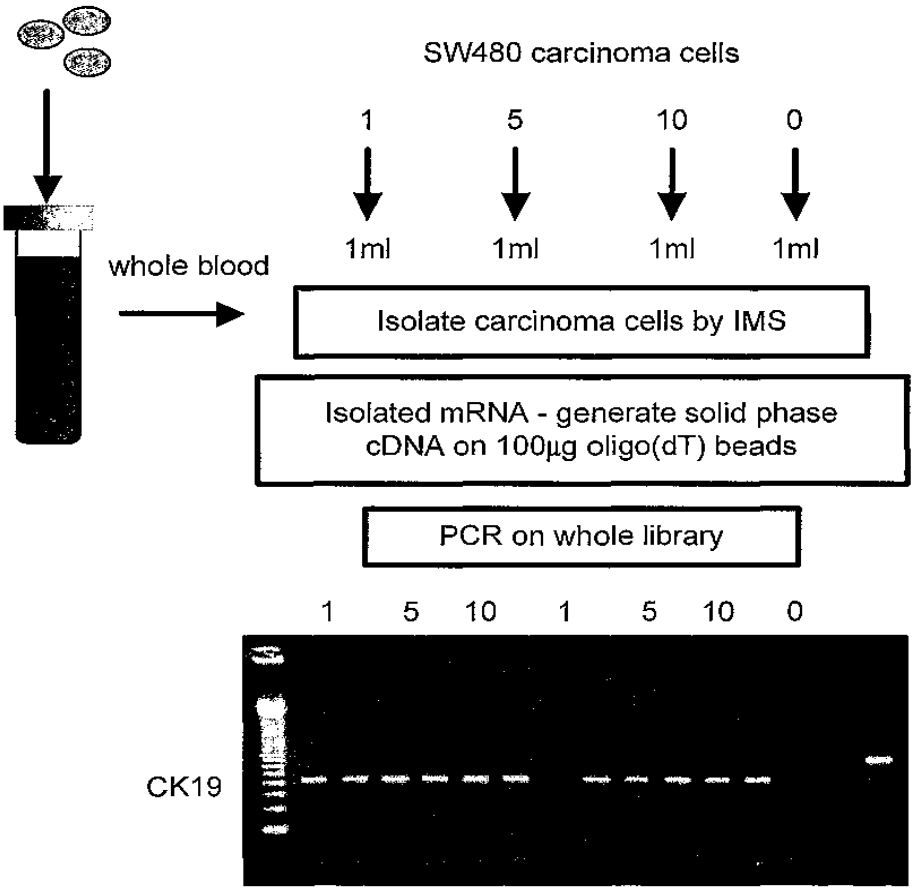


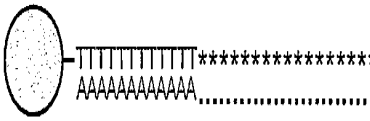
FIG. 10b

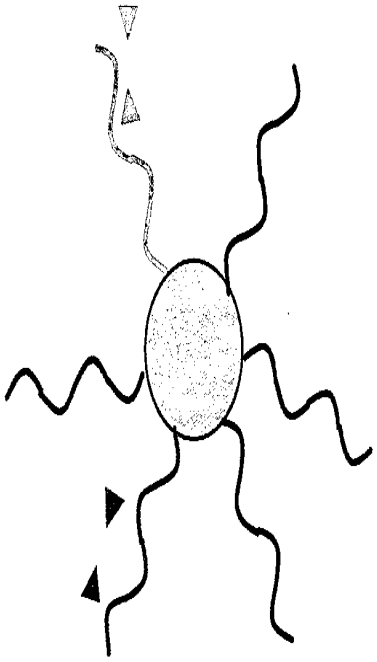
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Flexible technology

Re-Use of Solid Phase cDNA





Solid Phase cDNA library

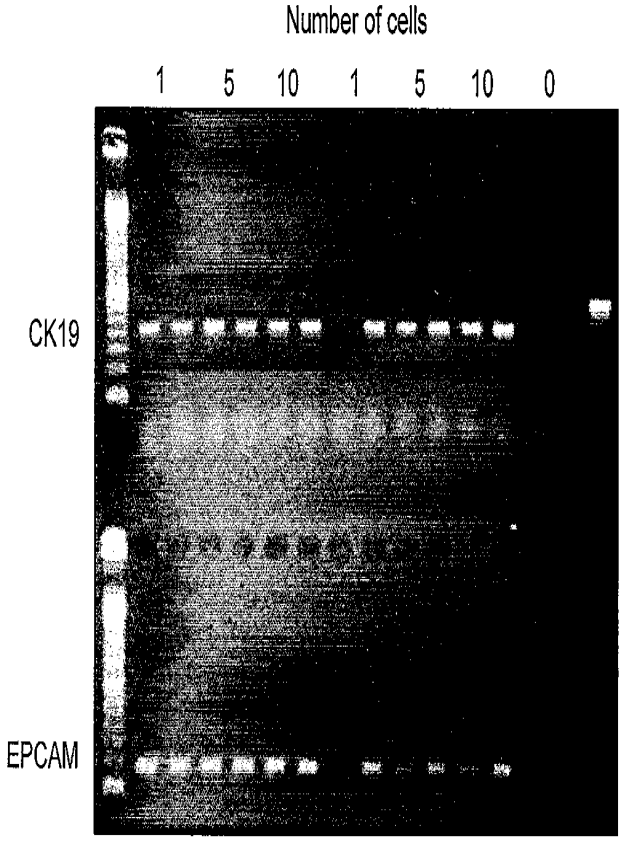


FIG. 11

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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High-resolution, high-throughput HLA genotyping by next-generation **sequencing**

G Bentley, R Higuchi, B Hoglund, D Goodridge... - Tissue ..., 2009 - Wiley Online Library

... We added 4-base **multiplex identifier** (MID) sequences (18) immediately following the library key tag to allow for multiplexed **sequencing** of HLA amplicons. ... image Figure 1. Schematic of 454 **sequencing** fusion primer pair with 4-base **multiplex identifier** (MIDs). ...

Cited by 114 Related articles All 8 versions Cite Save

Transcriptome **sequencing** with the Genome Sequencer FLX system

T Jarvie, T Harkins - Nature Methods, 2008 - nature.com

... Information on how to prepare small RNAs for 454 **Sequencing** analysis is available (Jarvie, T. & Harkins, T. Small RNA analysis using the Genome Sequencer FLX system. Nat. ... Multiplexing of samples is also supported by **Multiplex Identifier** tags. Full size image (27 KB). ...

Cited by 31 Related articles All 8 versions Cite Save

Digital PCR provides sensitive and absolute calibration for high throughput **sequencing**

RA White, PC Blainey, HC Fan, SR Quake - BMC genomics, 2009 - biomedcentral.com

... No titration or Sanger **sequencing** was performed. ... DNA; ssDNA: Single-stranded DNA; gsFLX: genome sequencer 'flex' from Roche/454; LOD: Limit of Detection; LOQ: Limit of Quantification; MDA: Multiple Displacement Amplification; MID: **Multiplex Identifier**; PCR: Polymerase ...

Cited by 111 Related articles All 31 versions Cite Save More

De novo 454 **sequencing** of barcoded BAC pools for comprehensive gene survey and genome analysis in the complex genome of barley

B Steuernagel, S Taudien, H Gundlach... - BMC ..., 2009 - biomedcentral.com

... Barcodes are specific short sequence tags that can be introduced either before the 454 **sequencing** library preparation [27] or by ligation of individual adaptors ("MID" = **Multiplex Identifier**", Roche Diagnostics) to fragmented BAC DNAs prior to **sequencing** in pools. ...

Cited by 59 Related articles All 12 versions Cite Save More

System and method for identification of individual samples from a multiplex mixture

MS Braverman, JF Simons, M Srinivasan... - US Patent App. 12/ ..., 2008 - Google Patents

... the invention relates to associating a unique identifier (UID) element, which is sometimes also referred to as a **multiplex identifier** (MID), with ... identifying each identifier and its associated sample from data generated by what are generally referred to as "**Sequencing**" techniques. ...

Cited by 22 Related articles All 4 versions Cite Save

High-resolution, high-throughput HLA DNA typing by massively parallel 454 **sequencing** with **multiplex identifier** tags

LG Bentley, B Hoglund, R Higuchi, ... - TISSUE ..., 2009 - ..., 380 MAIN ST, MALDEN 02148, MA ...

Cite Save

A NEW STRATEGY TO IDENTIFY THE DISEASE CAUSING MUTATION FOR NEURONAL CEROID LIPOFUSCINOSIS IN SOUTH HAMSPHIRE SHEEP

IFM Ismail, JAL Cavanagh, NL Mitchell, ... - Proc. Assoc. Advmt ..., 2009 - aaabg.org

... Targeted **sequencing** for mutation screening. Ten out of 15 long distance PCR products have been optimised for amplification on sheep genomic DNA. ... The resulting products will be individually **multiplex identifier** (MID) tagged and 454 sequenced. ...

Related articles All 4 versions Cite Save More

Mycobacteriophages BPs, Angel and Halo: comparative genomics reveals a novel class of ultra-small mobile genetic elements

T Sampson, GW Broussard, LJ Marinelli... - ..., 2009 - Soc General Microbiol

... adaptors were ligated to the fragments, which were then denatured and annealed to primer-coated beads; the 'A' adaptor contained a 10 bp **Multiplex Identifier** (MID) tag to enable subsequent extraction of Angel-specific sequences from a multiplexed **sequencing** run. ...

Cited by 39 Related articles All 9 versions Cite Save

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Page 00262

Phylotyping and functional analysis of two ancient human microbiomes

RY Tito, S Macmil, G Wiley, F Najar, L Cleeland, C Qu... - PLoS One, 2008 - dx.plos.org

... **Multiplex Identifier** (MID) 1 and 2 were used for samples Z1 and Z2, respectively. ... DNA sample preparation for **sequencing** on the 454/Roche GS-FLX were performed as described by the manufacturer [36] with modifications to improve the overall yields by replacing the Qiagen ...

Cited by 36 Related articles All 17 versions Cite Save More

Rapid high-throughput human leukocyte antigen typing by massively parallel pyrosequencing for high-resolution allele identification

C Gabriel, M Danzer, C Hackl, G Kopal, P Hufnagl , - Human immunology, 2009 - Elsevier

... The generated standard flowgram files (SFF) were analyzed with the latest version (2.0) of the GS-FLX amplicon variant analyzer (AVA) software supporting **multiplex identifier** sequence tags. In general, the AVA software maps the amplicon **sequencing** reads against a ...

Cited by 60 Related articles All 5 versions Cite Save

Two-dimensional navigation of multiplexed channels in a digital video distribution system

D O'callaghan - US Patent 7,561,214, 2009 - Google Patents

... Simply **sequencing** through all available channels, although an acceptable approach for systems with a limited number of channels, has become ... In this example, the on-screen display includes an anchor channel identifier, ABC, and a **multiplex identifier** 104 having a series of ...

Cited by 8 Related articles All 2 versions Cite Save

Transporting multiprotocol datagrams

SD Brueckheimer, LD Humphrey, DJ Stacey - US Patent 6,990,105, 2006 - Google Patents

... In particular, a **multiplex identifier** (MID) field is added to the SSCS to enable multiple messages to be interleaved in parallel within one minichannel. ... Alternatively, the PID can be provided in the first segment whereby the AAL2 SSCS SAR ensures **sequencing**, ie a non-parallel ...

Cited by 8 Related articles All 2 versions Cite Save

Identification of novel MHC class I sequences in pig-tailed macaques by amplicon pyrosequencing and full-length cDNA cloning and **sequencing**

CE O'Leary, RW Wiseman, JA Karl, BN Bimber, ... - Immunogenetics, 2009 - Springer

... 3'), which binds a conserved region of exon 3. Forward and reverse PCR primer pairs contained one of 12 **multiplex identifier** (MID) tags ... steps were performed following the manufacturer's GS FLX protocols at the University of Illinois—Urbana Champaign **Sequencing** Center. ...

Cited by 25 Related articles All 12 versions Cite Save

Major histocompatibility complex genotyping with massively parallel pyrosequencing

RW Wiseman, JA Karl, BN Bimber, CE O'Leary... - Nature medicine, 2009 - nature.com

... these amplicons into four pools, each containing products from 12 macaques that were distinguished by 10-bp **multiplex identifier** (MID) tags ... In addition, we detected five sequences not previously observed by cloning and Sanger **sequencing** (transcript levels between 0.3–2.2 ...

Cited by 85 Related articles All 10 versions Cite Save

SNP discovery via genomic reduction, barcoding, and 454-pyrosequencing in amaranth

PJ Maughan, SM Yoursione, EN Jellen... - The plant ..., 2009 - dl.sciencesocieties.org

... (2007) reduced genomic complexity in maize via construction of tissue-specific cDNA libraries and 454-based transcriptome **sequencing**. ... based on restriction site conservation (GR-RSC) with each sample labeled independently using a unique **multiplex identifier** (MID) barcode. ...

Cited by 61 Related articles All 9 versions Cite Save

System and method for improved processing of nucleic acids for production of sequencable libraries

GC Ferreri, JF Simons, MT Ronan ... - US Patent App. 12/ ..., 2009 - Google Patents

... 133. 28. The method of claim 25 , wherein: the step of associating comprises detection of up to two errors in the sequence from the **multiplex identifier** element and correction of up to one of the **sequencing** errors. 29. The method ...

Cited by 1 Related articles All 2 versions Cite Save

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Page 00263

A component-based architecture for software communication systems

M Jung, EW Bersack - Engineering of Computer Based ..., 2000 - ieeexplore.ieee.org

... and have hence different orders since fragmentation results in several pieces of data that all need individual processing (**sequencing**, buffering ... Data Order: A packet sent by the error-control- order or the retransmission-order contains an predefined de-**multiplex identifier** used to ...

Cited by 23 Related articles All 11 versions Cite Save

CO dehydrogenase genes found in metagenomic fosmid clones from the deep Mediterranean Sea

AB Martin-Cuadrado, R Ghai, A Gonzaga, ... - Applied and ..., 2009 - Am Soc Microbiol

... system; GATC, Constance, Germany), tagging each one individually using a **multiplex identifier** adaptor that contains a unique 10-base sequence that is recognized by the **sequencing** analysis software and allowing for automated sorting of **multiplex identifier** adaptor-containing ...

Cited by 19 Related articles All 12 versions Cite Save

Cost-effective sequence-based nonhuman primate MHC class I genotyping from RNA

JA Karl, RW Wiseman, DH O'Connor - Methods, 2009 - Elsevier

... full-length NHP class I cDNAs that are molecularly barcoded with **multiplex identifier** (MID) tags. This approach allows for ligation and transformation of pools of PCR amplicons from multiple animals, reducing cloning costs. Additionally, our method focuses **sequencing** on a ...

Cited by 10 Related articles All 7 versions Cite Save

Molecular analysis of the human mitochondrial DNA control region for forensic identity testing

MM Holland - Current Protocols in Human Genetics, 2001 - Wiley Online Library

... R429, TTA AAA GTG CAT ACC GCC A. 1. PCR amplify DNA extracts with first-round PCR (frPCR) primers (see Table 14.7.4). Each primer has a primer-binding sequence, a **multiplex identifier**, and an adaptor sequence for the 454 **sequencing** process. ...

Cited by 13 Related articles All 9 versions Cite Save

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
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(Not for submission under 37 CFR 1.99)

Application Number	14792094
Filing Date	2015-07-06
First Named Inventor	BRENNER, Sydney
Art Unit	~~1636~~ 1634
Examiner Name	Bhat N.
Attorney Docket Number	CGLC-020CON5

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	1	6723513		2004-04-20	LEXOW, Preben	
	2	7544473		2009-06-09	BRENNER, Sydney	
	3	7700325		2010-04-20	CANTOR, et al.	
	4	8101346		2012-01-24	TAKAHAMA, Shinichiro	
	5	8298767		2012-10-30	BRENNER, et al.	
	6	8318460		2012-11-27	CANTOR, et al.	

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	Examiner Name	Bhat N.
	Attorney Docket Number	CGLC-020CON5

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	1	20040224331		2004-11-11	CANTOR, et al.	
	2	20060008799		2006-01-12	CAI, et al.	
	3	20070020640		2007-01-25	MCCLOSKEY, et al.	
	4	20070238113		2007-10-11	KANDA, et al.	
	5	20070259357		2007-11-08	BRENNER, Sydney	
	6	20090155780		2009-06-18	XIAO, et al.	
	7	20100086914		2010-04-08	BENTLEY, et al.	
	8	20100113296		2010-05-06	MYERSON, Joel	
	9	20100120098		2010-05-13	GRUNENWALD, et al.	
	10	20110287435		2011-11-24	GRUNENWALD, et al.	
	11	20120208724		2012-08-16	STEEMERS, et al.	

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	Examiner Name	Bhat N.
	Attorney Docket Number	CGLC-020CON5

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	12	20120316074		2012-12-13	SAXONOV, Serge	
	13	20130059310		2013-03-07	BRENNER, et al.	

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	1	EP1841879	EP		2007-10-10	COMPASS GENETICS, LLC.		☐
	2	EP1672064	EP		2006-06-21	RIKEN		☐
	3	WO200190418	WO		2001-11-29	THE REGENTS OF THE UNIVERSITY OF CALIFORNIA		☐
	4	WO2004061083	WO		2004-07-22	AMGEN INC		☐
	5	WO2004065617	WO		2004-08-05	THE TRUSTEES OF BOSTON UNIVERSITY		☐
	6	WO2007018601	WO		2007-02-15	RUBICON GENOMICS, INC.		☐
	7	WO2008150432	WO		2008-12-11	454 LIFE SCIENCES CORPORATION		☐

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	8	WO2013036929	WO		2013-03-14	THE BOARD OF TRUSTEES OF THE LELAND STANFORD...		☐
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	1	BOULANGER, et al, "Massively parallel haplotyping on microscopic beads for the high-throughput phase analysis of single molecules", PLoS One, Vol 7:1-10, 2012.	☐
	2	CASBON, et al, "Reflex: intramolecular barcoding of long-range PCR products for sequencing multiple pooled DNAs", Nucleic Acids Res., Pages 1-6, 2013.	☐
	3	FAN, et al, "Whole-genome molecular haplotyping of single cells", Nat Biotechnol., 29: 51-59, 2011.	☐
	4	HIATT, et al, "Parallel, tag-directed assembly of locally derived short sequence reads", Nat Methods., 7:119-122, 2010.	☐
	5	IMBURGIO, et al, "Studies of promoter recognition and start site selection by T7 RNA polymerase using a comprehensive collection of promoter variants", Biochemistry., 39:10419-30, 2000.	☐
	6	KAPER, et al, "Whole-genome haplotyping by dilution, amplification, and sequencing", Proc Natl Acad Sci U. S. A., 110: 5552-7, 2013.	☐
	7	KIRKNESS, et al, "Sequencing of isolated sperm cells for direct haplotyping of a human genome", Genome Res., 23: 826-32, 2013.	☐

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	Art Unit	~~1636~~ 1634
	Examiner Name	Bhat N.
	Attorney Docket Number	CGLC-020CON5

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	8	KITZMAN, et al, "Haplotype-resolved genome sequencing of a Gujarati Indian individual", Nat Biotechnol., 29:59-63, 2011.	☐
	9	KOZAREWA, et al. "Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of GC-biased genomes", Nat Methods., 6: 291-5, 2009.	☐
	10	KONAREWA, et al, "96-plex molecular barcoding for the Illumina Genome Analyzer", Methods Mol Biol., 733:279-98, 2011.	☐
	11	KWOK, et al, "Single-molecule analysis for molecular haplotyping", Hum Mutat., 23:442-6, 2004.	☐
	12	LAIRD, et al, "Hairpin-bisulfite PCR: Assessing epigenetic methylation patterns on complementary strands of individual DNA molecules", 101:204-9, 2004.	☐
	13	LUNDIN, et al, "Hierarchical molecular tagging to resolve long continuous sequences by massively parallel sequencing", Sci Rep., 3:1186, 2003.	☐
	14	OYOLA, et al, "Optimizing Illumina next-generation sequencing library preparation for extremely AT-biased genomes", BMC Genomics.,13:1, 2012.	☐
	15	PCT/IB2010/002243, International Search Report and Written Opinion, Mailed February 9, 2011, 13 pages.	☐
	16	REISNER, et al, "Single-molecule denaturation mapping of DNA in nanofluidic channels", Proc Natl Acad Sci U.S.A., 107: 13294-9, 2010.	☐
	17	SCHWARTZ, et al, "Capturing native long-range contiguity by in situ library construction and optical sequencing", PNAS, 109:18749-54, 2012.	☐
	18	TURNER, et al, "Assaying chromosomal inversions by single molecule haplotyping", Nat Methods., 3:439-445, 2006.	☐

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	Examiner Name	Bhat N.
	Attorney Docket Number	CGLC-020CON5

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	19	TURNER, et al, "High-throughput haplotype determination over long distances by haplotype fusion PCR and ligation haplotyping", Nat Protoc., 4:1771-83, 2009.	☐
	20	USHIJIMA, "Detection and interpretation of altered methylation patterns in cancer cells", Nat Rev Cancer., 3:223-31, 2005.	☐
	21	VAN NIEUWERBURGH, et al, "Illumina mate-paired DNA sequencing-library preparation using Cre-Lox recombination", Nucleic Acids Res., 40:1-8, 2012.	☐
	22	WANG, et al, "Self-Formed Adaptor PCR: a Simple and Efficient Method for Chromosome Walking", Appl Environ Microbiol., 15: 5048–51, 2007.	☐
	23	XIAO, et al, "Determination of haplotypes from single DNA molecules: a method for single-molecule barcoding", Hum Mutat., 28:913-21, 2007.	☐
	24	YAN, et al, "Rapid one-step construction of hairpin RNA", Biochem Biophys Res Commun., 4:464-8, 2009.	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/NARAYAN K BHAT/	Date Considered	12/30/2015

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

EFS Web 2.1.17

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.K.B/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)	Application Number	14792094 ~~14792094 ~ GAU: 1634~~
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	
	Attorney Docket Number	CGLC-020CON5

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/James S. Keddie, Reg. No. 48,920/	Date (YYYY-MM-DD)	2015-11-09
Name/Print	James S. Keddie	Registration Number	48920

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/792,094	07/06/2015	Sydney Brenner	CGLC-020CON5

CONFIRMATION NO. 9697

93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

PUBLICATION NOTICE

OC000000080911788

Title: Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Publication No.US-2016-0053303-A1
Publication Date:02/25/2016

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

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Electronically Filed

AMENDMENT UNDER 37 C.F.R. §1.111		
	Attorney Docket No.	CGLC-020CON5
	Confirmation No.	9697
	First Named Inventor	Brenner, Sydney
	Application Number	14/792,094
Address to: Mail Stop Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Filing Date	July 6, 2015
	Group Art Unit	1634
	Examiner Name	BHAT, Narayan Kameshwar
	Title: "*Compositions and Methods for Intramolecular Nucleic Acid Rearrangement*"	

Sir:

This amendment is responsive to the Office Action dated January 13, 2016 for which a three-month period for response was given making this response due by April 13, 2016. A request for a three month time extension is filed herewith. As such, this response is timely filed. In view of the amendments to the claims and the remarks put forth below, reconsideration and allowance are respectfully requested.

AMENDMENTS TO THE SPECIFICATION

Please amend the Abstract on page 43 as shown below. A clean copy of the abstract is provided after the signature page on the last page of this response.

Aspects of the present invention are drawn to processes for **analyzing nucleic acids from a single cell source** ~~moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method".~~ In certain embodiments, the ~~reflex~~ method **involves tagging the plurality of nucleic acids from the single cell source with multiplex identifier (MID) sequences and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences** ~~results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID)~~. Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

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AMENDMENTS TO THE CLAIMS

Please amend the claims as shown below. A complete listing of the claims, including their current status identifiers, is set forth below.

1-20 (Cancelled)

21. (Currently amended) A method of analyzing nucleic acids, comprising:

(a) providing a plurality of nucleic ~~acids~~ **acid samples, each made** from a single **isolated** cell ~~source~~;

(b) tagging [[the]] **a plurality of segments of the nucleic acid molecules in the samples of (a)** ~~plurality of nucleic acids acids from the single cell source~~ with multiplex identifier (MID) sequences, **wherein:**

i. the MID sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and

ii. all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence; and

(c) sequencing the tagged sample of (b) to obtain the nucleotide sequences ~~determining a nucleotide sequence~~ of the plurality of nucleic acids and attached MID sequences.

22-23. (Cancelled)

24. (Currently amended) The method of claim 21, further comprising amplifying the **product of step (b)** ~~plurality of nucleic acids and attached MID sequences~~ before determining the nucleotide sequence of the plurality of nucleic acids and attached MID sequences.

25. (Cancelled)

26. (Previously Presented) The method of claim 21, wherein the plurality of nucleic acids is selected from DNA and RNA.

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27. (Previously Presented) The method of claim 26, wherein the plurality of nucleic acids comprises mRNA.

28. (Currently amended) The method of claim 23, further comprising identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within **an isolated single cell** ~~a single cell source~~ from a number of unique MID sequences identified.

29. (Currently amended) The method of claim 21, wherein the tagging step comprises ligating a sequence comprising the MID sequences to the **segments of the** plurality nucleic acids.

30. (Previously Presented) The method of claim 21, wherein the tagging step comprises performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

31. (Currently amended) The method of claim 21, wherein:

the providing step comprises providing a plurality of nucleic acids from each of a plurality of **the samples** ~~single cell sources~~;

the attaching step comprises attaching different MID sequences to **segments of** the nucleic acids from each of the plurality of **the samples** ~~single cell sources~~; and

wherein nucleic acid sequences are attributed to different **isolated cells** ~~individual cell sources~~ based upon the MID sequences attached in the attaching step.

32. (Previously Presented) The method of claim 21, wherein the MID sequences further comprise a sequencing primer sequence.

33. (Previously Presented) The method of claim 21, wherein the MID sequences are from 2 to 100 bases in length.

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34. (Previously Presented) The method of claim 31, wherein the plurality of different MID sequences comprises at least 100 different MID sequences.

35. (Currently amended) A method of analyzing nucleic acids, comprising:

providing a plurality of nucleic ~~acids~~ **acid samples, each made** from a single **isolated** cell ~~source~~;

tagging [[the]] **segments of the nucleic acid molecules in the samples of (a)** ~~plurality of nucleic acids acids from the single cell source~~ with multiplex identifier (MID) sequences, **wherein:**

i. the MID sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and

ii. all of the tagged segments of a particular nucleic acid molecule are associated with the same MID sequence ~~wherein each of the plurality of molecules is tagged with a unique MID sequence;~~ and

analyzing the plurality of nucleic acids and attached MID sequences.

36. (Currently amended) The method of claim 35, wherein the analyzing step comprises sequencing the **tagged segments of a** plurality of nucleic acids and attached MID sequences.

37. (Currently amended) A method of analyzing nucleic acids, comprising:

providing a plurality of nucleic ~~acids~~ **acid samples, each made** from a single **isolated** cell ~~source~~;;

tagging [[the]] **segments of the nucleic acid molecules in the samples of (a)** ~~plurality of nucleic acids acids from the single cell source~~ with multiplex identifier (MID) sequences, **wherein:**

i. the MID sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and

ii. all of the tagged segments of a particular nucleic acid molecule are associated with the same MID sequence ~~wherein each of the plurality of molecules is tagged with a unique MID sequence;~~ and

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amplifying the **tagged segments of the** plurality of nucleic acids and associated MID sequences to provide amplified nucleic acids; and

determining a nucleotide sequence of amplified nucleic acids.

38. (Previously Presented) The method of claim 37, wherein the amplifying step comprises performing a polymerase chain reaction amplification.

39. (Previously Presented) The method of claim 37, wherein the amplifying step comprises a linear amplification step.

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REMARKS

FORMAL MATTERS:

Claims 21, 24 and 26-39 are pending after entry of the amendments set forth herein.

Claims 22, 23 and 25 are canceled without prejudice.

Claims 21, 24, 28, 29, 31 and 35-37 are amended. Claims 21, 25 and 37 are amended to recite single isolated cell (support for which is found at page 8, lines 3-6), to require that the MID sequence uniquely identifies a sample and (support for which is found at page 8, lines 1-3), and to require that the tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence (support for which is found in Fig. 5 where A, B and C are the segments, and the tagged segments from, e.g., nuclei acid 1, all have the same MID, i.e., MID1 or MID2).

No new matter is added.

Reconsideration of this application is respectfully requested.

OBJECTIONS TO THE SPECIFICATION

The abstract of the disclosure was objected to as failing to be in line with the claims and for allegedly containing the acronym "MID".

The Abstract has been amended and it is understood that this objection may be withdrawn.

Withdrawal of this objection is requested.

DOUBLE PATENTING REJECTION

Claims 21-39 were rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 1-21 of U.S. Patent No. 7,544,473 in view of Braverman, et al. (U.S. Publication no. 20090105959, hereinafter "Braverman").

Claims 21-39 were rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 1-22 of U.S. Patent No. 8,685,678 in view of Braverman, et al. (U.S. Publication no. 20090105959, hereinafter "Braverman").

Claims 21-39 were rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 1-25 of U.S. Patent No. 8,722,368 in view of Braverman, et al. (U.S. Publication no. 20090105959, hereinafter "Braverman").

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Claims 21-39 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 25-45 of U.S. Patent Application No. 14/273,197 in view of Braverman, et al. (U.S. Publication no. 20090105959, hereinafter "Braverman").

These rejections are traversed on the basis that none of the claims of US7,544,473, US8,685,678, US8,722,368 or US14/273,197 teaches or suggest tagging nucleic acid molecules in a way that results in the segments of a particular nucleic acid molecule being tagged with the same tag sequence. Braverman, which is cited solely to provide isolated cells, fails to provide such a method and, as such, a *prima facie* case of obviousness-type double patenting has not been established.

Withdrawal of this rejection is requested.

REJECTIONS UNDER §102

Claims 21-25 and 28-39 were rejected under 35 U.S.C. §102(b), for allegedly being unpatentable over Brenner (U.S. Publication No. 20070259357, hereinafter "Brenner").

The standard for anticipation under section 102 is one of strict identity. An anticipation rejection requires a showing that each limitation of a claim be found in a single reference, Atlas Powder Co. v. E.I. DuPont de Nemours & Co., 224 U.S.P.Q. 409, 411 (Fed. Cir. 1984).

This rejection is traversed on the basis that Brenner fails to disclose a method for tagging nucleic acid molecules in a way that results in the segments of a particular nucleic acid molecule being tagged with the same tag sequence. Because Brenner fails to disclose this element of the rejected claims, Brenner cannot anticipate the claims.

Withdrawal of this rejection is requested.

Claims 21-25 and 28-39 were rejected under 35 U.S.C. §102(b), for allegedly being anticipated over Braverman, et al. (U.S. Publication no. 20090105959, hereinafter "Braverman").

This rejection is traversed on the basis that Braverman fails to disclose a method for tagging nucleic acid molecules in a way that results in the segments of a particular nucleic acid molecule being tagged with the same tag sequence. Because Braverman fails to disclose this element of the rejected claims, Brenner cannot anticipate the claims.

Withdrawal of this rejection is requested.

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REJECTIONS UNDER §103(A)

Claims 21-39 were rejected under 35 U.S.C. §103(a) for allegedly being unpatentable over Braverman, et al. (U.S. Publication no. 20090105959, hereinafter "Braverman") in view of Bosnes (International Publication No. WO2003062462, hereinafter "Bosnes").

In order to meet its burden in establishing a rejection under 35 U.S.C. §103(a), the Office must first demonstrate that a prior art reference, or references when combined, teach or suggest all claim elements. See, *e.g.*, *Süd Chemie, Inc. v. Multisorb Technologies, Inc.* 554 F. 3d 1001 (Fed. Cir. 2009); *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1740 (2007); *Pharmastem Therapeutics v. Viacell et al.*, 491 F.3d 1342, 1360 (Fed. Cir. 2007); MPEP § 2143(A)(1).

Braverman is deficient because it fails to teach or suggest a method for tagging nucleic acid molecules in a way that results in the segments of a particular nucleic acid molecule being tagged with the same tag sequence.

Bosnes, which is cited solely to provide a way to analyze mRNA, fails to meet Braverman's deficiency and, as such, the cited references, taken in any combination, fail to provide each and every element of the rejected claims.

The Applicants submit that this rejection has been adequately addressed. Withdrawal of this rejection is requested.

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

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The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number CGLC-020CON5.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: June 29, 2016

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie
Registration No. 48,920

Enclosure(s): IDS to cite WO2012148497, US9,290,808

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ABSTRACT

Aspects of the present invention are drawn to processes for analyzing nucleic acids from a single cell source. In certain embodiments, the method involves tagging the plurality of nucleic acids from the single cell source with multiplex identifier (MID) sequences and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences. Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

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Electronic Patent Application Fee Transmittal

Application Number:	14792094
Filing Date:	06-Jul-2015
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	James Samuel Keddie
Attorney Docket Number:	CGLC-020CON5

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	2253	1	700	700
Miscellaneous:				
Submission- Information Disclosure Stmt	2806	1	90	90
		Total in USD ($)		**790**

Electronic Acknowledgement Receipt	
EFS ID:	26213420
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	29-JUN-2016
Filing Date:	06-JUL-2015
Time Stamp:	18:37:53
Application Type:	Utility under 35 USC 111(a)

Payment information:

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Warnings:					
Information:					
2	Foreign Reference	WO2012148497--101-200.pdf	16327197 [illegible]	no	100
Warnings:					
Information:					
3	Foreign Reference	WO2012148497--201-309.pdf	10271551 [illegible]	no	109
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Form (SB08)	CGLC-020CON5-IDS-1449.pdf	612288 [illegible]	no	4
Warnings:					
Information:					
5		CGLC-020CON5_Resp_OA_dated_January_13_2016.pdf	70002 [illegible]	yes	11

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Abstract	2	2
Claims	3	6

	Applicant Arguments/Remarks Made in an Amendment		7	10	
	Abstract		11	11	
Warnings:					
Information:					
6	Fee Worksheet (SB06)	fee-info.pdf	32521 633c27737ca5fcc0e76516986779923e7c9c3931	no	2
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National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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(54) Title: IDENTIFICATION OF POLYNUCLEOTIDES ASSOCIATED WITH A SAMPLE

(57) Abstract: Disclosed herein are compositions and methods for sequencing, analyzing, and utilizing samples such as single samples. Also disclosed herein are compositions and methods for matching together two or more sequences from a sample. Also disclosed herein are compositions and methods for expressing and screening molecules of interest.

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TITLE

Identification of Polynucleotides Associated With a Sample.

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is related to U.S. provisional patent application No. 61/517,976, filed April 28, 2011, U.S. provisional patent application No. 61/575,652, filed August 24, 2011, U.S. provisional patent application No. 61/599,870, filed February 16, 2012, and U.S. provisional patent application No. 61/608,571, filed March 8, 2012; the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under N01-HV-28183 NHLBI Proteomics Center and N01-HV-00242 NHLBI Proteomics Center awarded by the National Heart Lung and Blood Institute of the U.S. National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] This instant application contains a "lengthy" Sequence Listing which has been submitted via CD-R, in lieu of a printed paper copy, and is hereby incorporated by reference in its entirety. Said CD-R, recorded on April 25, 2012, are labeled "CRF," "Copy 1 – SEQUENCE LISTING PART, " "Copy 2 – SEQUENCE LISTING PART" and "Copy 3 - SEQUENCE LISTING PART," respectively, and each contains only one single self-extracting file named 20786PCT_CRF_Sequencelising.EXE (138,571,776 bytes), which subsequently contains one uncompressed ASCII text file named 20786PCT_CRF_Sequencelisting.TXT (795,566,080 bytes).

BACKGROUND

[0004] Producing therapeutic monoclonal antibodies from human sources is biologically and technically challenging. To date, several approaches have been described, including generating human hybridomas, using transgenic mice expressing human immunoglobulins, and using human immunoglobulin phage display libraries.

[0005] Human hybridomas can be difficult to generate because human myeloma fusion partners, unlike their mouse counterparts, are inefficient at generating hybridomas. Human hybridomas also have a tendency to spontaneously lose the expressed antibody genes after prolonged culture. Epstein-Barr virus (EBV) transformation immortalizes B cells, but only extremely small fractions of all of the EBV-transformed B cells are affinity matured or recognize the target

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antigen. The generation of hybridomas typically includes large screens to obtain therapeutic monoclonal antibodies. None of the therapeutic monoclonal antibodies currently approved by the U.S. F.D.A. were created through the generation of human hybridomas or EBV transformation of B cells, attesting to the technical difficulties and challenges posed by these methods.

[0006] Phage display libraries of human antibody sequences represent another method for producing therapeutic human monoclonal antibodies. This method utilizes recombinant DNA technology to randomly express human antibody heavy- and light-chain sequences together to enable screening for combinations that bind to the target antigen. However, this strategy does not produce affinity-matured antibodies, and antibodies produced in this way usually bind to antigen with low affinity and avidity. Successive mutation and selection/screening steps are then needed to generate high-affinity antibodies.

[0007] Another way to produce therapeutic human monoclonal antibodies is by creating or using transgenic mice that possess a human antibody repertoire. When immunized, such mice produce antibodies that target the immunizing antigen, and hybridomas can then be generated for the production of therapeutic human monoclonal antibodies. Such transgenic mice are proprietary and not commonly available for use in generating human antibodies.

[0008] Thus the inventors have identified a need for compositions, kits, and methods that can, e.g., produce large numbers of affinity-matured human antibodies, avoiding the need for laborious and time-consuming humanization of an antibody, or the need to conduct extensive screens. The compositions, kits, and methods described herein address this need. In addition, the compositions, kits, and methods described herein are broadly applicable outside the human antibody space and can be used in a number of different applications including, e.g., matching together two or more polynucleotides of interest that are derived from a single sample and present in a library of polynucleotides.

SUMMARY

[0009] Disclosed herein is a composition comprising a polynucleotide, wherein the polynucleotide comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises at least one identification region, and wherein the first region is coupled to the second region.

[0010] In some aspects, the variable immunoglobulin region comprises a VDJ region of an IgG immunoglobulin nucleotide sequence isolated from an activated human B cell greater than or equal to 8 μm in diameter, and wherein the 5' end of the immunoglobulin region is coupled to

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the 3' end of the identification region. In some aspects, the composition is comprised in a clonal family.

[0011] In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is an activated B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasmablast. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell located in the blood of a subject. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a human activated B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a memory B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasma cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is an antigen-specific B cell. In some aspects, the immunoglobulin region is isolated from a mammalian B cell. In some aspects, the immunoglobulin region is isolated from a human B cell. In some aspects, the immunoglobulin region is isolated from a mouse B cell. In some aspects, the immunoglobulin region is isolated from a B cell from a subject with a disease or condition of interest. In some aspects, the immunoglobulin region is isolated from a B cell from a subject recovering from or recovered from a disease or condition of interest. In some aspects, the immunoglobulin region is isolated from a B cell from a subject administered with at least one antigen of interest. In some aspects, the immunoglobulin region is isolated from a B cell from a subject administered with at least one antigen of interest and an adjuvant. In some aspects, the immunoglobulin region is isolated from a B cell located in the blood of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in the bone marrow of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in the spleen of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in at least one lymph node of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in lymphoid tissue of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in the gut of a subject. In some aspects, the immunoglobulin region is isolated from an activated B cell that is about 8-20 μm in diameter. In some aspects, the immunoglobulin region is isolated from an activated B cell that is 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or greater than 20 μm in diameter. In some aspects, the immunoglobulin region is isolated from an activated B cell that is about 60, 70, 80, 90, 100, 120, 130, 140, 150, 200, 250, 300, 350, or greater than 350 μm^2 in area. In some

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aspects, the immunoglobulin region is isolated from an activated B cell that is about 250, 268, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, or greater than 4000 μm^3 in volume. In some aspects, the immunoglobulin region is isolated from an activated B cell that has a diameter of 10% or greater in size than the median diameter of a control resting B cell. In some aspects, the immunoglobulin region is isolated from an activated B cell that has a diameter of 15% or greater in size than the median diameter of a control resting B cell. In some aspects, the immunoglobulin region is isolated from an activated B cell that has a diameter of 20% or greater in size than the median diameter of a control resting B cell. In some aspects, the immunoglobulin region is isolated from an activated B cell capable of secreting immunoglobulin. In some aspects, the immunoglobulin region is isolated from a B cell in the gap 1 (G1), synthesis (S), gap 2 (G2), or mitosis (M) phase of the cell cycle. In some aspects, the immunoglobulin region is isolated from a B cell is not in the gap 0 (G0) phase of the cell cycle. In some aspects, the immunoglobulin region is isolated from a B cell characterized as having an FSC greater than 1.2x of the FSC mean of resting B lymphocytes by flow cytometry. In some aspects, the immunoglobulin region is isolated from a B cell characterized as having an FSC mean between 0.7 - 1.15x of the FSC mean of human monocytes by flow cytometry. In some aspects, the immunoglobulin region is isolated from a single CD19 positive B cell. In some aspects, the immunoglobulin region is isolated from a single CD38 positive B cell. In some aspects, the immunoglobulin region is isolated from a single CD27 positive B cell. In some aspects, the immunoglobulin region is isolated from a single CD20 negative B cell. In some aspects, the immunoglobulin region is isolated from a single $CD19^{+}CD20^{-}CD27^{+}CD38^{hi}$ B cell.

[0012] In some aspects, the 5' end of the immunoglobulin region is coupled to the 3' end of the identification region.

[0013] In some aspects, the variable immunoglobulin region comprises a VDJ region of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises a VJ region of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises a V, D, and/or J region of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises a heavy and/or light chain of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises an IgG, IgM, IgD, IgE, or IgA immunoglobulin sequence. In some aspects, the variable immunoglobulin region comprises a human IgG1, IgG2, IgG3, or IgG4 immunoglobulin sequence. In some aspects, the variable immunoglobulin region comprises a mouse IgG1, IgG2a, IgG2b, or IgG3 immunoglobulin sequence. In some aspects,

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the immunoglobulin region is about 200-2000 nucleotides in length. In some aspects, the immunoglobulin region is less than 200, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or greater than 2000 nucleotides in length.

[0014] In some aspects, the identification region comprises a plurality of identification regions. In some aspects, the identification region comprises a plurality of identification regions, and wherein each identification region in the plurality has a distinct sequence. In some aspects, the identification region comprises at least one sample identification region and at least one plate identification region. In some aspects, the identification region comprises a sequence distinct from the sequence of the immunoglobulin region. In some aspects, the identification region is about 2 - 100 nucleotides in length. In some aspects, the identification region is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, or greater than 100 nucleotides in length. In some aspects, the identification region is 2 – 1,000 nucleotides in length. In some aspects, the identification region is equal to or greater than 100 nucleotides in length. In some aspects, the identification region comprises a contiguous non-coding nucleotide sequence. In some aspects, the identification region comprises a non-coding nucleotide sequence. In some aspects, the identification region comprises a non-contiguous, non-coding nucleotide sequence. In some aspects, the length of the sequence of the identification region is less than the length of the sequence of the immunoglobulin region.

[0015] In some aspects, a composition described herein can include a third region, wherein the third region comprises an adapter region. In some aspects, the third region comprises an adapter region, and wherein the third region is located between the first region and the second region. In some aspects, the third region comprises an adapter region, and wherein the adapter region comprises at least one G nucleotide located at its 3' end.

[0016] In some aspects, the identification region is 2-100 nucleotides long and has a sequence distinct from the immunoglobulin region sequence, and wherein the adaptor region comprises at least one G nucleotide at its 3' end and is located 3' of the sample identification region and 5' of the immunoglobulin region, and wherein the immunoglobulin variable region has undergone hypermutation and differs from the germline sequence of a naïve B cell.

[0017] In some aspects, the composition is present in a container. In some aspects, a plurality of the compositions are present in a container. In some aspects, a plurality of the compositions are present in a single well of a single plate comprising a plurality of wells.

[0018] In some aspects, the composition is in a library of compositions, wherein each composition is present in a separate container, wherein each composition comprises a

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polynucleotide comprising a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region o is distinct from the nucleotide sequence of the other identification regions present in the library, and wherein the last nucleotide sequences of a plurality of variable immunoglobulin regions in the library share at least 80-99% sequence identity.

[0019] In some aspects, the composition is comprised in a library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide, wherein the polynucleotide comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, and wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in each separate container in the library.

[0020] Also described herein is a polynucleotide composition library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide, wherein the polynucleotide comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, and wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in each separate container in the library.

[0021] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide of the plurality is present in a separate container, wherein each polynucleotide of the plurality comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in the library, and wherein at least two variable immunoglobulin regions in the plurality share at least 80-99% sequence identity.

[0022] Also described herein is a polynucleotide library comprising a clonal family of polynucleotides, wherein each polynucleotide in the family comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region

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and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in the family, and wherein each of the variable immunoglobulin regions in the family exhibit at least 80-99% sequence identity. In some aspects, the library comprises a plurality of clonal families.

[0023] Also described herein is a clonal family of immunoglobulin sequences wherein each sequence in the family is coupled to an identification region. In some aspects, each identification region is distinct from the other identification regions. In some aspects, the immunoglobulin sequences comprise heavy chain immunoglobulin sequences. In some aspects, the immunoglobulin sequences comprise light chain immunoglobulin sequences. In some aspects, the immunoglobulin sequences comprise heavy chain and light chain immunoglobulin sequences. In some aspects, one or more of the identification regions comprise a light chain immunoglobulin sequence. In some aspects, one or more of the identification regions comprise a heavy chain immunoglobulin sequence.

[0024] Also described herein is a set of two or more of the clonal families described herein.

[0025] Also described herein is a set of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more of the clonal families described herein.

[0026] Also described herein is a clonal family of immunoglobulin sequences wherein each sequence in the family is operatively coupled to at least one contiguous nucleotide sequence. In some aspects, the immunoglobulin sequences comprise heavy chain immunoglobulin sequences and the at least one contiguous nucleotide sequence comprises a light chain immunoglobulin sequence. In some aspects, the immunoglobulin sequences comprise light chain immunoglobulin sequences and the at least one contiguous nucleotide sequence comprises a heavy chain immunoglobulin sequence.

[0027] Also described herein is a method of producing a clonal family of immunoglobulin sequences comprising obtaining a plurality of immunoglobulin sequences each having V, D, and/or J regions and each coupled to an identification region; and grouping two or more sequences from the plurality to produce the clonal family wherein each sequence in the clonal family is a mutated version of the same germline immunoglobulin sequence having a V, D, and/or J region or the germline immunoglobulin sequence having the V, D, and/or J region.

[0028] In some aspects, each identification region is distinct from the other identification regions.

[0029] Also described herein is a method of producing a clonal family of immunoglobulin sequences comprising obtaining a plurality of immunoglobulin sequences each having V, D,

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and/or J regions and each coupled to an identification region, and wherein each identification region is distinct from the other identification regions; removing one or more identification regions; and grouping two or more sequences from the plurality to produce the clonal family wherein each sequence in the clonal family is a mutated version of the same germline immunoglobulin sequence having a V, D, and/or J region or the germline immunoglobulin sequence having the V, D, and/or J region.

[0030] Also described herein is a method of identifying a second cDNA coupled to a first identification region comprising selecting a first cDNA coupled to the first identification region and identifying the second cDNA based on the shared identity of the identification region coupled to each cDNA.

[0031] Also described herein is a method of producing a 3' tail on a second nucleotide sequence comprising obtaining a first nucleotide sequence and contacting the first nucleotide sequence with a thermal stable RNase H- reverse transcriptase having template switching activity at less than 50°C, wherein the contacting produces the 3' tail and the second nucleotide sequence. In some aspects, the first nucleotide sequence is contacted at about less than 50, 49, 48, 47, 46, 45 ,44, 43, 42, or less than 42°C. In some aspects, the first nucleotide sequence is contacted at 42°C. In some aspects, the first nucleotide sequence is contacted at 45.5°C. In some aspects, the transcriptase is a Moloney Murine Leukemia Virus (MMLV) RNase H- reverse transcriptase. In some aspects, the transcriptase is SuperScript III.

[0032] Also described herein is a method for determining the naturally occurring sequence of a first sequence of interest comprising obtaining a plurality of sequences related to the first sequence and each coupled to a first identification region, wherein each first identification region is identical, and wherein one or more of the sequences in the plurality is distinct from the naturally occurring sequence; and comparing the sequences in the plurality to determine the naturally occurring sequence of the first sequence of interest. In some aspects, the plurality of sequences comprise immunoglobulin sequences. In some aspects, the plurality of sequences comprise immunoglobulin sequences. In some aspects, the plurality of sequences comprise immunoglobulin sequences. In some aspects, the plurality of sequences are each coupled to a second identification region and each second identification region is identical. In some aspects, the first sequence of interest is an immunoglobulin sequence. In some aspects, the plurality of sequences are immunoglobulin sequences.

[0033] Also described herein is a composition comprising a polynucleotide comprising a first region and a second region, wherein the first region comprises a B cell-derived variable

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immunoglobulin region and the second region comprises an identification region, and wherein the first region is coupled to the second region.

[0034] Also described herein is a polynucleotide composition library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide comprising a B cell-derived variable immunoglobulin region and an identification region, wherein the variable immunoglobulin region is coupled to the identification region, wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in each separate container in the library.

[0035] Also described herein is a method for producing a polynucleotide composition, comprising: obtaining a polynucleotide comprising a first region, wherein the first region comprises an expressed B cell variable immunoglobulin region associated with a subject; and generating the polynucleotide composition comprising the first region and a second region by coupling the first region to the second region, wherein the second region comprises an identification region.

[0036] In some aspects, obtaining the polynucleotide comprises obtaining a B cell associated with the subject and processing the cell to prepare the polynucleotide. In some aspects, obtaining the polynucleotide comprises receiving the polynucleotide directly or indirectly from a third party that has processed a B cell associated with the subject to prepare the polynucleotide. In some aspects, obtaining the polynucleotide comprises receiving the polynucleotide directly or indirectly from a third party that has solubilized a B cell associated with the subject to prepare the polynucleotide. In some aspects, obtaining the polynucleotide comprises obtaining a B cell using a flow cytometer. In some aspects, obtaining the polynucleotide comprises obtaining a B cell using a microfluidic device.

[0037] In some aspects, the variable immunoglobulin region comprises a VDJ region of an IgG immunoglobulin nucleotide sequence isolated from an activated human B cell greater than or equal to 8 μm in diameter, and wherein the 5' end of the immunoglobulin region is coupled to the 3' end of the identification region. In some aspects, the composition is comprised in a clonal family.

[0038] In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is an activated B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasmablast. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell. In some

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aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell located in the blood of a subject. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a human activated B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a memory B cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasma cell. In some aspects, the immunoglobulin region is isolated from a B cell, and wherein the B cell is an antigen--specific B cell. In some aspects, the immunoglobulin region is isolated from a mammalian B cell. In some aspects, the immunoglobulin region is isolated from a human B cell. In some aspects, the immunoglobulin region is isolated from a mouse B cell. In some aspects, the immunoglobulin region is isolated from a B cell from a subject with a disease or condition of interest. In some aspects, the immunoglobulin region is isolated from a B cell from a subject recovering from or recovered from a disease or condition of interest. In some aspects, the immunoglobulin region is isolated from a B cell from a subject administered with at least one antigen of interest. In some aspects, the immunoglobulin region is isolated from a B cell from a subject administered with at least one antigen of interest and an adjuvant. In some aspects, the immunoglobulin region is isolated from a B cell located in the blood of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in the bone marrow of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in the spleen of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in at least one lymph node of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in lymphoid tissue of a subject. In some aspects, the immunoglobulin region is isolated from a B cell located in the gut of a subject. In some aspects, the immunoglobulin region is isolated from an activated B cell that is about 8-20 μm in diameter. In some aspects, the immunoglobulin region is isolated from an activated B cell that is 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or greater than 20 μm in diameter. In some aspects, the immunoglobulin region is isolated from an activated B cell that is about 60, 70, 80, 90, 100, 120, 130, 140, 150, 200, 250, 300, 350, or greater than 350 μm^2 in area. In some aspects, the immunoglobulin region is isolated from an activated B cell that is about 250, 268, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, or greater than 4000 μm^3 in volume. In some aspects, the immunoglobulin region is isolated from an activated B cell that has a diameter of 10% or greater in size than the median diameter of a control resting B cell. In some aspects, the immunoglobulin region is isolated from an activated B cell that has a diameter of 15% or greater in size than the median diameter of a control resting B cell. In some aspects, the immunoglobulin region is isolated from an activated B cell that has a diameter of 20% or greater

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in size than the median diameter of a control resting B cell. In some aspects, the immunoglobulin region is isolated from an activated B cell capable of secreting immunoglobulin. In some aspects, the immunoglobulin region is isolated from a B cell in the gap 1 (G1), synthesis (S), gap 2 (G2), or mitosis (M) phase of the cell cycle. In some aspects, the immunoglobulin region is isolated from a B cell is not in the gap 0 (G0) phase of the cell cycle. In some aspects, the immunoglobulin region is isolated from a B cell characterized as having an FSC greater than 1.2x of the FSC mean of resting B lymphocytes by flow cytometry. In some aspects, the immunoglobulin region is isolated from a B cell characterized as having an FSC mean between 0.7 - 1.15x of the FSC mean of human monocytes by flow cytometry. In some aspects, the immunoglobulin region is isolated from a single CD19 positive B cell. In some aspects, the immunoglobulin region is isolated from a single CD38 positive B cell. In some aspects, the immunoglobulin region is isolated from a single CD27 positive B cell. In some aspects, the immunoglobulin region is isolated from a single CD20 negative B cell. In some aspects, the immunoglobulin region is isolated from a single $CD19^{+}CD20^{-}CD27^{+}CD38^{hi}$ B cell.

[0039] In some aspects, the 5' end of the immunoglobulin region is coupled to the 3' end of the identification region.

[0040] In some aspects, the variable immunoglobulin region comprises a VDJ region of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises a VJ region of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises a V, D, and/or J region of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises a heavy and/or light chain of an immunoglobulin nucleotide sequence. In some aspects, the variable immunoglobulin region comprises an IgG, IgM, IgD, IgE, or IgA immunoglobulin sequence. In some aspects, the variable immunoglobulin region comprises a human IgG1, IgG2, IgG3, or IgG4 immunoglobulin sequence. In some aspects, the variable immunoglobulin region comprises a mouse IgG1, IgG2a, IgG2b, or IgG3 immunoglobulin sequence. In some aspects, the immunoglobulin region is about 200-2000 nucleotides in length. In some aspects, the immunoglobulin region is less than 200, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or greater than 2000 nucleotides in length.

[0041] In some aspects, the identification region comprises a plurality of identification regions. In some aspects, the identification region comprises a plurality of identification regions, and wherein each identification region in the plurality has a distinct sequence. In some aspects, the

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identification region comprises at least one sample identification region and at least one plate identification region. In some aspects, the identification region comprises a sequence distinct from the sequence of the immunoglobulin region. In some aspects, the identification region is about 2 - 100 nucleotides in length. In some aspects, the identification region is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, or greater than 100 nucleotides in length. In some aspects, the identification region is 2 – 1,000 nucleotides in length. In some aspects, the identification region is equal to or greater than 100 nucleotides in length. In some aspects, the identification region comprises a contiguous non-coding nucleotide sequence. In some aspects, the identification region comprises a non-coding nucleotide sequence. In some aspects, the identification region comprises a non-contiguous, non-coding nucleotide sequence. In some aspects, the length of the sequence of the identification region is less than the length of the sequence of the immunoglobulin region.

[0042] In some aspects, a composition described herein can include a third region, wherein the third region comprises an adapter region. In some aspects, the third region comprises an adapter region, and wherein the third region is located between the first region and the second region. In some aspects, the third region comprises an adapter region, and wherein the adapter region comprises at least one G nucleotide located at its 3' end.

[0043] In some aspects, the identification region is 2-100 nucleotides long and has a sequence distinct from the immunoglobulin region sequence, and wherein the adaptor region comprises at least one G nucleotide at its 3' end and is located 3' of the sample identification region and 5' of the immunoglobulin region, and wherein the immunoglobulin variable region has undergone hypermutation and differs from the germline sequence of a naïve B cell.

[0044] In some aspects, the composition is present in a container. In some aspects, a plurality of the compositions are present in a container. In some aspects, a plurality of the compositions are present in a single well of a single plate comprising a plurality of wells.

[0045] In some aspects, the composition is in a library of compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide comprising a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region o is distinct from the nucleotide sequence of the other identification regions present in the library, and wherein the last nucleotide sequences of a plurality of variable immunoglobulin regions in the library share at least 80-99% sequence identity.

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[0046] In some aspects, the composition is comprised in a library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide, wherein the polynucleotide comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, and wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in each separate container in the library.

[0047] Also described herein is a method for producing a polynucleotide composition, comprising: obtaining a B cell associated with a subject; isolating polynucleotides from the cell comprising an expressed B cell variable immunoglobulin region; and generating the polynucleotide composition comprising the variable immunoglobulin region and an identification region by coupling the variable immunoglobulin region to the identification region.

[0048] Also described herein is a method for producing a polynucleotide composition, comprising: obtaining a polynucleotide comprising a B cell-derived variable immunoglobulin region associated with a subject; and generating the polynucleotide composition comprising the variable immunoglobulin region and an identification region by coupling the variable immunoglobulin region to the identification region.

[0049] In some aspects, obtaining the polynucleotide comprises obtaining a B cell and processing the cell to prepare the polynucleotide. In some aspects, obtaining the polynucleotide comprises receiving the polynucleotide directly or indirectly from a third party that has processed a B cell to prepare the polynucleotide.

[0050] Also described herein is a method for producing two or more polynucleotide compositions, comprising: obtaining a polynucleotide library comprising a plurality of polynucleotides associated with a plurality of samples obtained from one or more subjects, wherein one or more polynucleotides comprises an expressed B cell variable immunoglobulin region, wherein each sample is associated with a B cell, and wherein each polynucleotide associated with each sample is present in a separate container; and generating two or more polynucleotide compositions each comprising a polynucleotide from the plurality of polynucleotides and an identification region by coupling the polynucleotide to the identification region, wherein the sequence of each identification region is distinct from the sequence of the identification regions coupled to the other polynucleotides in the library.

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[0051] In some aspects, obtaining the polynucleotide library comprises obtaining a plurality of B cells and processing the cells to prepare the polynucleotide library. In some aspects, obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has processed a plurality of B cells to prepare the polynucleotide library.

[0052] Also described herein is a method for producing two or more polynucleotide compositions, comprising:obtaining a polynucleotide library comprising a plurality of polynucleotides associated with a plurality of samples obtained from one or more subjects, wherein one or more polynucleotides comprises a B cell-derived variable immunoglobulin region, and wherein each polynucleotide associated with each sample is present in a separate container; and generating two or more polynucleotide compositions each comprising a polynucleotide from the plurality of polynucleotides and an identification region by coupling the polynucleotide to the identification region, wherein the sequence of each identification region is distinct from the sequence of the identification regions coupled to the other polynucleotides in the library.

[0053] In some aspects, obtaining the polynucleotide library comprises obtaining a plurality of B cells and processing the cells to prepare the polynucleotide library. In some aspects, obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has processed a plurality of B cells to prepare the polynucleotide library.

[0054] Also described herein is a polynucleotide composition library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a single sample-derived cDNA region comprising the nucleotide C at the 3' end of the cDNA region and a sample identification-adapter region comprising a sample identification region coupled to an adapter region, wherein the nucleotide sequence of the sample identification region of each sample identification-adapter region is distinct from the nucleotide sequence of the sample identification region of the other sample identification-adapter regions present in each separate container in the library, wherein the adapter region comprises the nucleotide G at the 3' end of the adapter region, and wherein the sample identification-adapter region is attached to the cDNA region by binding between the C and G.

[0055] In some aspects, the cDNA region comprises an RNA polynucleotide hybridized to a DNA polynucleotide. In some aspects, the cDNA region comprises an mRNA polynucleotide hybridized to a cDNA polynucleotide. In some aspects, the cDNA region comprises at least one C at the 3' end and wherein the adapter region comprises at least one G at the 3' end.

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[0056] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a sample identification region, an adapter region, and a single sample-derived cDNA region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the cDNA region is coupled to the 3' end of the adapter region, wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample, and wherein the sample identification region is double-stranded. In some aspects, each polynucleotide comprises a plurality of sample identifications regions.

[0057] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[0058] In some aspects, the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises the sequence CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G. In some aspects, the sequence of the universal primer region is not fully complementary to any human gene exon, and wherein the universal primer region has minimal secondary structure that does not interfere with the adapter region. In some aspects, the universal primer region is the sequence CACGACCGGTGCTCGATTTAG. In some aspects, the amplicon region comprises a cDNA region comprising a cDNA nucleotide sequence. In some aspects, the sequence of the sample identification region of each polynucleotide from a first single sample differs by at least 1 nucleotide from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample. In some aspects, the sequence of the sample identification region of each polynucleotide from a first single sample differs by at least 2 nucleotides from the sequence of the sample identification region of

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the other polynucleotides in the library from one or more samples distinct from the first single sample. In some aspects, the sample identification region is at least 10 nucleotides in length. In some aspects, the sample identification region is at least 1 nucleotide in length. In some aspects, the sequence of each sample identification region is selected from Tables 2 and 7. In some aspects, the sequence of the adapter region comprises at least one G nucleotide at its 3' end. In some aspects, the amplicon region comprises an immunoglobulin heavy chain amplicon sequence, an immunoglobulin light chain amplicon sequence, a T cell receptor alpha amplicon sequence, or a T cell receptor beta amplicon sequence.

[0059] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-3', wherein A is a universal primer region, wherein B is a sample identification region, wherein C is an adapter region, wherein D is an amplicon region from a single sample, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[0060] Also described herein is a polynucleotide comprising a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region.

[0061] In some aspects, the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

[0062] Also described herein is a polynucleotide comprising the sequence 5'-A-B-C-D-3', wherein A is a universal primer region, wherein B is a sample identification region, wherein C is an adapter region, and wherein D is an amplicon region from a single sample.

[0063] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a first plate identification region, a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first plate identification region is

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operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[0064] In some aspects, the sequence of the first plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the first plate identification region of each polynucleotide from the first set of single samples differs by at least 1 nucleotide from the sequence of the first plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the first plate identification region of each polynucleotide from the first set of single samples differs by at least 2 nucleotides from the sequence of the first plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples. In some aspects, the first plate identification region is at least 10 nucleotides in length. In some aspects, the sequence of the first plate identification region is selected from Tables 3 and 6. In some aspects, the 3' end of the first plate identification region is coupled to the 5' end of the universal primer region, wherein the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, wherein the adapter region comprises at least one G, wherein each polynucleotide further comprises a second plate identification region, a first sequencing region, and a second sequencing region, wherein the 5' end of the second plate identification region is coupled to the 3' end of the amplicon region, wherein the 3' end of the first sequencing region is coupled to the 5' end of the first plate identification region, and wherein the 5' end of the second sequencing region is coupled to the 3' end of the second plate identification region. In some aspects, the sequence of the second plate identification region is identical to the sequence of the first plate identification region on each polynucleotide. In some aspects, the sequence of the second plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the second plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the second plate identification region of each polynucleotide from the first set of single samples

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differs by at least 1 nucleotide from the sequence of the second plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the second plate identification region of each polynucleotide from the first set of single samples differs by at least 2 nucleotides from the sequence of the second plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples. In some aspects, the second plate identification region is at least 10 nucleotides in length. In some aspects, the sequence of the second plate identification region is selected from Tables 3 and 6. In some aspects, the first sequencing region comprises GAGAGACTGACAGCGTATCGCCTCCCTCGCGCCATCAG. In some aspects, the second sequencing region comprises CTATGCGCCTTGCCAGCCCGCTCAG.

[0065] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-E-3', wherein A is a plate identification region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, wherein E is an amplicon region from a single sample, and wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample

[0066] Also described herein is a polynucleotide comprising a first plate identification region, a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first plate identification region is operatively coupled to the universal primer region, and wherein the amplicon region is operatively coupled to the adapter region.

[0067] In some aspects, the 3' end of the first plate identification region is coupled to the 5' end of the universal primer region, wherein the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, wherein the adapter region comprises at least one G, wherein each polynucleotide further comprises a second plate identification region, a first sequencing region, and a second sequencing region, wherein the 5' end of the second plate identification region is coupled to the 3' end of the amplicon region, wherein the 3' end of the

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first sequencing region is coupled to the 5' end of the first plate identification region, and wherein the 5' end of the second sequecing region is coupled to the 3' end of the second plate identification region.

[0068] Also described herein is a polynucleotide comprising the sequence 5'-A-B-C-D-E-3', wherein A is a plate identification region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, and wherein E is an amplicon region from a single sample.

[0069] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a first restriction site region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second restriction site region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first restriction site region is operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, wherein the second restriction site region is operatively coupled to the amplicon region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[0070] In some aspects, the first restriction site region comprises one or more restriction sites. In some aspects, the first restriction site region comprises one or more restriction sites selected from the group consisting of: NheI, XhoI, BstBI, EcoRI, SacII, BbvCI, PspXI, AgeI, ApaI, KpnI, Acc65I, XmaI, BstEII, DraIII, PacI, FseI, AsiSI and AscI. In some aspects, the second restriction site region comprises one or more restriction sites. In some aspects, the second restriction site region comprises one or more restriction sites selected from the group consisting of: NheI, XhoI, BstBI, EcoRI, SacII, BbvCI, PspXI, AgeI, ApaI, KpnI, Acc65I, XmaI, BstEII, DraIII, PacI, FseI, AsiSI and AscI. In some aspects, the 3' end of the first restriction site region is coupled to the 5' end of the universal primer region, wherein the 3' end of the adapter region is coupled to the 5' end of the amplicon region, wherein the 3' end of the amplicon region is coupled to the 5' end of the second restriction site region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

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[0071] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-E-F-3', wherein A is a first restriction site region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, wherein E is an amplicon region from a single sample, wherein F is a second restriction site region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[0072] Also described herein is a polynucleotide for insertion into a vector, comprising a first restriction site region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second restriction site region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first restriction site region is operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, and wherein the second restriction site region is operatively coupled to the amplicon region.

[0073] In some aspects, the 3' end of the first restriction site region is coupled to the 5' end of the universal primer region, wherein the 3' end of the adapter region is coupled to the 5' end of the amplicon region, wherein the 3' end of the amplicon region is coupled to the 5' end of the second restriction site region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

[0074] Also described herein is a polynucleotide for insertion in a vector, comprising the sequence 5'-A-B-C-D-E-F-3', wherein A is a first restriction site region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, wherein E is an amplicon region from a single sample, and wherein F is a second restriction site region.

[0075] Also described herein is a polynucleotide adapter molecule, comprising a universal primer region, a sample identification region, and an adapter region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, and wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region. In some aspects, the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

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[0076] Also described herein is a polynucleotide primer, comprising a universal primer region and a plate identification region, and wherein the 3' end of the plate identification region is coupled to the 5' end of the universal primer region. In some aspects, the universal primer region comprises CACGACCGGTGCTCGATTTAG, wherein the primer further comprises a sequencing region, and wherein the 3' end of the sequencing region is coupled to the 5' end of the plate identification region.

[0077] Also described herein is a vector comprising a polynucleotide described herein. In some aspects, the vector comprises a plurality of polynucleotides. In some aspects, the vector is selected from the group consisting of: pEE6.4 and pEE12.4

[0078] Also described herein is an isolated host cell comprising a vector described herein or a polynucleotide described herein. In some aspects, the host cell is selected from the group consisting of: CHO cells, CHO-K1 cells, CHO-S cells, NS0 cells, CHO cells that are dhfr-, CHO-dhfr-, DUKX-B11 CHO cells, and DG44 CHO cells.

[0079] Also described herein is a method for producing one or more polynucleotides of interest, comprising: obtaining a cDNA library comprising a plurality of cDNAs associated with a plurality of samples obtained from one or more subjects, wherein each cDNA is associated with a single sample in the plurality of samples, and wherein each cDNA associated with each sample is present in a separate container; and adding an adapter molecule to the cDNA associated with each sample to produce the one or more polynucleotides of interest, wherein the adapter molecule comprises a sample identification region and an adapter region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the sequence of the sample identification region of each adapter molecule is distinct from the sequence of the sample identification region of the other adapter molecules added to each cDNA in the library.

[0080] In some aspects, the method further includes allowing the 3' end of the adapter region to attach to the 3' end of each cDNA in the library to produce the one or more polynucleotides of interest. In some aspects, obtaining the cDNA library comprises obtaining the plurality of samples and processing the samples to prepare the cDNA library. In some aspects, the adapter molecule further comprises a universal primer region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region. In some aspects, each cDNA region comprises an mRNA polynucleotide hybridized to a cDNA polynucleotide. In some aspects, each sample comprises a cell. In some aspects, the cell is a B cell. In some aspects, the B cell is a plasmablast, memory B cell, or a plasma cell. In some aspects, each sample comprises a plurality of cells. In some aspects, obtaining the cDNA library comprises receiving

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the cDNA library directly or indirectly from a third party that has processed the plurality of samples to prepare the cDNA library. In some aspects, the adaptor is added by annealing the adaptor to the '3 tail of a cDNA generated during a reverse transcription reaction. In some aspects, each cDNA comprises at least one C nucleotide, wherein C is located at the 3' end of each cDNA, wherein the adapter region comprises at least one G nucleotide, wherein G is located at the 3' end of the adapter region, and wherein the adapter region is attached to each cDNA via binding between the G and C. In some aspects, the adapter molecule is single-stranded, and further comprising incorporating the adapter molecule into each cDNA by allowing an enzyme to make the adapter molecule double-stranded. In some aspects, the adapter molecule is incorporated into each cDNA to produce the polynucleotide of interest by an MMLV H- reverse transcriptase.

[0081] Also described herein is a method of producing one or more polynucleotides of interest for sequencing, comprising: obtaining a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample; and amplifying the polynucleotide library with a set of primers to produce the one or more polynucleotides of interest for sequencing, wherein the one or more polynucleotides of interest for sequencing comprises a first sequencing region, a first plate identification region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second sequencing region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first plate identification region is operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, wherein the first sequencing region is located at the 5' end of the polynucleotide of interest, and wherein the second sequencing region is located at the 3' end of the polynucleotide of interest.

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[0082] In some aspects, the method further includes sequencing the one or more polynucleotides of interest. In some aspects, the method further includes sequencing the one or more polynucleotides of interest with 454 sequencing. In some aspects, the method further includes sequencing the one or more polynucleotides of interest with SMRT sequencing. In some aspects, the method further includes sequencing the one or more polynucleotides of interest with SOLiD sequencing. In some aspects, the method further includes sequencing the one or more polynucleotides of interest with SOLEXA sequencing. In some aspects, the method further includes sequencing the one or more polynucleotides of interest with tSMS sequencing. In some aspects, the set of primers is selected from the primers shown in Tables 1 and 5. In some aspects, obtaining the polynucleotide library comprises preparing the polynucleotide library in a laboratory. In some aspects, obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has prepared the polynucleotide library.

[0083] Also described herein is a method for analyzing sequencing data, comprising: obtaining a dataset associated with a plurality of polynucleotides, wherein the dataset comprises sequencing data for the plurality of polynucleotides, wherein each polynucleotide in the plurality of polynucleotides comprises a sample identification region, and wherein each sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the plurality of polynucleotides from one or more samples distinct from the first single sample; and analyzing the dataset to match together polynucleotides with identical sample identification regions, wherein a match indicates that the polynucleotides originated from the same sample.

[0084] In some aspects, each polynucleotide in the plurality of polynucleotides further comprises a first plate identification region, wherein each combination of each first plate identification region and sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the first plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the plurality of polynucleotides from one or more single sample sets distinct from the first set of single samples, and further comprising analyzing the dataset to match together polynucleotides with identical first plate identification regions and identical sample identification regions, wherein a match between both regions indicates that the polynucleotides originated from the same sample. In some aspects, obtaining the dataset comprises obtaining the plurality of polynucleotides and sequencing the plurality of

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polynucleotides to experimentally determine the dataset. In some aspects, obtaining the dataset comprises receiving the dataset directly or indirectly from a third party that has sequenced the plurality of polynucleotides to experimentally determine the dataset. In some aspects, the dataset is stored on an electronic storage medium. In some aspects, the single sample is a single cell. In some aspects, the single sample comprises a single cell. In some aspects, the single sample comprises a single B cell. In some aspects, the single sample comprises a plurality of B cells. In some aspects, further comprising selecting one or more polynucleotides for cloning.

[0085] Also described herein is a method for identifying a second polynucleotide of interest based on selection of a first polynucleotide of interest, comprising: obtaining a dataset associated with a plurality of polynucleotides, wherein the dataset comprises sequencing data for the plurality of polynucleotides, wherein each polynucleotide in the plurality of polynucleotides comprises a sample identification region, and wherein each sample identification region on each polynucleotide is unique to a single sample thereby associating each polynucleotide in the plurality of polynucleotides with a distinct single sample, wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the plurality of polynucleotides from one or more samples distinct from the first single sample; and selecting a first polynucleotide of interest associated with a first single sample from the dataset and identifying a second polynucleotide of interest in the first single sample based on the sample identification region of the first polynucleotide of interest.

[0086] In some aspects, each polynucleotide in the plurality of polynucleotides further comprises a first plate identification region, wherein each combination of each first plate identification region and sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the first plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the plurality of polynucleotides from one or more single sample sets distinct from the first set of single samples, and further comprising identifying a second polynucleotide of interest in the first single sample based on the sample identification region and first plate identification region of the first polynucleotide of interest. In some aspects, the first single sample comprises a B cell. In some aspects, the first single sample comprises a plurality of B cells. In some aspects, the first single sample comprises a B cell, wherein the first polynucleotide of interest comprises an antibody heavy chain nucleotide sequence, and wherein the second polynucleotide of interest comprises an antibody light chain nucleotide sequence. In some aspects, the first single sample comprises a B cell, wherein the

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first polynucleotide of interest comprises an antibody light chain nucleotide sequence, and wherein the second polynucleotide of interest comprises an antibody heavy chain nucleotide sequence. In some aspects, obtaining the dataset comprises obtaining the plurality of polynucleotides and sequencing the plurality of polynucleotides to experimentally determine the dataset. In some aspects, obtaining the dataset comprises receiving the dataset directly or indirectly from a third party that has sequenced the plurality of polynucleotides to experimentally determine the dataset. In some aspects, the dataset is stored on an electronic storage medium.

[0087] Also described herein is a method of producing one or more polynucleotides of interest for cloning, comprising: obtaining a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample; and amplifying the polynucleotide library with a set of primers to produce the one or more polynucleotides of interest for cloning, wherein the one or more polynucleotides of interest for cloning comprises a first restriction site region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second restriction site region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the amplicon region is operatively coupled to the adapter region, wherein the first restriction site region is located at the 5' end of the polynucleotide of interest, and wherein the second restriction site region is located at the 3' end of the polynucleotide of interest.

[0088] In some aspects, obtaining the polynucleotide library comprises preparing the polynucleotide library in a laboratory. In some aspects, obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has prepared the polynucleotide library.

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[0089] Also described herein is a method of producing a molecule of interest, comprising: obtaining a host cell comprising a polynucleotide comprising a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region; and culturing the host cell under conditions sufficient to produce the molecule of interest. In some aspects, obtaining the host cell comprises preparing the host cell comprising the polynucleotide in a laboratory. In some aspects, obtaining the host cell comprises receiving the host cell comprising the polynucleotide directly or indirectly from a third party that has prepared the host cell. In some aspects, the molecule of interest is a protein. In some aspects, the molecule of interest is an antibody. In some aspects, the molecule of interest is a human monoclonal antibody. In some aspects, further comprising collecting the molecule of interest.

[0090] Also described herein is a kit, comprising a polynucleotide, a polynucleotide library, a vector, or a host cell described herein and instructions for use.

[0091] Also described herein is a method of linking and barcoding a plurality of non-contiguous polynucleotide sequences of interest, said method comprising: (a) providing a plurality of cDNA molecules; (b) physically linking cDNA molecules of interest; and (c) adding a barcode sequence to the cDNAs of interest prior to, during, or after physical linkage.

[0092] In some aspects, the physical linking is by ligation. In some aspects, the physical linking is by recombination. In some aspects, the physical linking comprises using an overlap-extension sequence. In some aspects, the barcode sequence is located at one or both of the ends of the physically linked cDNAs. In some aspects, the barcode sequence is located in between the physically linked cDNAs. In some aspects, the ligation is performed by annealing and ligation of compatible ends. In some aspects, the compatible ends are a restriction site. In some aspects, the ligation is performed by blunt end ligation. In some aspects, the overlap-extension sequence is added during the course of amplification using a primer comprising the overlap-extension tail. In some aspects, the overlap-extension sequence is added during the course of reverse transcription using a primer comprising the overlap-extension tail. In some aspects, the overlap-extension sequence is added by annealing an adaptor to the 3' tail of a cDNA generated during a reverse transcription reaction. In some aspects, the barcode sequence is added by ligation. In some aspects, the ligation is performed by annealing and ligation of compatible ends. In some aspects, the compatible ends are a restriction site. In some aspects, the ligation is performed by blunt end ligation of an adaptor comprising the barcode sequence. In some aspects, the barcode sequence is added during the course of an amplification reaction using a primer comprising the

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barcode sequence. In some aspects, the barcode sequence is added during the course of a reverse transcription reaction using a primer comprising the barcode sequence. In some aspects, the barcode sequence is added by annealing an adaptor to the 3' tail of a cDNA generated during a reverse transcription reaction. In some aspects, the 3' end of the cDNA comprises at least one C nucleotide, and wherein the 3' end of the adaptor comprises at least one G nucleotide, and wherein the adaptor is attached to each cDNA via binding between the C and G. In some aspects, the adaptor is single-stranded, and further comprising incorporating the adaptor into each cDNA by allowing an enzyme to make the adaptor double-stranded. In some aspects, the adaptor is incorporated into each cDNA by an MMLV H- reverse transcriptase. In some aspects, the overlap-extension sequence comprises a barcode sequence. In some aspects, the polynucleotide sequences of interest comprise antibody heavy and light chains. In some aspects, further comprising (d) adding a sequencing region to the cDNAs of interest prior to, during, or after physical linkage. In some aspects, the sequencing region is added with an adaptor. In some aspects, further comprising (e) sequencing of the physically linked cDNA molecules of interest using a NextGen sequencing platform. In some aspects, the NextGen sequencing platform is 454 sequencing. In some aspects, the NextGen sequencing platform is SMRT sequencing. In some aspects, the NextGen sequencing platform is SOLiD sequencing. In some aspects, the NextGen sequencing platform is SOLEXA sequencing. In some aspects, the NextGen sequencing platform is tSMS sequencing. In some aspects, the plurality of cDNA molecules is from single samples contained in a plate with at least 6 wells, at least 12 wells, at least 24 wells, at least 48 wells, at least 96 wells, at least 384 wells, at least 1536 wells, or more wells. In some aspects, the plurality of cDNA molecules is from single samples contained in at least one, two, three, four, five, six, seven, eight, nine, ten, twenty, thirty, forty, fifty, seventy five, one hundred, or more plates with at least 96 wells each.

[0093] Also described herein is a method of linking and barcoding a plurality of samples containing polynucleotide sequences of interest, said method comprising: (a) distributing the samples into a plurality of containers; (b) synthesizing polynucleotide sequences of interest using templates from the sample, wherein said synthesis results in the addition of a barcode sequence; and (c) effecting linkage of the polynucleotide sequences of interest synthesized in step (b).

[0094] In some aspects, each sample comprises a cell. In some aspects, the cell is a B cell. In some aspects, the B cell is a plasmablast, memory B cell, or a plasma cell. In some aspects, each sample comprises a plurality of cells. In some aspects, the polynucleotide sequences of interest comprise antibody heavy and light chains. In some aspects, said synthesis comprises an

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RT-PCR amplification. In some aspects, said RT-PCR amplification is performed in a single step. In some aspects, said linkage of the polynucleotide of interest is performed during the course of an RT-PCR amplification using an overlap-extension primer. In some aspects, further comprising (d) adding a sequencing region to the polynucleotide sequences of interest prior to, during, or after barcode sequence addition or linkage. In some aspects, the sequencing region is added with an adaptor. In some aspects, further comprising (e) sequencing of the linked polynucleotide sequences of interest using a NextGen sequencing platform. In some aspects, the NextGen sequencing platform is 454 sequencing. In some aspects, the NextGen sequencing platform is SMRT sequencing. In some aspects, the NextGen sequencing platform is SOLiD sequencing. In some aspects, the NextGen sequencing platform is SOLEXA sequencing. In some aspects, the NextGen sequencing platform is tSMS sequencing. In some aspects, the plurality of samples are single samples contained in a plate with at least 6 wells, at least 12 wells, at least 24 wells, at least 48 wells, at least 96 wells, at least 384 wells, at least 1536 wells, or more wells. In some aspects, the plurality of samples are single samples contained in at least one, two, three, four, five, six, seven, eight, nine, ten, twenty, thirty, forty, fifty, seventy five, one hundred, two hundred, five hundred or more plates with at least 96 wells each.

[0095] Also described herein is a method of linking and barcoding a plurality of non-contiguous polynucleotide sequences of interest, said method comprising: (a) distributing cells into a plurality of containers to obtain isolated one or more cells; (b) amplifying polynucleotide sequences of interest using templates from said isolated one or more cells, wherein said amplification results in the addition of a barcode sequence; and (c) effecting linkage of the polynucleotide sequences of interest amplified in step (b).

[0096] In some aspects, the nucleotide sequences of interest comprise antibody heavy and light chains. In some aspects, said amplification comprises an RT-PCR amplification. In some aspects, said RT-PCR amplification is performed in a single step. In some aspects, said linkage of the nucleotide of interest is performed during the course of an RT-PCR amplification using an overlap-extension primer. In some aspects, further comprising (d) adding a sequencing region to the polynucleotide sequences of interest prior to, during, or after barcode sequence addition or linkage. In some aspects, the sequencing region is added with an adaptor. In some aspects, further comprising (e) sequencing of the linked polynucleotide sequences of interest using a NextGen sequencing platform. In some aspects, the NextGen sequencing platform is 454 sequencing. In some aspects, the NextGen sequencing platform is SMRT sequencing. In some aspects, the NextGen sequencing platform is SOLiD sequencing. In some aspects, the NextGen sequencing platform is SOLEXA sequencing. In some aspects, the NextGen sequencing

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platform is tSMS sequencing. In some aspects, the one or more cells are contained in a plate with at least 6 wells, at least 12 wells, at least 24 wells, at least 48 wells, at least 96 wells, at least 384 wells, at least 1536 wells, or more wells. In some aspects, the one or more cells are contained in at least one, two, three, four, five, six, seven, eight, nine, ten, twenty, thirty, forty, fifty, seventy five, one hundred, or more plates with at least 96 wells each.

[0097] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-3', wherein A is a sample identification region (barcode sequence), wherein B is a first cDNA region from a single sample, wherein C is a linker region, wherein D is a second cDNA region from the same single sample, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[0098] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-3', wherein A is a first cDNA region from a single sample, wherein B is a linker region, wherein C is a second cDNA region from the same single sample, wherein D is a sample identification region (barcode sequence), and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[0099] Also described herein is a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-3', wherein A is a first cDNA region from a single sample, wherein B is a linker region comprising a sample identification region (barcode sequence), wherein C is a second cDNA region from the same single sample, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

[00100] In some aspects, said first cDNA region comprises an antibody heavy chain and said second cDNA region comprises an antibody light chain. In some aspects, the library comprises at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, at least 100,000, at least 300, 000, at least 1,000,000, at least 3,000,000, at least 10,000,000, at least 30,000,000, or more members. In some aspects, the

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library comprises at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, or more genes of a cell sample's whole transcriptome. In some aspects, the library comprises at least 1, at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 10,000, at least 100,000, at least 1,000,000, at least 10,000,000, at least 100,000,000 or more of the different antibody species present in the blood of an individual. In some aspects, the antibodies are expressed by plasmablasts, plasma cells, memory B cells, long-lived plasma cells, other B lineage cells or combinations thereof.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[00101] These and other features, aspects, and advantages will become better understood with regard to the following description, and accompanying drawings, where:

[00102] **Figure 1. B cell differentiation.** Mature naive B cells are $CD19^+$ and can be activated to proliferate and differentiate upon antigenic challenge in secondary lymphoid tissues such as lymph nodes and spleen. They proliferate and differentiate in either extra-follicular foci or in germinal centers. Differentiating B cells in extrafollicular foci typically differentiate to become short-lived plasma cells and usually reside in the secondary lymphoid tissue they originated from. B cells differentiating in germinal centers can either become memory B cells, which can be further stimulated to differentiate via subsequent antigenic challenge, or become plasmablasts that have the potential to become long-lived plasma cells. These plasmablasts can enter the circulation and are trafficked to the various tissues in which long-lived plasma cells reside, such as the bone marrow, mucosal tissues (for IgA+ plasma cells) and inflamed tissues. Some transiting plasma cells are also present in blood. All of the above mentioned cell types can also be found in circulation in the blood.

[00103] **Figure 2. Schematic of high-throughput sequencing, cloning, and expression of paired genes from single sorted cells.** Desired cell populations are single cell sorted based on their expression of cell surface markers into 96-well PCR plates. During reverse transcription, barcoded (sample-IDs) DNA adaptor molecules are added onto synthesized 1st strand cDNA utilizing the template-switching property of MMLV H^- reverse transcriptases. RT products from each plate are then pooled separately and 2 rounds of PCR performed to amplify specific amplicon regions (amplicons). PCR is done with primers with 5' flanking barcodes to add plate identification regions (plate-IDs) to amplicons. Amplicons are then sent for 454 sequencing (a). Obtained sequences are subset by plate-IDs and sample-IDs before sequence assembly. Amplicons from the same cell are paired together using plate-IDs and sample-IDs and desired clones selected for cloning and expression (b). Specific amplicons can be amplified from each

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pooled plate of amplicons by using cloning primers that are complementary to the sample-ID of that particular amplicon. Cloning primers also add restriction site regions (RS) which are then used to insert the clone into mammalian expression vectors for downstream expression and screening. In this example, the amplicons are immunoglobulin (Ig) heavy and light chain genes which code for antibody. Expressed antibodies can then be used for downstream screening (c). 5' RS and 3' RS: 5' and 3' restriction sites respectively. HC and LC: heavy chain and light chain respectively.

[00104] Figure 3. Schematic of reverse transcription and PCR to add sample-IDs and plate-IDs to Ig amplicons. Reverse transcription (RT) was performed with Superscript II or Superscript III, which are MMLV H- reverse transcriptases. These transcriptases have a 3' tailing activity and add a couple of cytosines to the 3' end of newly synthesized 1st strand cDNAs. An oligonucleotide ending with –GGG (an adapter region) can complement base-pair to this and the reverse transcriptase then switches template to the oligonucleotide and carry on transcription, resulting in the incorporation of the sample-ID adaptor to the 3' end of the cDNA (a). As the sample-ID adaptor contains a 5' invariable sequence (universal primer region), forward primers complementary to this sequence can be used for subsequent PCRs. The 1st PCR was done with Fw Long Primer1, Fw Short Primer1 and GSP1. The Fw Long Primer1 has a 5' flanking region containing a plate-ID barcode and the 454 Titanium Primer A for 454 sequencing, which were incorporated into the amplicon. The Fw Short Primer1 has a Tm similar to the GSP1 primer and was included to slightly increase the efficiency of the PCR. Each GSP1 (gene-specific primer 1) has a complementary gene specific sequence and was used to amplify a specific gene. Here, the gene-specific primers are for the kappa and lambda light chains and the gamma heavy chain to amplify these genes. Sequences for primers are shown (b). The second PCR is a nested PCR and was done with Fw Primer2, Long GSP2 Primer and RV Primer2. Sequences for primers are as shown. Long GSP2 Primer has a 5' flanking region containing a plate-ID barcode and the 454 Titanium Primer B for 454 sequencing, which were incorporated into the amplicon. Long GSP2 primers again amplify the kappa and lambda light chains and the gamma heavy chain. The RV Primer2 has a Tm similar to Fw Primer2 and was included to slightly increase PCR efficiency. Sequences for primers are shown. After RT and 2 PCRs, each amplicon will have 454 Titanium Primers A and B for 454 sequencing, two identical plate-IDs, each identifying the amplicon as coming from a particular single cell-sorted 96-well plate and a sample-ID determining its position on the 96-well plate (c).

[00105] Figure 4. Successful amplification of single-cell sorted B cells using sequencing and cloning primers. A 96-well plate of single-cell sorted B cells were reverse transcribed,

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pooled and amplified as shown in the schematics in Fig. 1a and Fig. 2. Bands for the kappa light chain, lambda light chain, and gamma heavy chain were visualized on an agarose gel at the expected sizes: ~600bp for kappa and lambda and ~700bp for gamma (a). DNA chromatogram of Sanger sequencing of the gamma heavy chain from the 5' end showed a 'variable' sequence corresponding to multiple sample-IDs for the pooled plate (b). Sanger sequencing of the kappa light chain from the 3' end showed a 'variable' sequence after the constant region and beginning at the VJ junction corresponding to multiple light chains (c). A cloning primer pair specific for well A1 was used to amplify the kappa light chain. Sanger sequencing showed that in contrast to (c), only one clean sequence was amplified (d). All results are representative of the other amplified immunoglobulin genes. M: 100bp DNA ladder, K: kappa light chain, L: lambda light chain, G: gamma heavy chain.

[00106] Figure 5. CCP+ RA patients have peripheral blood plasmablasts percentages that correlated with disease activity and secreted anti-citrulline autoantibodies. Plasmablasts are $CD19^{+}CD20^{-}CD27^{+}CD38^{++}$ and were first gated on CD3- cells then gated as shown (a). Peripheral blood was obtained from consented RA patients and plasmablasts plotted as a percentage of total PBMCs. A Mann-Whitney test showed that CCP+ RA patients possess significantly higher ($p<0.05$) plasmablast percentages than CCP- RA patients (b). Plasmablast percentages were significantly correlated with clinical disease activity index (CDAI) in CCP+ patients by linear regression. Linear regression was performed on log transformed plasmablasts percentages to achieve normality of dataset (c). CCP+ plasmablasts were also either mock-sorted or underwent a plasmablast-depletion sort with >95% elimination of plasmablasts (d) and cultured for 7 days in RPMI supplemented with 10% FBS. Supernatant were collected and analyzed for anti-citrullinated peptide reactivity with Luminex. Mean fluorescence intensities for antibody reactivity against each peptide were plotted as peptide reactivities (e).

[00107] Figure 6. Strategy for selection and screening of clones for neutralizing antibodies. Paired LC and HC antibody sequences are obtained from bioinformatic analysis of 454-sequenced amplicons and grouped into clonal families based on their LC and HC V(D)J usage. The clone(s) occurring at the highest frequency in each clonal family will be selectively cloned, expressed, and screened for binding to the target antigen of interest using ELISA. Representative clone(s) from the entire clonal family which secrete binding antibodies will then be cloned and expressed for screening of neutralizing antibodies. Each antibody diagram represents a clone.

[00108] Figure 7. Characterization of immunoglobulin heavy chain V(D)J sequences and clonal families derived from individual human subjects. Blood was obtained from

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humans with the following conditions: (i) chronic *Staphylococcus aureus* osteomyelitis in a human who was not taking antibiotics (due to non-compliance) but whose immune system effectively suppressed the infection and prevented fulminant infection for several months off antibiotics; (ii) a human with acute and fulminant *Staphylococcus aureus* bacteremia requiring transfer to the intensive care unit and aggressive intravenous antibiotic treatment; (iii) 3 humans with chronic active rheumatoid arthritis (RA) (with disease activity scores (DAS) > 5); (iv) a human 7 days following receipt of the trivalent influenza vaccine (Fluzone, Sanofi); and (v) a human with metastatic lung adenocarcinoma who was expected to expire but following chemotherapy went into a state of long-term non-progression. In all cases, the human patients exhibited elevations in their peripheral blood plasmablast levels (ranging from 1.5 – 6% of peripheral blood B cells being plasmablasts [$CD20^{-}CD19^{+}CD38^{++}CD27^{+}$], with levels in normal humans being 0.1-0.2% of peripheral blood B cells), indicating an activated immune response. Plasmablasts were single-cell sorted into 96-well plates, and barcoding and 454 sequencing of the expressed immunoglobulin cDNA was performed as described in Figures 2 and 3. Bioinformatic analysis was used to pair the heavy and light chain immunoglobulins expressed by individual plasmablasts. Pie chart diagrams of the percent of heavy chain V(D)J usage for the individual patients are presented – each wedge represents the percent of plasmablasts expressing a distinct heavy chain V(D)J sequence rearrangement.

[00109] Figure 8. Clustering of immunoglobulin heavy chain V(D)J sequences from human subjects demonstrates clonal families and clonal subfamilies. The immunoglobulin heavy chain sequence datasets generated in the studies described in Figure 7 were subject to hierarchical clustering using the program Clustal. Hierarchical clustering yielded evolutionary trees representing the antibody response in each individual human.

[00110] Figure 9. Schematic of RT and PCR to add sample-IDs and plate-IDs to any amplicon and downstream utility. Individual samples comprising either single cells or multiple cells are separately reverse transcribed in wells. Reverse transcription adds a sample-ID and a 5' universal primer region to all 1st strand cDNA as previously described (a). cDNA from all wells of a plate are pooled and undergo 2 rounds of PCR. The 1st PCR uses Fw Short Primer1, Fw Long Primer 1 as forward primers and adds a 454 Titanium Primer A for 454 sequencing and a plate-ID to the 5' end of the sequence. The Fw Short Primer1 has a Tm similar to the GSP1 primer and was included to slightly increase the efficiency of the PCR. Each GSP1 primer has a gene specific sequence and can specifically amplify that gene. Sequences for primers are shown. Note that regardless of which gene is amplified, the forward primers remain constant (b). The second PCR is a nested PCR. Fw Primer 2 is the forward

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primer, and the reverse primers are Long GSP2 Primer and Rv Primer 2. Long GSP2 is gene-specific and only amplifies a specific gene. It also adds the 454 Titanium Primer B for 454 sequencing and a plate-ID to the 3' end of the amplicon. RV Primer2 has a Tm similar to Fw Primer2 and was included to slightly increase PCR efficiency. Sequences for primers are shown. After RT and 2 PCRs, amplicons from all plates are pooled and 454-sequenced. The combination of plate-IDs and sample-IDs allows for identification of sequences that originate from the same sample. This allows for comparison of sequences between multiple samples. Sequences from the same origin may also be expressed in pairs to obtain the exact protein from the original cell, such as the T-cell receptor and other Ig isotypes such as IgM, IgE and IgA (c).

[00111] Figure 10. Gene-specific primers for reverse transcription (RT-GSPs) of immunoglobulin heavy and light chains. RT-GSPs were used instead of oligo(dT)s as primers in reverse transcription of heavy and light chain genes. cDNA were then amplified by PCR and visualized on an agarose gel. RT-GSP primers IgKC_v3(a), IgLC_v5, IgLC_v6, IgLC_v7 and IgLC_v8 in lanes 1-4 respectively (b), IgHGC_v10, IgHGC_v11, IgHGC_v13 and IgGC_v15 in lanes 1-4 respectively (c) and IgHGC_v16 (d). KC, LC and GC in the primer names indicate that the primer is specific for the kappa chain, lambda chain and gamma heavy chain respectively. White bands in gel photos indicate where non-relevant lanes had been cropped out.

[00112] Figure 11. Adaptor region sequences. RNA was reversed transcribed with oligonucleotides comprising a universal primer region and an adaptor region at the 3' terminal end. cDNA was then amplified using the universal primer region sequence as a forward primer and gene-specific sequences as reverse primers. Amplified products were visualized on an agarose gel. Adaptor region consists of G (a), GGGGG and rGrGrG in lanes 1 and 2 respectively (b). rG indicates RNA nucleotides instead of DNA nucleotides.

[00113] Figure 12. Universal primer sequences. RNA was reverse transcribed with oligonucleotides comprising a universal primer sequence and an adaptor region at the 3' terminal. cDNA were then amplified by PCR using a forward primer complementary to the universal primer region and a reverse primer complementary to the gene specific sequence. Univ_seq_4 (a), univ_seq_5 (b) and univ_seq_f (c). Vertical white bands in gel photos indicate where non-relevant lanes have been cropped out. Otherwise lanes belong to the same gel photo.

[00114] Figure 13. Gene-specific primer sequences for 1st PCR reaction. Gene-specific reverse primers used in amplification of sequences in the first PCR reaction. Either the 1st PCR reaction or the subsequent 2nd nested PCR products were run and visualized on an agarose gel. Reverse primers used are IgKC_v4, IgLC_v5, IgHGC_v13 on lanes 1-3 respectively (a), K_GSP1, L_GSP1, G_GSP1 on lanes 1-3 respectively (b), K_GSP1c, L_GSP1c on lanes 1-2

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respectively (c), G_GSP1 (d), L_GSP1d, G_GSP1g on lanes 1-2 respectively (e), G_GSP1h, G_GSP1k, L_GSP1f, L_GSP1g on lanes 1-4 respectively (f), G_GSP1d (g) L_GSP1h-o on lanes 1-8 respectively (h), G_GSP1m-q and G_GSP1t on lanes 1-6 respectively (i). K, L and G in the primer names indicate that the primers are specific for the kappa, lambda and gamma immunoglobulin constant regions respectively. Each gel starts with a lane marker on the left followed by sample lanes. White bars between lanes on the same gel photo indicate where non-relevant lanes in-between have been cropped out.

[00115] Figure 14. Gene-specific sequences for the 2nd PCR reaction. Gene-specific reverse primers used in amplification of sequences in the 2nd PCR reaction. PCR products were run and visualized on an agarose gel. Reverse primers used are K_GSP2, L_GSP2, G_GSP2 in lanes 1-3 respectively (a), K_GSP2v2a, K_GSP2v2b, L_GSP2v2 in lanes 1-3 respectively (b), K_GSP2v2c, K_GSP2v2c, G_GSP2v2c1, G_GSP2v2c2 in lanes 1-4 respectively (c), K_GSP2v2d-f in lanes 1-3 respectively (d),K_GSP2v2g, L_GSP2v2d and G_GSP2b in lanes 1-3 respectively (e). K, L, G in the primer names indicates that they are specific for the kappa, lambda and gamma immunoglobulin constant regions respectively. Each gel starts with a lane marker on the left followed by sample lanes. White bars between lanes on the same gel photo indicate that non-relevant lanes in-between have been cropped out.

[00116] Figure 15. Potential locations of barcode sequences to identify a linked pair of polynucleotide sequences. The schematic illustrates the physical linkage of two nucleic acid segments, A and B (e.g., two cDNAs). A barcode (BC) is appended to any one of the ends, or both ends, or anywhere in the sequence linking A and B. In one embodiment, A and B represent immunoglobulin heavy and light chain sequences.

[00117] Figure 16. Different types of overlap-extension tails. The bold line corresponds to a gene specific sequence and the thin line corresponds to the overlapping tail. As indicated, the overlap can be entirely due to the overlap of the primer sequence or else due to partial or total overlap with a gene specific sequence. As indicated, the overlap can also contain a barcode sequence. Structures I, II, and III indicate potential locations of the overlaps.

[00118] Figure 17. Schematic overview of external barcode addition to a linked pair of antibody light and heavy chains. Shown are the products of a reverse transcription reaction. The LC gene specific PCR primer contains a bar code, sequencing primer site, and restriction site (RE1) to allow these elements to be added to the 3' end of the resulting PCR product. Primers specific for LC and HC with overlap-extensions and encoding a restriction site (RE3) are indicated. A reverse primer specific for HC containing a sequencing primer site and a

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restriction site (RE2) is also indicated. Amplification results in a nucleic acid with the linked structure shown with a bar code at one end.

[00119] Figure 18. Schematic overview of internal barcode addition to a linked pair of antibody light and heavy chains. Shown is a method of using adaptors containing extension overlap and barcode sequences to join cDNAs resulting from reverse transcription of mRNAs using oligo (dT) primers. The method shown takes advantage of the 3' tailing and template switching activities of reverse transcriptase to add overlap-extension sequences to the cDNAs to be joined. In this example, one of the adaptors adds both a barcode and overlap-extension sequence to one of the cDNAs to be joined, while only the overlap-extension sequence is added to the other cDNA to be joined. After amplification, a linked structure carrying one barcode sequence in between the linked cDNAs is generated.

[00120] Figure 19. Schematic overview of addition of two internal barcodes to a linked pair of antibody light and heavy chains using universal sequence overlap-extension primers. Shown is a method of using adaptors containing both a universal sequence and a barcode to join cDNAs resulting from reverse transcription of mRNAs using oligo (dT) primers. In this example, PCR primers to the universal sequence add an overlap-extension sequence to each of the cDNAs to be joined. After the amplification scheme shown, a linked structure carrying two barcodes in between the linked cDNAs is generated.

[00121] Figure 20. Schematic overview of addition of two internal barcodes to a linked pair of antibody light and heavy chains using overlap-extension adaptors. Shown is a method of using adaptors containing both a barcode and overlap-extension sequence to join cDNAs resulting from reverse transcription of mRNAs using gene specific primers (GSP). In this example, the overlap extension sequences on the adaptors added to each of the cDNAs allow for joining of the cDNAs by annealing. After the amplification scheme shown, a linked structure carrying two barcodes in between the linked cDNAs is generated

[00122] Figure 21. Use of barcoded GSPs during reverse transcription in combination with template-switch added adaptors. RT was performed with total PBMC RNA and univ_seq_2 template-switching oligo and IgKC_v3 GSP (lanes 1-2) and IgLC_v5 GSP (lanes 3-4) with an additional 5' flanking sequence, of which the first part is the Fixed_PCR3 sequence, and the last 8 bp is a barcode. Aliquots of the RT reaction were used in subsequent PCR reactions, with either a 5' V_K (lane 1) or V_L (lane 3) primer or the Univ_seq_2 (lanes 2 and 4) as the 5' primer, and Fixed_PCR3 as the 3' primer. The PCR products in lanes 2 and 4 ran as a smear, showing that the barcoded GSPs are non-specific in the RT reaction, and are not suitable for use with template-switch added adaptors.

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[00123] Figure 22. Gating scheme for flow cytometry sorting of single cells into 96-well plates. Plasmablasts are defined as $CD19^{+}CD20^{-}CD27^{+}CD38^{++}$. Single PBMCs were first gated on based on their FSC and SSC profile (not shown). Live $CD19^{+}$ B cells were then gated on (left panel), and further narrowed down to CD20- B cells (2nd panel from left), and refined to $CD27^{+}CD38^{++}$ cells. From this, IgG^{+} plasmablasts were determined as IgA^{-} and IgM^{-}, as IgG^{+} plasmablasts do not express cell surface IgG. This population was single cell sorted into 96-well plates.

[00124] Figure 23. Plasmablasts are present in people undergoing immunological challenge. Plasmablasts constituted 0.15% of peripheral blood B cells in a representative healthy donor, and range from 0.5%-16.4% in people undergoing a variety of immunological challenges including infections (*Staphylococcus aureus* and *Clostridium difficile* infections), cancer (a patient with metastatic melanoma who was a non-progressor for >4 years due to treatment with ipilimumab and a patient with metastatic adenocarcinoma of the lung who was a long-term non-progressor for >3 years after receipt of chemotherapy), and vaccination (receipt of influenza virus vaccine). This shows that plasmablasts are elevated in and obtainable from a range of subjects mounting immune responses of interest for isolation of individual plasmablasts for high-throughput sequencing of the antibody repertoire to characterize the active humoral response.

[00125] Figure 24. Expressed recombinant antibodies were secreted for 2-3 weeks in transient transfections. As outlined in Figure 2, the paired heavy and light chain immunoglobulin cDNA were cloned by PCR and co-transfected into 293T cells at the 48-well scale. Supernatants were collected every other day for 18 days. Anti-human IgG ELISA was performed to determine the amount of secreted antibodies in the collected supernatants, and the concentration of the antibodies in the supernatants of a panel of individual co-transfectants are graphed. Secretion tended to peak by day 9 and was substantially diminished by day 18.

[00126] Figure 25. Paired antibody heavy chain (HC) and light chain (LC) from an influenza vaccinated human exhibit variation across the complement determining regions (CDRs). Figure 25A: Partial dendrogram of flu antibodies. After pairing of heavy and light chains, a multiple sequence alignment was generated for heavy chains, and another multiple sequence alignment was generated for light chains. Both multiple sequence alignments were generated using Clustalw2 2.1 with default parameters. The two alignments were concatenated together and used to build a tree in CLC Sequence Viewer v. 6.5.2 using the neighbor joining method with 100 bootstrap replicates. Figure 25B: Heavy chain CDRs for a clonal family from flu-vaccinated patient. Identifiers in figure correspond to sequence names in Sequence Listing

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as follows: 51.A11.1 = NA.51.11.A11.1.454.heavy.3.nb-aa, 49.A08.1 = NA.49.8.A08.1.454.heavy.3.nb-aa, 51.D07.1 is the amino acid sequence obtained by translating NA.51.40.D07.1.454.heavy.3.nb in frame 1. Figure 25C: Light chain CDRs for a clonal family from flu-vaccinated patient. Identifiers in figure correspond to sequence names in Sequence Listing as follows: 51.A11.1 = NA.51.11.A11.1.454.light.4.nb-aa, 49.A08.1 = NA.49.8.A08.1.454.light.4.nb-aa, 51.D07.1 = NA.51.40.D07.1.454.light.4.zerom50-aa.

[00127] Figure 26. Recombinant anti-influenza antibodies bound to Fluzone influenza virus vaccine. Analysis of the evolutionary tree (Figure 8) of the heavy and light chain antibody repertoire dataset generated for the influenza vaccinated human described in Figure 7 was performed to select antibodies representative of the clonal families identified. The heavy and light chains for the selected antibodies representing both clonal families as well as several singlet branches were cloned by PCR and co-transfected into 293T cells (as outlined in Figure 2), and supernatants collected from transfectants as described in Figure 24. The recombinant antibodies were then tested for reactivity against the Fluzone influenza virus vaccine (Sanofi) by ELISA, with the Fluzone vaccine coated on the ELISA plate. The recombinant influenza virus antibodies were incubated in the ELISA plate at 100ng/ml, and a horse radish peroxidase (HRP)-conjugated anti-human IgG antibody used to detect antibody binding. The TMB substrate reaction was allowed to go for 30 minutes before quenching with acid stop. Readout is displayed as 450nm absorbance as no standards were available. Multiple recombinant antibodies representing the identified clonal families bound to the influenza virus vaccine, while recombinant antibodies representative of other clonal families and the "dead ends" did not bind influenza vaccine.

[00128] Figure 27. Recombinant anti-influenza antibodies representative of clonal families bind influenza virus hemaglutinins with picomolar affinities. The recombinant anti-influenza virus antibodies representative of clonal families from the Fluzone-vaccinated human (Figure 7) that bound influenza vaccine in an ELISA assay (Figure 26) were tested using a surface plasmon resonance (SPR) instrument (ProteOn System, Bio-Rad Laboratories) to determine their binding affinities for influenza hemagglutinin (both the H3N2 A/Perth/16/2009 and H1N1 A/California/07/2009 strains present in the vaccine). The recombinant anti-influenza virus antibodies were bound to the surface using EDAC-NHS chemistry, and the H3N2 Perth and H1N1 California hemaglutinins were independently tested as the ligands, with hemagglutinin as the analyte. Ka column denotes the on-rates, Kd column the off-rates and K_D the dissociation constant. Multiple recombinant antibodies bound either the H3N2 Perth or the H1N1 California hemagglutinin with picomolar affinities.

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[00129] Figure 28. Recombinant anti-influenza antibodies neutralize influenza virus infectivity in microneutralization assays. Six antibodies exhibiting reactivity on the Fluzone ELISA (Figure 26) were sent to the contract research organization (CRO) Virapur, LLC, (San Diego, CA) for testing in a microneutralization assay using the H1N1 California/07/2009 influenza virus strain and the H3N2 A/Perth/16/2009 influenza virus strain, 2 of the three stains of influenza virus in the Fluzone vaccine. 5 out of the 6 recombinant antibodies neutralized influenza virus in the microneutralization assay, preventing infectivity at microgram per milliliter levels and possibly sub-microgram per milliliter concentrations. The recombinant antibody F21 neutralized H3N2 Perth, and although it bound Fluzone in the ELISA assay it did not show binding in the SPR analysis (Figure 27) likely because the concentrations of the hemagglutinin analyte that were used were too low for binding to be detectable.

[00130] Figure 29. Recombinant anti-*Staph. aureus* antibodies bound to fixed *S. aureus* by flow cytometry. Analysis of the evolutionary tree (Figure 8) of the heavy and light chain antibody repertoire dataset generated from the human who controlled (without antibiotics) a chronic *Staph. aureus* osteomyelitis (as described in Figure 7) enabled selection of antibodies representative of the clonal families identified. The heavy and light chains for the selected antibodies representing both clonal families as well as several singlet branches were cloned by PCR and co-transfected into 293T cells (as outlined in Figure 2), and supernatants collected from transfectants as described in Figure 24. The recombinant anti-*Staph. aureus* antibodies were then tested for reactivity against fixed *S. aureus*. The secondary antibody used was a FITC-conjugated mouse anti-human IgG, and samples were analyzed on a BD LSR II or LSR Fortessa. The percentage of positive staining is shown, with 2 anti-influenza antibodies used as negative controls. The 2° antibody alone did not result in binding over background, as protein A binds weakly to mouse IgG1, which is the isotype of the 2° antibody. The staining observed above background is due to the binding of the recombinant anti-*Staph. aureus* antibodies to the small percentage of the Wood strain of *S. aureus* that express protein A. (a). Flow cytometry plots are shown for the 2 positive binding anti-*Staph. aureus* antibodies, S6 and S11, along with the isotype-matched negative control anti-influenza antibodies (b). The level of binding of the anti-*Staph. aureus* antibodies to *S. aureus* was proportional to the amount of antibody used. The dark solid line represents an antibody concentration of 10ug/ml, the dark dotted line 5ug/ml, and grey dotted line 1ug/ml (c).

[00131] Figure 30. Anti-*Staph. aureus* antibodies reduced the number of *S. aureus* colony forming units. Recombinant anti-*Staph.* aureus antibodies were incubated with *S. aureus* in combination with baby rabbit serum as a complement source, before being serially

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diluted and grown overnight on 5% trypticase soy agar (TSA) blood agar plates. Colony forming units (CFUs) were then counted and graphed. Two recombinant anti-*Staph. aureus antibodies* (Ab-a and Ab-b) resulted in killing of the *Staph. aureus* and thus a reduced number of CFU/ml.

[00132] Figure 31. Identification of *S. aureus* antigen targets of recombinant anti-*Staph. aureus* antibodies generated from a human mounting an effective immune response against a chronic *Staph. aureus* infection. A protein lysate was generated from a clinical *Staph. aureus* isolate. Recombinant anti-*Staph. aureus* antibodies representative of clonal families identified in the antibody repertoire from a human mounting an immune response that was preventing progression of a chronic *Staph. aureus* osteomyelitis infection were used to immunoprecipitate proteins from a *Staph. aureus* Spa$^-$ clinical isolate. The immunoprecipitates were separated by SDS-PAGE, identified bands excised, and mass spectrometry (an Agilent XCT-Plus ion trap mass spectrometer) used to identify the immunoprecipiated proteins which are presented in the Figure.

[00133] Figure 32. Generation of anti-lung adenocarcinoma antibodies from a human with metastatic lung adenocarcinoma who was a long-term non-progressor. A human with metastatic lung adenocarcinoma who became a long-term non-progressor following chemotherapy exhibited persistently elevated blood plasmablasts, indicating a persistently activated immune response (Figure 7). The patient's peripheral blood plasmablasts were sorted, the antibody repertoire sequenced, and antibodies representative of clonal families (Figure 8) were cloned and expressed recombinantly. One of the expressed recombinant antibodies, which is representative of one of the identified clonal families, bound to an independent lung adenocarcinoma in immunohistochemical stains. Tissue arrays were then used to further characterize the reactivity of this antibody. Tissue arrays containing multiple independent lung adenocarcinomas, squamous cell carcinoma and healthy lung tissue were blocked overnight with 100ug/ml of F(ab) goat anti-human antibody. Slides were stained with anti-lung adenocarcinoma antibodies or an anti-influenza antibody as a negative control, and visualized with Vector Red. Slides were counterstained with hematoxylin. Hematoxylin blue color was removed using Photoshop so that only nuclei and Vector Red (red) staining shows up as darker grey in the image. This recombinant antibody bound to 4 out of 5 independent lung adenocarcinoma tissue samples tested (contained in tissue arrays), but did not bind to lung squamous cell carcinoma or to healthy lung tissue.

[00134] Figure 33. The identified anti-lung adenocarcinoma antibody (Figure 32) binds to the surface of a lung adenocarcinoma cell line. The recombinant anti-lung

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adenocarcinoma antibody (Figure 32) strongly stained the surface of the lung adenocarcinoma cell line H1650, and exhibited only low-level staining of a kidney epithelial and a lung squamous tumor cell lines. This anti-lung adenocarcinoma antibody did not bind a second lung adenocarcinoma cell line (H2009), consistent with our observation that this antibody bound 4 out of the 5 independent lung adenocarcinoma tissue samples tested by immunohistochemistry (Figure 32).

[00135] Figure 34. Generation of rheumatoid factor antibodies from rheumatoid arthritis (RA) patients. Recombinant antibodies representative of clonal families identified in the evolutionary trees of antibody repertoires generated from humans with RA (Figure 8) were selected, cloned and recombinantly expressed. Recombinant antibodies derived from RA patients were used as the primary antibody in a direct ELISA and anti-human IgG-HRP was used as the secondary antibody, and binding visualized with TMB substrate. Recombinant antibodies RA2 and RA3 exhibited reactivity, and thus represent rheumatoid factor antibodies.

[00136] Figure 35. Generation of anti-CCP and anti-histone 2A antibodies from RA patients. Additional recombinant antibodies generated from RA patients with active disease were characterized using a histone 2A ELISA and a cyclic-citrullinated peptide (CCP) ELISA (using the CCP2 ELISA kit [Axis Shield]). Recombinant antibodies were used at 125ug/ml. Panel (a) presents the results from a histone 2A ELISA, and multiple recombinant antibodies bound to histone 2A. Panel (b) present the results of the CCP2 ELISA, and several recombinant antibodies exhibited positive reactivity. The anti-CCP2 ELISA included a seronegative and 2 seropositive controls. For both assays, absorbance was recorded as the readout. Absorbance values above the background (dotted line) were considered to be positive.

[00137] Figure 36. Confirmatory independent experiment demonstrating generation of anti-histone 2A antibodies from active RA patients. Recombinant antibodies derived from RA patient evolutionary trees (Figure 8 and Figure 35) were further tested in a histone 2A ELISA assay. Antibodies were used at 30ug/ml, a 4-fold lower concentration that that used in Figure 35. Absorbance was recorded as the readout. Absorbance values above the background were considered to be positive.

[00138] Figure 37. Identification of anti-histone and anti-citrullinated protein antibodies using RA antigen arrays. Antibodies derived from RA patients were used to probe an RA antigen array containing a spectrum of native and citrullinated proteins and peptides. Following incubation with a Cy-3-labeled anti-human IgG secondary antibody, recombinant antibody binding was quantitated by scanning with an Axon Instruments GenePix microarray scanner.

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Reactivities are displayed as a heatmap. Recombinant antibodies derived from RA bound to several distinct citrullinated or native antigens.

[00139] Figure 38. Pacific Biosciences sequencing provides full-length sequencing reads of IgG heavy chain amplicon. IgG heavy chain amplicons from plate 44 were provided to Pacific Biosciences for SMRT sequencing. The number of circular consensus sequence (CCS) reads with barcodes corresponding to selected wells are shown.

[00140] Figure 39. Use of alternative cell surface markers and other cellular features to identify blood plasmablasts. Plasmablasts can be identified and sorted through use of a variety of cell surface markers and/or cellular features. Panel (a) demonstrates that plasmablasts exhibit higher forward scatter (FSC) than resting B cells. Plasmablasts were identified based on $CD19^{+}CD20^{-}CD27^{+}CD38^{hi}$ staining, and these results demonstrate that B cells (grey) are smaller than plasmablasts (black). Panel (b) demonstrates that use of anti-CD19 staining combined with FSC identifies a population of B cells that contains 72% plasmablasts. Panel (c) demonstrates that, for a population of $CD19^{+}$ B cells (cells were pre-gated as being CD19 positive), side scatter (SSC) and FSC can be used to identify a population of B cells that contains 37% plasmablasts. Panels (d-f) present several approaches to identify plasmablasts within the $CD19^{+}$ B cell population. Gating on FSC^{hi} cells give 37% purity of plasmablasts (c). Gating on $FSC^{hi}CD20^{-}$ cells gave 71% purity in plasmablasts (d). Gating on $FSC^{hi}CD38^{+}$ cells gave 80% purity in plasmablasts (e). Gating on $FSC^{hi}CD27^{+}$ cells gave 44% purity in plasmablasts (f).

[00141] Figure 40. Human blasting B cells (plasmablasts) are larger than resting B cells but smaller than monocytes on average. Singlet monocytes, B cells and plasmblasts were gated and compared for side- and forward-scatter parameters. Monocytes were defined by their characteristic FSC and SSC profile, and as $CD19^{-}CD3^{-}$. B cells were defined as $CD19^{+}CD20^{+}$. Plasmablasts were defined as $CD19^{+}CD20^{-}CD27^{+}CD38^{++}$. Cells shown on the FSC-A (forward scatter area) and SSC-A (side scatter area) axes (a). Cells shown on the FSC-W (forward scatter width) and SSC-W (side scatter width) axes (b). The median of the FSC-A, SSC-A, FSC-W, SSC-W of plasmablasts were divided by that of resting B cells or monocytes to obtain a ratio which represent the size relationship between the cell types (c). The median of the FSC-A, SSC-A, FSC-W, SSC-W of the 20th percentile of plasmablasts were divided by that of the median of resting B cells or monocytes to obtain a ratio which represent the size relationship between the cell types wherein at least 80% of plasmablasts are larger than the ratio (d). Error bars indicate 95% confidence interval.

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[00142] Figure 41. Size of human plasmablasts compared to resting B cells by microscopy. Plasmablasts and resting B cells were sorted as $CD19^{+}CD20^{-}CD27^{+}CD38^{++}$ and $CD19^{+}CD20^{+}$ respectively. Cells were then imaged using an Olympus microscope at 200x. Cell area was measured using ImageJ and diameter determined using area=π x r^2 where diameter = 2 x radius, and volume determined by 4/3 x πr^3. Error bars denote the inter-quartile range. A cut-off of $\geq$8uM or $\geq$50uM^2 or $\geq$268uM^3 will include 96% of plasmablasts and exclude 92% of resting B cells.

[00143] Figure 42. Superscript III has template switching activity at temperatures at and below 50°C. Reverse transcription (RT) was performed for 90 minutes using the temperatures indicated above the lanes using an adaptor ending with rGrGrG, and 1 round of PCR was done using GAPDH as the 3' primer (sequence ATGGTTCACACCCATGACG). As can be seen, no template switching activity to add on the adaptor could be seen at 55°C, and template switching activity increases from minimal at 50°C to highest at 42°C, the lowest temperature tested, as indicated by the brightness of the band at ~450bp. Marker is a 100bp marker. Superscript III is an MMLV reverse transcriptase that has specific mutations that result in a loss of RNAse H activity, and also has mutations made to the polymerase domain to increase thermal stability and has a half-life of 220 minutes at an RT temperature of 50oC. Other MMLV H- enzymes that have been engineered for higher thermal stability are expected to exhibit similar activity.

[00144] Figure 43. Additional primers for human kappa, lambda and gamma constant regions. These primers were used for the 1st PCR, and then the 2nd PCR was performed using the primers from Table 1 and PCR products separated on a 2% agarose gel and the image was taken. Primers used for 1st PCR are Kappa GSP1, kappa GSP1e, kappa GSP1f, lambda GSP1, lambda GSP1x and lambda GSP1y respectively. Sequences are in Table 10. White bars between lanes on the same gel photo indicate that non-relevant lanes in-between have been cropped out.

[00145] Figure 44. Additional primers for other human constant regions and genes. 1st and 2nd PCR were done and products ran on a 2% agarose-gel and imaged. Lanes are from left: marker, mu, alpha constant regions, TCR alpha (a) and marker, TCR beta (b). Primers used and sequences are in Table 10. White bars between lanes on the same gel photo indicate that non-relevant lanes in-between have been cropped out.

[00146] Figure 45. Additional primers for mouse genes. 1st and 2nd PCR were done and products ran on a 2% agarose gel and imaged. Lanes are from left: marker, kappa, lambda, lambda, lambda, lambda light chains and mu heavy chain. The 4 lambda lanes had this combination of primers used: mouse_lambda_GSP1a with mouse_lambda_GSP2a,

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mouse_lambda_GSP1a with mouse_lambda_GSP2b, mouse_lambda_GSP1b with mouse_lambda_GSP2a, and mouse_lambda_GSP1b with mouse_lambda GSP2a (a). Marker and alpha heavy chain (b). Gamma1, 2a, 2c heavy chains with 2nd PCR using mo_g12_GSP2d and mo_g12_GSP2e respectively, marker (c). Marker, gamma 3 heavy chain with 2nd PCR using mo_g3_GSP2d, mo_g3_GSP2e respectively followed by gamma 2b heavy chain with 2nd PCR using mo_g2b_GSP2d, mo_g2b_GSP2e respectively (d). Marker, TCR alpha (e). Marker, TCR beta (f). White bars between lanes on the same gel photo indicate that non-relevant lanes in-between have been cropped out.

[00147] Figure 46. Anti-*S. aureus* antibody-mediated killing of *S. aureus* by the HL-60 neutrophil cell line. Various recombinant anti-*S. aureus* antibodies (staph 1, staph 4, staph 6, staph 7, staph 9, staph 12) were incubated at 4°C with *S. aureus* for 30 minutes, following which non-bound antibody was washed away, and the *S. aureus* incubated with activated HL-60 cells and baby rabbit complement for 45 minutes at 37°C. Cells were then washed twice and extracellular bacteria were serially plated on 5% TSA blood agar plates, incubated overnight, and colony forming units (CFUs) counted. Recombinant antibodies staph 6, stepha 9 and staph 12 induced greater than 20% killing of *S. aureus*.

DETAILED DESCRIPTION

Compositions

Polynucleotides

[00148] In some aspects, a composition can include a polynucleotide. The term "polynucleotide(s)" refers to nucleic acids such as DNA molecules and RNA molecules and analogs thereof (e.g., DNA or RNA generated using nucleotide analogs or using nucleic acid chemistry). As desired, the polynucleotides may be made synthetically, e.g., using art-recognized nucleic acid chemistry or enzymatically using, e.g., a polymerase, and, if desired, can be modified. Typical modifications include methylation, biotinylation, and other art-known modifications. In addition, a polynucleotide can be single-stranded or double-stranded and, where desired, linked to a detectable moiety. In some aspects, a polynucleotide can include hybrid molecules, e.g., comprising DNA and RNA.

[00149] "G," "C," "A," "T" and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine and uracil as a base, respectively. However, it will be understood that the term "ribonucleotide" or "nucleotide" can also refer to a modified nucleotide or a surrogate replacement moiety. The skilled person is well aware that guanine, cytosine, adenine, and uracil may be replaced by other moieties without substantially altering the base

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pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base may base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine may be replaced in nucleotide sequences by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods described herein.

[00150] As used herein, and unless otherwise indicated, the term "complementary," when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of a polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with a polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions may include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing. Other conditions, such as physiologically relevant conditions as may be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

[00151] Complementary sequences include base-pairing of a region of a polynucleotide comprising a first nucleotide sequence to a region of a polynucleotide comprising a second nucleotide sequence over the length or a portion of the length of one or both nucleotide sequences. Such sequences can be referred to as "complementary" with respect to each other herein. However, where a first sequence is referred to as "substantially complementary" with respect to a second sequence herein, the two sequences can be complementary, or they may include one or more, but generally not more than about 5, 4, 3, or 2 mismatched base pairs within regions that are base-paired. For two sequences with mismatched base pairs, the sequences will be considered "substantially complementary" as long as the two nucleotide sequences bind to each other via base-pairing.

[00152] "Complementary" sequences, as used herein, may also include, or be formed entirely from, non-Watson-Crick base pairs and/or base pairs formed from non-natural and modified nucleotides, in as far as the above embodiments with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs includes, but are not limited to, G:U Wobble or Hoogstein base pairing.

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[00153] The term percent "identity," in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described below (e.g., BLASTP and BLASTN or other algorithms available to persons of skill) or by visual inspection. Depending on the application, the percent "identity" can exist over a region of the sequence being compared, e.g., over a functional domain, or, alternatively, exist over the full length of the two sequences to be compared.

[00154] For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[00155] Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel et al., infra).

[00156] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information web-site.

[00157] Identical sequences include 100% identity of a polynucleotide comprising a first nucleotide sequence to a polynucleotide comprising a second nucleotide sequence over the entire length of one or both nucleotide sequences. Such sequences can be referred to as "fully identical" with respect to each other herein. However, in some aspects, where a first sequence is referred to as "substantially identical" with respect to a second sequence herein, the two sequences can be fully complementary, or they may have one or more, but generally not more than about 5, 4, 3, or 2 mismatched nucleotides upon alignment. In some aspects, where a first sequence is referred to as "substantially identical" with respect to a second sequence herein, the

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two sequences can be fully complementary, or they may be about 50, 60, 70, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to each other.

[00158] Where a first sequence is referred to as "distinct" with respect to the identity of a second sequence herein, the two sequences have at least one or more mismatched nucleotides upon alignment. In some aspects, distinct sequences can have 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more mismatched nucleotides upon alignment. In some aspects, distinct sequences can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or less than 100% identical to each other. In some aspects, where a first sequence is referred to as "distinct" with respect to a second sequence herein, the two sequences can have substantially or fully identical sequences, but instead differ from one another based upon differing patterns of modification within the sequences. Such modifications are generally known in the art, e.g., methylation.

[00159] In some aspects, a polynucleotide can be present in a library of polynucleotides. In some aspects, a polynucleotide library can include a plurality of polynucleotides. In some aspects, each polynucleotide in the plurality of polynucleotides can be derived from a single sample. In some aspects, a single sample can include a single cell such as a B cell.

[00160] Conventional notation is used herein to describe nucleotide sequences: the left-hand end of a single-stranded nucleotide sequence is the 5'-end; the left-hand direction of a double-stranded nucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand;" sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences;" sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences."

[00161] The term "messenger RNA" or "mRNA" refers to an RNA that is without introns and that can be translated into a polypeptide.

[00162] The term "cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

[00163] The term "amplicon" refers to the amplified product of a nucleic acid amplification reaction, e.g., RT-PCR.

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[00164] The term "hybridize" refers to a sequence specific non-covalent binding interaction with a complementary nucleic acid. Hybridization may occur to all or a portion of a nucleic acid sequence. Those skilled in the art will recognize that the stability of a nucleic acid duplex, or hybrids, can be determined by the Tm. Additional guidance regarding hybridization conditions may be found in: Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 1989, 6.3.1-6.3.6 and in: Sambrook et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, Vol. 3.

[00165] As used herein, "region" refers to a contiguous portion of the nucleotide sequence of a polynucleotide. Examples of regions are described herein an include identification regions, sample identification regions, plate identification regions, adapter regions, and the like. In some aspects, a polynucleotide can include one or more regions. In some aspects, a polynucleotide can include less than 2, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more regions. In some aspects, regions can be coupled. In some aspects, regions can be operatively coupled. In some aspects, regions can be physically coupled.

[00166] As used herein, "variable region" refers to a variable nucleotide sequence that arises from a recombination event, for example, it can include a V, J, and/or D region of an immunoglobulin or T cell receptor sequence isolated from a T cell or B cell of interest, such as an activated T cell or an activated B cell.

[00167] As used herein "B cell variable immunoglobulin region" refers to a variable immunoglobulin nucleotide sequence isolated from a B cell. For example, a variable immunoglobulin sequence can include a V, J, and/or D region of an immunoglobulin sequence isolated from a B cell of interest such as a memory B cell, an activated B cell, or plasmablast.

[00168] As used herein "identification region" refers to a nucleotide sequence label (e.g., a unique barcode sequence) that can be coupled to at least one nucleotide sequence for, e.g., later identification of the at least one nucleotide sequence.

[00169] As used herein "immunoglobulin region" refers to a contiguous portion of nucleotide sequence from one or both chains (heavy and light) of an antibody.

[00170] As used herein "adapter region" refers to a linker that couples a first nucleotide sequence to a second nucleotide sequence. In some aspects, an adapter region can include a contiguous portion of nucleotide sequence that acts as a linker. For example, an adapter region can have the sequence GGG and couples a first sequence to a second sequence via binding between GGG and CCC.

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[00171] In some aspects, a polynucleotide can include a cDNA region. In some aspects, a polynucleotide can include a sample identification-adapter region. In some aspects, a polynucleotide can include a sample identification region. In some aspects, a polynucleotide can include an adapter region. In some aspects, a polynucleotide can include a universal primer region. In some aspects, a polynucleotide can include an amplicon region. In some aspects, a polynucleotide can include a plate identification region. In some aspects, a polynucleotide can include a first plate identification region. In some aspects, a polynucleotide can include a second plate identification region. In some aspects, a polynucleotide can include a restriction site region. In some aspects, a polynucleotide can include a first restriction site region. In some aspects, a polynucleotide can include a second restriction site region. In some aspects, a polynucleotide can include a sequencing region. In some aspects, a polynucleotide can include a first sequencing region. In some aspects, a polynucleotide can include a second sequencing region.

[00172] In some aspects, a polynucleotide can include a plurality of any region described herein. For example, a polynucleotide can include a first sample identification region and a second sample identification region. In some aspects, the first sample identification region and the second sample identification region are identical or substantially identical. In some aspects, the first sample identification region and the second sample identification region are distinct. In some aspects, an identification region is coupled to a variable immunoglobulin region.

[00173] In some aspects the sequence of a region will be at least long enough to serve as a target sequence for a primer or a probe in a PCR reaction. In some aspects, a region can be 1 to greater than 5000 base pairs in length. For example, a region can be from 1-10,000 nucleotides in length, e.g., 2-30 nucleotides in length, including all sub-ranges therebetween. As non-limiting examples, a region can be from 1-30 nucleotides, 1-26 nucleotides, 1-23 nucleotides, 1-22 nucleotides, 1-21 nucleotides, 1-20 nucleotides, 1-19 nucleotides, 1-18 nucleotides, 1-17 nucleotides, 18-30 nucleotides, 18-26 nucleotides, 18-23 nucleotides, 18-22 nucleotides, 18-21 nucleotides, 18-20 nucleotides, 19-30 nucleotides, 19-26 nucleotides, 19-23 nucleotides, 19-22 nucleotides, 19-21 nucleotides, 19-20 nucleotides, 20-30 nucleotides, 20-26 nucleotides, 20-25 nucleotides, 20-24 nucleotides, 20-23 nucleotides, 20-22 nucleotides, 20-21 nucleotides, 21-30 nucleotides, 21-26 nucleotides, 21-25 nucleotides, 21-24 nucleotides, 21-23 nucleotides, or 21-22 nucleotides. In some aspects, a region can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more nucleotides in length. In some aspects, a region can be less than 50, 50-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800,

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800-900, 900-1000, or greater than 1000 nucleotides in length. In some aspects, a region can be less than 1000, 1000-2000, 2000-3000, 3000-4000, 4000-5000, 5000-6000, 6000-7000, 7000-8000, 8000-9000, 9000-10000, or greater than 10000 nucleotides in length. In some aspects, a region can include at least two nucleotides, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20 or more nucleotides of a polynucleotide disclosed herein.

[00174] The term "sample" can include RNA, DNA, a single cell or multiple cells or fragments of cells or an aliquot of body fluid, taken from a subject (e.g., a mammalian subject, an animal subject, a human subject, or a non-human animal subject). Samples can be selected by one of skill in the art using any means now known or later discovered including centrifugation, venipuncture, blood draw, excretion, swabbing, ejaculation, massage, biopsy, needle aspirate, lavage sample, scraping, surgical incision, laser capture microdissection, gradient separation, or intervention or other means known in the art. Samples can also be selected by one of skill in the art using one or more markers known to be associated with a sample of interest. Samples can also be selected using methods known in the art such as cell sorting and FACS. Further examples of sample selection methods are described in the Examples section below.

[00175] In some aspects a polynucleotide can be derived from or associated with a single sample. In some aspects a region can be derived from or associated with a single sample. In some aspects, a cDNA region can be derived from or associated with a single sample. In some aspects, an amplicon region can be derived from or associated with a single sample. A "single sample" includes a sample comprising polynucleotides that is taken from a single source. In some aspects, a single source includes a sample taken at a particular time point or at a particular location, e.g., in a subject or flask of cells or plate of cells. In some aspects, a first single sample is taken from a first subject at a first time point and a second single sample is taken from the first subject at a second time point that is distinct from the first time point. In some aspects, a first single sample is taken from a first subject at a first location and a second sample is taken from the first subject at a second location that is distinct from the first location. In some aspects, a first single sample is taken from a first subject at a time point and a second single sample is taken from a second subject at a time point. In some aspects, a first single sample is taken from a first subject at a location and a second sample is taken from a second subject at a location. In one embodiment, a sample comprises polynucleotides that include mRNA derived from one or more B cells. In another embodiment, a sample comprises polynucleotides including cDNA derived from one or more B cells. In another embodiment, a single sample comprises mRNA

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derived from one or more B cells sorted into a single well of a 96-well or 384-well plate. Samples are generally derived from a prokaryotic cell(s) (e.g., a bacterial cell(s)), a eukaryotic cell(s) (e.g., a mammalian and yeast cell(s)), or other sources of genetic material such as a virus or phage. The term "mammal" or "mammalian" as used herein includes both humans and non-humans and include but is not limited to humans, non-human primates, canines, felines, murines, bovines, equines, and porcines. In some aspects, the methods of the invention are applied to single samples in a plate with at least 96 wells, at least 384 wells, at least 1536 wells, or more wells. In further aspects, the methods of the invention are applied to single samples in at least one, two, three, four, five, six, seven, eight, ten, fifteen, twenty, thirty or more plates with at least 96 wells each.

[00176] In some aspects a 5' adaptor region sequence and/or a sample identification region are added to all cDNAs from a single sample, e.g., during RT and not just to Ig genes. In some aspects, 3' gene specific primers (GSPs) can be used to amplify any expressed gene in the single sample. In some aspects, genes are amplified that have a 5' variable region, e.g., T cell receptors and B cell receptors without needing multiple degenerate 5' primers to amplify the gene(s) of interest. GSPs can include primers specific for IgG, IgM, IgD, IgA, IgE, TCR chains, and other genes of interest.

[00177] In some aspects, multiple rounds of PCR can also be performed, e.g., using nested GSPs. For such nested GSPs, the GSP for the second round of PCR hybridizes to its target gene sequence at a position 5' along that sequence relative to the position hybridized to by the GSP used in the first round of PCR.

[00178] In some aspects, cDNA region or an amplicon region can include a DNA polynucleotide. In some aspects, cDNA region or an amplicon region can include a cDNA polynucleotide. In some aspects, cDNA region or an amplicon region can include an RNA polynucleotide hybridized to a DNA polynucleotide. In some aspects, cDNA region or an amplicon region can include an mRNA polynucleotide hybridized to a cDNA polynucleotide.

[00179] In some aspects, a universal primer region is not fully complementary to any human exon. In some aspects, a universal primer region is not fully complementary to any expressed human gene. In some aspects, a universal primer region has minimal secondary structure.

[00180] In some aspects, an amplicon region comprises an immunoglobulin heavy chain amplicon sequence. In some aspects, an amplicon region comprises an immunoglobulin light chain amplicon sequence. In some aspects, an amplicon region comprises a T cell receptor alpha amplicon sequence. In some aspects, an amplicon region comprises a T cell receptor beta amplicon sequence.

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[00181] In some aspects, a polynucleotide is present in a library of polynucleotides and can be differentiated from other polynucleotides present in the library based on a region of the polynucleotide.

[00182] In some aspects, the sequence of the sample identification region of each polynucleotide in a library derived from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library derived from one or more samples distinct from the first single sample. In some aspects, the sequence of the sample identification region of each polynucleotide in a library derived from a first single sample differs by at least 1 nucleotide from the sequence of the sample identification region of the other polynucleotides in the library derived from one or more samples distinct from the first single sample. In some aspects, the sequence of the sample identification region of each polynucleotide in a library derived from a first single sample differs by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more nucleotides from the sequence of the sample identification region of the other polynucleotides in the library derived from one or more samples distinct from the first single sample. In some aspects, the sequence of the sample identification region of each polynucleotide in a library derived from a first single sample can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or less than 100% identical to the sequence of the sample identification region of the other polynucleotides in the library derived from one or more samples distinct from the first single sample. In some aspects, the sequence of the sample identification region of each polynucleotide in a library derived from a first single sample is less than 100% identical to the sequence of the sample identification region of the other polynucleotides in the library derived from one or more samples distinct from the first single sample. In some aspects, a sample-identification region acts as a digital barcode on all 1st strand cDNA reverse transcribed from a single sample. In some aspects, the sample identification region is at least 1 nucleotide in length. In some aspects, a sample-identification region can comprise at least 3 nucleotides, and sample-identification regions can differ from each other by at least 1 nucleotide. In one embodiment, sample-identification regions are 3-15 nucleotides in length and differ from each other by at least 1 nucleotide. In some aspects, sample-identification regions can comprise at least 64 variants (using sample-identification regions 3 nucleotides in length with each sample-ID differing from each other by at least 1 nucleotide), or in some aspects larger numbers of

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variants. In some aspects, the sequence attached 3' to the sample-identification region can be an adapter region comprising at least 1 G. In a preferred embodiment, the sequence attached 3' to the sample-identification region can be an adapter region comprising at least 2 G's. In one embodiment, a sequence attached to the 5' end of a sample-identification region is a universal primer sequence that can be used during PCR amplification to avoid the need for the subsequent addition of a 5' universal primer sequence (by ligation or another method) or the use of multiple degenerate 5' primers to amplify genes with variable 5' regions. Examples of sample identification regions are shown in Tables 2 and 8.

[00183] In some aspects, the sequence of the first plate identification region of each polynucleotide in a library derived from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the first plate identification region of each polynucleotide in a library derived from the first set of single samples differs by at least 1 nucleotide from the sequence of the first plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the first plate identification region of each polynucleotide in a library derived from the first set of single samples differs by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more nucleotides from the sequence of the first plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the first plate identification region of each polynucleotide in a library derived from the first set of single samples can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or less than 100% identical to sequence of the first plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the first plate identification region of each polynucleotide in a library derived from the first set of single samples is less than 100% identical to sequence of the first plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. Examples of first plate identification regions are shown in Tables 3 and 7.

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[00184] In some aspects, the sequence of the second plate identification region of each polynucleotide in a library derived from a first set of single samples is distinct from the sequence of the second plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the second plate identification region of each polynucleotide in a library derived from the first set of single samples differs by at least 1 nucleotide from the sequence of the second plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the second plate identification region of each polynucleotide in a library derived from the first set of single samples differs by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more nucleotides from the sequence of the second plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the second plate identification region is identical to the sequence of the first plate identification region on a polynucleotide. In some aspects, the sequence of the second plate identification region of each polynucleotide in a library derived from the first set of single samples can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or less than 100% identical to sequence of the second plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. In some aspects, the sequence of the second plate identification region of each polynucleotide in a library derived from the first set of single samples is less than 100% identical to sequence of the second plate identification region of the other polynucleotides in the library derived from one or more single sample sets distinct from the first set of single samples. Examples of second plate identification regions are shown in Tables 3 and 7.

[00185] In some aspects, a plate-identification region (e.g., a first plate identification region or a second plate identification region) can comprise at least 2 nucleotides, and plate-identification regions differ from each other by at least 1 nucleotide. In one embodiment, plate-identification regions are 2-10 nucleotides in length and differ from each other by at least 1 nucleotide. In some aspects, use of plate-identification regions is found in only some embodiments, as the use of a larger number of different sample-identification regions (one per single sample to be analyzed) can eliminate the need for plate-identification regions. In some

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aspects, plate-identification regions are used to reduce the number of unique oligonucleotides containing a sample-identification region that need to be synthesized.

[00186] In some aspects, a polynucleotide includes one or more adapter regions. In some aspects, an adapter region includes one or more G's. In some aspects, an adapter region includes 2, 3, 4, 5, 6, 7, 8, 9, 10 or more G's. In some aspects, adapter regions are attached to the 3' ends of cDNAs using the template switching property of MMLV H- reverse transcriptases. Different methods to attach adaptor regions exist, including but not limited to, doing PCR with primers with 5' flanking adaptor region sequences, sticky and blunt end ligations, template-switching-mediated addition of nucleotides, or other methods to covalently attach nucleotides to the 5' end, to the 3' end, or to the 5' and 3' ends of the polynucleotides. These methods can employ properties of enzymes commonly used in molecular biology. PCR can use, e.g., thermophilic DNA polymerase. Sticky ends that are complementary or substantially complementary are created through either cutting dsDNA with restriction enzymes that leave overhanging ends or through 3' tailing activities of enzymes such as TdT (terminal transferase). Sticky and blunt ends can then be ligated with a complementary adaptor region using ligases such as T4 ligase. Template-switching utilizes the 3' tailing activity of MMLV H- reverse transcriptase to add one or more cytosines (C's) to the 3' end of cDNAs and its ability to switch template from mRNA to an adaptor region with complementary G's. In some aspects, a cDNA includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more C's on its 3' end.

[00187] In some aspects, a polynucleotide includes one or more restriction site regions. Restriction site regions include one or more restriction sites. Restrictions sites can include: NheI, XhoI, BstBI, EcoRI, SacII, BbvCI, PspXI, AgeI, ApaI, KpnI, Acc65I, XmaI, BstEII, DraIII, PacI, FseI, AsiSI, and AscI. In some aspects, any rare 8-cutter enzyme restriction site can be used.

[00188] In some aspects, one or more regions of a polynucleotide described herein can be operatively coupled to one or more other regions of the polynucleotide. In some aspects, two or more distinct regions of a single polynucleotide can be operatively coupled. For example, a universal primer region can be operatively coupled to an adapter region. In some aspects two or more regions can be operatively coupled together that are substantially identical to each other in sequence or identical in description. For example, a first sample identification region can be operatively coupled to a second sample identification region. In some aspects, the sequences of the first sample identification region and the second sample identification region are identical or substantially identical. In some aspects, the sequences of the first sample identification region and the second sample identification region are different or distinct.

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[00189] In some aspects, one or more regions of a polynucleotide described herein can be coupled to one or more other regions of the polynucleotide. In some aspects, two or more distinct regions of a single polynucleotide can be coupled. For example, a universal primer region can be coupled to an adapter region. In some aspects two or more regions can be coupled together that are substantially identical to each other in sequence or identical in description. For example, a first sample identification region can be coupled to a second sample identification region. In some aspects, the sequences of the first sample identification region and the second sample identification region are identical or substantially identical. In some aspects, the sequences of the first sample identification region and the second sample identification region are different or distinct.

[00190] In some aspects, a polynucleotide includes the sequence 5'-A-B-3', wherein A is a sample identification region, and wherein B is an adapter region. In some aspects, a polynucleotide includes the sequence 5'-A-B-C-3', wherein A is a universal primer region, wherein B is a sample identification region, and wherein C is an adapter region. In some aspects, a polynucleotide includes the sequence 5'-A-B-C-3', wherein A is a sample identification region, wherein B is an adapter region, and wherein C is an amplicon region derived from a single sample. In some aspects, a polynucleotide includes the sequence 5'-A-B-C-D-3', wherein A is a universal primer region, wherein B is a sample identification region, wherein C is an adapter region, and wherein D is an amplicon region derived from a single sample. In some aspects, a polynucleotide includes the sequence 5'-A-B-C-D-E-3', wherein A is a plate identification region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, and wherein E is an amplicon region derived from a single sample. In some aspects, a polynucleotide includes the sequence 5'-A-B-C-D-E-F-3', wherein A is a first restriction site region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, wherein E is an amplicon region derived from a single sample, and wherein F is a second restriction site region.

[00191] In some aspects, the regions of each of the above sequences can be rearranged in a different order, e.g., 5'-C-A-D-B-3' or 5'-E-A-C-B-D-F-3' or 5'-B-A-3'. In some aspects, one or more regions of the above sequences can be deleted, e.g., 5'-A-D-3' or 5'-B-C-3'. In some aspects, one or more additional regions can be added to the above sequences, e.g., 5'-A-A_2-B-3' or 5'-A-B-C-D-E-F-G-3'. In such examples the one or more additional regions can be any region disclosed herein or equivalents thereof. In some aspects, one or more regions of the sequences above can be modified, e.g., methylated.

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[00192] In some aspects, a polynucleotide can include an adapter molecule. In some aspects, a polynucleotide adapter molecule can include a universal primer region, a sample identification region, and an adapter region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, and wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region. In some aspects, an adapter molecule includes a polynucleotide comprising at least 2 nucleotides that bind to C's added by a reverse transcriptase at the 3' end of a 1st strand cDNA. In some aspects, an adapter molecule includes a deoxyribose polynucleotide comprising 3-6 G's (DNA G's). In another embodiment, an adapter molecule includes a ribose polynucleotide consisting of 3-6 G's (RNA G's). In other embodiments, the adapter molecule can utilize nucleotide analogues, such locked nucleic acids (LNAs), e.g., LNA G's. In other embodiments, the nucleotide base may also be a universal or degenerate base such as 5-nitroindole and 3-nitropyrrole that can base-pair to C's as well as other nucleotides, in any combination.

[00193] In some aspects, a polynucleotide can include a primer or a probe. In some aspects, a primer can include a universal primer region and a plate identification region, and wherein the 3' end of the plate identification region is coupled to the 5' end of the universal primer region.

[00194] In some aspects, a composition can include a polynucleotide composition library. In some aspects, a polynucleotide composition library includes a plurality of polynucleotide compositions. In some aspects each composition is present in a separate container. In some aspects, a container can be a test tube. In some aspects, a container can be a well in a plate. In some aspects, a container can be a well in a 96-well plate. In some aspects, a container can be a well in a 384-well plate. In some aspects, each composition comprises a cDNA region derived from a single sample. In some aspects, each composition comprises a sample identification-adapter region comprising a sample identification region coupled to an adapter region. In some aspects the sequence of the sample identification region of each sample identification-adapter region in a library is distinct from the nucleotide sequence of the sample identification region of the other sample identification-adapter regions present in each separate container in the library. In some aspects the sample identification-adapter region is attached to the cDNA region. In some aspects the sample identification-adapter region is attached to the cDNA region by binding between their 3' regions. In some aspects the sample identification-adapter region is attached to the cDNA region by G:C binding. In some aspects, the cDNA region comprises an RNA polynucleotide hybridized to a DNA polynucleotide. In some aspects, the cDNA region comprises an mRNA polynucleotide hybridized to a cDNA polynucleotide.

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[00195] In some aspects, the plurality of polynucleotide compositions in a polynucleotide library can comprise at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, at least 100,000, at least 300, 000, at least 1,000,000, at least 3,000,000, at least 10,000,000, at least 30,000,000, or more members. In other aspects, the plurality of polynucleotide compositions in a polynucleotide library can comprise at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, or more genes of a cell sample's whole transcriptome. In other aspects, the plurality of polynucleotide compositions in a polynucleotide library comprises at least 1, at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 10,000, at least 100,000, at least 1,000,000, at least 10,000,000, at least 1,000,000,000 or more of the different antibody species present in the blood of an individual. These the antibody species can be expressed by plasmablasts, plasma cells, memory B cells, long-lived plasma cells, naïve B cells, other B lineage cells, or combinations thereof.

Vectors

[00196] In some aspects, a composition can include a vector. Vectors can be used in the transformation of a host cell with a nucleic acid sequence. In some aspects, a vector can include one or more polynucleotides described herein. In one embodiment, a library of nucleic acid sequences encoding target polypeptides may be introduced into a population of cells, thereby allowing screening of a library. The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous" or "heterologous" which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, and viruses (e.g., bacteriophage). One of skill in the art may construct a vector through standard recombinant techniques, which are described in Maniatis et al., 1988 and Ausubel et al., 1994, both of which references are incorporated herein by reference. In some aspects, a vector can be a vector with the constant regions of an antibody pre-engineered in. In this way, one of skill can clone just the VDJ regions of an antibody of interest and clone those regions into the pre-engineered vector.

[00197] The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences for the

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transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well.

[00198] In some aspects, a vector can include a promoter. In some aspects, a vector can include an enhancer. A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[00199] A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic cell, and promoters or enhancers not "naturally occurring," i.e., containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR, in connection with the compositions disclosed herein (see U.S. Pat. No. 4,683,202, U.S. Pat. No. 5,928,906, each incorporated herein by reference).

[00200] In some aspects, a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type chosen for expression. One example of such promoter that may be used is the E. coli arabinose or T7 promoter. Those of skill in the art of molecular biology generally are familiar with the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook et al. (1989), incorporated herein by

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reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

[00201] In some aspects, vectors can include initiation signals and/or internal ribosome binding sites. A specific initiation signal also may be included for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

[00202] In some aspects, a vector can include sequences that increase or optimize the expression level of the DNA segment encoding the gene of interest. An example of such sequences includes addition of introns in the expressed mRNA (Brinster, R.L. et al. (1988) Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA 85, 836--40; Choi, T. et al. (1991) A generic intron increases gene expression in transgenic mice. Mol. Cell. Biol. 11, 3070--4). Another example of a method for optimizing expression of the DNA segment is "codon optimization". Codon optimization involves insertion of silent mutations in the DNA segment to reduce the use of rare codons to optimize protein translation (Codon engineering for improved antibody expression in mammalian cells. Carton JM, Sauerwald T, Hawley-Nelson P, Morse B, Peffer N, Beck H, Lu J, Cotty A, Amegadzie B, Sweet R. Protein Expr Purif. 2007 Oct;55(2):279-86. Epub 2007 Jun 16.).

[00203] In some aspects, a vector can include multiple cloning sites. Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see Carbonelli et al., 1999, Levenson et al., 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the

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vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

[00204] In some aspects, a vector can include a termination signal. The vectors or constructs will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments, a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary in vivo to achieve desirable message levels.

[00205] Terminators contemplated for use include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, rho dependent or rho independent terminators. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

[00206] In some aspects, a vector can include an origin of replication.

[00207] In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated.

[00208] In some aspects, a vector can include one or more selectable and/or screenable markers. In certain embodiments, cells containing a nucleic acid construct may be identified in vitro or in vivo by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

[00209] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would

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also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

[00210] In one aspect, the vector can express DNA segments encoding multiple polypeptides of interest. For example, DNA segments encoding both the immunoglobulin heavy chain and light chain can be encoded and expressed by a single vector. In one aspect, both DNA segments can be included on the same expressed RNA and internal ribosome binding site (IRES) sequences used to enable expression of the DNA segments as separate polypeptides (Pinkstaff JK, Chappell SA, Mauro VP, Edelman GM, Krushel LA., Internal initiation of translation of five dendritically localized neuronal mRNAs., Proc Natl Acad Sci U S A. 2001 Feb 27;98(5):2770-5. Epub 2001 Feb 20.). In another aspect, each DNA segment has its own promoter region resulting in expression of separate mRNAs (Andersen CR, Nielsen LS, Baer A, Tolstrup AB, Weilguny D. Efficient Expression from One CMV Enhancer Controlling Two Core Promoters. Mol Biotechnol. 2010 Nov 27. [Epub ahead of print]).

Host cells and Expression Systems

[00211] In some aspects, a composition can include a host cell. In some aspects, a host cell can include a polynucleotide or vector described herein. In some aspects, a host cell can include a eukaryotic cell (e.g., insect, yeast, or mammalian) or a prokaryotic cell (e.g., bacteria). In the context of expressing a heterologous nucleic acid sequence, "host cell" can refer to a prokaryotic cell, and it includes any transformable organism that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

[00212] In particular embodiments, a host cell is a Gram negative bacterial cell. These bacteria are suited for use in that they possess a periplasmic space between the inner and outer membrane and, particularly, the aforementioned inner membrane between the periplasm and cytoplasm, which is also known as the cytoplasmic membrane. As such, any other cell with such a periplasmic space could be used. Examples of Gram negative bacteria include, but are not limited to, E. coli, Pseudomonas aeruginosa, Vibrio cholera, Salmonella typhimurium, Shigella flexneri, Haemophilus influenza, Bordotella pertussi, Erwinia amylovora, Rhizobium sp. The Gram negative bacterial cell may be still further defined as bacterial cell which has been

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transformed with the coding sequence of a fusion polypeptide comprising a candidate binding polypeptide capable of binding a selected ligand. The polypeptide is anchored to the outer face of the cytoplasmic membrane, facing the periplasmic space, and may comprise an antibody coding sequence or another sequence. One means for expression of the polypeptide is by attaching a leader sequence to the polypeptide capable of causing such directing.

[00213] Numerous prokaryotic cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials. An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote host cell for replication of many vectors. Bacterial cells used as host cells for vector replication and/or expression include DH5-alpha, JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE™ Competent Cells and SOLOPACK™ Gold Cells (STRATAGENE™, La Jolla). In some aspects, other bacterial cells such as E. coli LE392 are contemplated for use as host cells.

[00214] Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with a prokaryotic host cell, particularly one that is permissive for replication or expression of the vector. Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

[00215] In some aspects, a host cell is mammalian. Examples include CHO cells, CHO-K1 cells, or CHO-S cells. Other mammalian host cells include NS0 cells and CHO cells that are dhfr-, e.g., CHO-dhfr-, DUKX-B11 CHO cells, and DG44 CHO cells.

[00216] Numerous expression systems exist can that comprise at least a part or all of the compositions disclosed herein. Expression systems can include eukaryotic expression systems and prokaryotic expression systems. Such systems could be used, for example, for the production of a polypeptide product identified as capable of binding a particular ligand. Prokaryote-based systems can be employed to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available. Other examples of expression systems comprise of vectors containing a strong prokaryotic

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promoter such as T7, Tac, Trc, BAD, lambda pL, Tetracycline or Lac promoters, the pET Expression System and an E. coli expression system.

Polypeptides

[00217] In some aspects, a composition can include a polypeptide. In some aspects, a polypeptide encoded by a polynucleotide described herein can be expressed, e.g., from a host cell. The terms "polypeptide" or "protein" include a macromolecule having the amino acid sequence of a native protein, that is, a protein produced by a naturally-occurring and non-recombinant cell; or it is produced by a genetically-engineered or recombinant cell, and comprise molecules having the amino acid sequence of the native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence. The term also includes amino acid polymers in which one or more amino acids are chemical analogs of a corresponding naturally-occurring amino acid and polymers. The terms "polypeptide" and "protein" encompass antigen binding proteins, antibodies, or sequences that have deletions from, additions to, and/or substitutions of one or more amino acids of antigen-binding protein. The term "polypeptide fragment" refers to a polypeptide that has an amino-terminal deletion, a carboxyl-terminal deletion, and/or an internal deletion as compared with the full-length native protein. Such fragments can also contain modified amino acids as compared with the native protein. In certain embodiments, fragments are about five to 500 amino acids long. For example, fragments can be at least 5, 6, 8, 10, 14, 20, 50, 70, 100, 110, 150, 200, 250, 300, 350, 400, or 450 amino acids long. Useful polypeptide fragments include immunologically functional fragments of antibodies, including binding domains. In the case of a binding antibody, useful fragments include but are not limited to a CDR region, a variable domain of a heavy and/or light chain, a portion of an antibody chain or just its variable region including two CDRs, and the like.

[00218] The term "isolated protein" means that a subject protein (1) is free of at least some other proteins with which it would normally be found, (2) is essentially free of other proteins from the same source, e.g., from the same species, (3) is expressed by a cell from a different species, (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature, (5) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature, or (6) does not occur in nature. Typically, an "isolated protein" constitutes at least about 5%, at least about 10%, at least about 25%, or at least about 50% of a given sample. Genomic DNA, cDNA, mRNA or other RNA, nucleic acids of synthetic origin, or any combination

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thereof can encode such an isolated protein. Preferably, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural environment that would interfere with its therapeutic, diagnostic, prophylactic, research or other use.

[00219] In some aspects, a polypeptide can include an antigen binding protein (ABP). An "antigen binding protein" ("ABP") as used herein means any protein that binds a specified target antigen. "Antigen binding protein" includes but is not limited to antibodies and binding parts thereof, such as immunologically functional fragments. Peptibodies are another example of antigen binding proteins. The term "immunologically functional fragment" (or simply "fragment") of an antibody or immunoglobulin chain (heavy or light chain) antigen binding protein, as used herein, is a species of antigen binding protein comprising a portion (regardless of how that portion is obtained or synthesized) of an antibody that lacks at least some of the amino acids present in a full-length chain but which is still capable of specifically binding to an antigen. Such fragments are biologically active in that they bind to the target antigen and can compete with other antigen binding proteins, including intact antibodies, for binding to a given epitope. In some embodiments, the fragments are neutralizing fragments. These biologically active fragments can be produced by recombinant DNA techniques, or can be produced by enzymatic or chemical cleavage of antigen binding proteins, including intact antibodies. Immunologically functional immunoglobulin fragments include, but are not limited to, Fab, a diabody (heavy chain variable domain on the same polypeptide as a light chain variable domain, connected via a short peptide linker that is too short to permit pairing between the two domains on the same chain), Fab', F(ab')2, Fv, domain antibodies and single-chain antibodies, and can be derived from any mammalian source, including but not limited to human, mouse, rat, camelid or rabbit. It is further contemplated that a functional portion of the antigen binding proteins disclosed herein, for example, one or more CDRs, could be covalently bound to a second protein or to a small molecule to create a therapeutic agent directed to a particular target in the body, possessing bifunctional therapeutic properties, or having a prolonged serum half-life. As will be appreciated by one of skill in the art, an antigen binding protein can include nonprotein components. Additional details about antigen binding proteins and antibodies such as modifications, variants, methods of making, and methods of screening can be found in U.S. Pat. Pub. 20110027287, herein incorporated by reference in its entirety for all purposes.

[00220] In some aspects, a polypeptide can include an antibody. The term "antibody" refers to an intact immunoglobulin of any isotype, or a fragment thereof that can compete with the intact antibody for specific binding to the target antigen, and includes, for instance, chimeric, humanized, fully human, and bispecific antibodies. An "antibody" is a species of an antigen

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binding protein. An intact antibody will generally comprise at least two full-length heavy chains and two full-length light chains, but in some instances can include fewer chains such as antibodies naturally occurring in camelids which can comprise only heavy chains. Antibodies can be derived solely from a single source, or can be "chimeric," that is, different portions of the antibody can be derived from two different antibodies. The antigen binding proteins, antibodies, or binding fragments can be produced in hybridomas, by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact antibodies. Unless otherwise indicated, the term "antibody" includes, in addition to antibodies comprising two full-length heavy chains and two full-length light chains, derivatives, variants, fragments, and muteins thereof. Furthermore, unless explicitly excluded, antibodies include monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies, synthetic antibodies (sometimes referred to herein as "antibody mimetics"), chimeric antibodies, humanized antibodies, human antibodies, antibody fusions (sometimes referred to herein as "antibody conjugates"), and fragments thereof, respectively. In some embodiments, the term also encompasses peptibodies.

[00221] A therapeutically effective amount of an ABP can be administered to a subject in need thereof. ABPs can be formulated in pharmaceutical compositions. These compositions can comprise, in addition to one or more of the ABPs, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material can depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

[00222] Pharmaceutical compositions for oral administration can be in tablet, capsule, powder or liquid form. A tablet can include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol can be included.

[00223] For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives can be included, as required.

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[00224] ABP administration is preferably in a "therapeutically effective amount" or "prophylactically effective amount" (as the case can be, although prophylaxis can be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of disease being treated. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

[00225] A composition can be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Immune cells

[00226] A sample can include immune cells. The immune cells can include T cells and B cells. T-cells (T lymphocytes) include, for example, cells that express T cell receptors. B-cells include, for example, activated B cells, blasting B cells, plasma cells, plasmablasts, memory B cells, B1 cells, B2 cells, marginal-zone B cells, and follicular B cells. T cells include activated T cells, blasting T cells, Helper T cells (effector T cells or Th cells), cytotoxic T cells (CTLs), memory T cells, central memory T cells, effector memory T cells and regulatory T cells. A sample can include a single cell in some applications (e.g., a calibration test to define relevant T or B cells) or more generally at least 1,000, at least 10,000, at least 100,000, at least 250,000, at least 500,000, at least 750,000, or at least 1,000,000 cells.

B cells

[00227] As used herein a "B cell" refers to any cell that has at least one rearranged immunoglobulin gene locus. A B cell can include at least one rearranged immunoglobulin heavy chain locus or at least one rearranged immunoglobulin light chain locus. A B cell can include at least one rearranged immunoglobulin heavy chain locus and at least one rearranged immunoglobulin light chain locus. B cells are lymphocytes that are part of the adaptive immune system. B cells can include any cells that express antibodies either in the membrane-bound form as the B-cell receptor (BCR) on the cell surface or as secreted antibodies. B cells can express immunoglobulins (antibodies, B cell receptor). Antibodies can include heterodimers formed from the heavy and light immunoglobulin chains. The heavy chain is formed from gene rearrangements of the variable, diversity, and junctional (VDJ) genes to form the variable

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region, which is joined to the constant region. The light chain is formed from gene rearrangements of the variable and junctional (VJ) genes to form the variable region, which is then joined to the constant region. Owing to a large possible number of junctional combinations, the variable regions of the antibody gene (which is also the BCR) have huge diversity, enabling B cells to recognize any foreign antigen and mount a response against it.

B-cell activation and differentiation

[00228] B cells are activated and differentiate when they recognize an antigen in the context of an inflammatory immune response. They usually include 2 signals to become activated, one signal delivered through BCR (a membrane-bound form of the rearranged immunoglobulin), and another delivered through CD40 or another co-stimulatory molecule. This second signal can be provided through interaction with helper T cells, which express the ligand for CD40 (CD40L) on their surface. B cells then proliferate and may undergo somatic hypermutation, where random changes in the nucleotide sequences of the antibody genes are made, and B cells whose antibodies have a higher affinity B cells are selected. They may also undergo "class-switching", in which the constant region of the heavy chain encoding the IgM isotype is switched to the constant region encoding the IgG, IgA, or IgE isotype. Differentiating B cells may end up as memory B cells, which are usually of higher affinity and classed switched, though some memory B cells are still of the IgM isotype. Memory B cells can also become activated and differentiate into plasmablasts and ultimately, into plasma cells. Differentiating B cells may also first become plasmablasts, which then differentiate to become plasma cells.

Affinity maturation and clonal families

[00229] A clonal family is generally defined by the use of related immunoglobulin heavy chain and/or light chain V(D)J sequences by 2 or more samples. Related immunoglobulin heavy chain V(D)J sequences can be identified by their shared usage of V(D)J gene segments encoded in the genome. Within a clonal family there are generally subfamilies that vary based on shared mutations within their V(D)J segments, that can arise during B cell gene recombination and somatic hypermutation.

[00230] Activated B cells migrate and form germinal centers within lymphoid or other tissues, where they undergo affinity maturation. B cells may also undergo affinity maturation outside of germinal centers. During affinity maturation, B cells undergo random mutations in their antibody genes, concentrated in the complementary determining regions (CDRs) of the genes, which encode the parts of the antibody that directly bind to and recognize the target antigen against which the B cell was activated. This creates sub-clones from the original

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proliferating B cell that express immunoglobulins that are slightly different from the original clone and from each other. Clones compete for antigen and the higher-affinity clones are selected, while the lower-affinity clones die by apoptosis. This process results in the "affinity maturation" of B cells and consequently in the generation of B cells expressing immunoglobulins that bind to the antigen with higher affinity. All the B cells that originate from the same 'parent' B cell form clonal families, and these clonal families include B cells that recognize the same or similar antigenic epitopes. In some aspects, we expect that clones present at higher frequencies represent clones that bind to antigen with higher affinity, because the highest-affinity clones are selected during affinity maturation. In some aspects, clones with different V(D)J segment usage exhibit different binding characteristics. In some aspects, clones with the same V(D)J segment usage but different mutations exhibit different binding characteristics.

Memory B cells

[00231] Memory B cells are usually affinity-matured B cells, and may be class-switched. These are cells that can respond more rapidly to a subsequent antigenic challenge, significantly reducing the time included for affinity-matured antibody secretion against the antigen from ~14 days in a naive organism to ~7 days.

Plasmablasts and plasma cells

[00232] Plasma cells can be either long-lived or short-lived. Long-lived plasma cells may survive for the lifetime of the organism, whereas short-lived plasma cells can last for 3-4 days. Long-lived plasma cells reside either in areas of inflammation, in the mucosal areas (in the case of IgA-secreting plasma cells), in secondary lymphoid tissues (such as the spleen or lymph nodes), or in the bone marrow. To reach these divergent areas, plasmablasts fated to become long-lived plasma cells may first travel through the bloodstream before utilizing various chemokine gradients to traffic to the appropriate areas. Plasmablasts are cells that are affinity matured, are typically classed-switched, and usually secrete antibodies, though generally in lower quantities than the quantity of antibody produced by plasma cells. Plasma cells are dedicated antibody secretors.

Characteristics of TCR and BCR genes

[00233] Since identifying recombinations are present in the DNA of each individual adaptive immune cell as well as their associated RNA transcripts, either RNA or DNA can be sequenced. A recombined sequence from a T-cell or B-cell can also be referred to as a clonotype. The DNA

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or RNA can correspond to sequences from T-cell receptor (TCR) genes or immunoglobulin (Ig) genes that encode antibodies. For example, the DNA and RNA can correspond to sequences encoding alpha, beta, gamma, or delta chains of a TCR. In a majority of T-cells, the TCR is a heterodimer consisting of an alpha-chain and beta-chain. The TCR-alpha chain is generated by VJ recombination, and the beta chain receptor is generated by V(D)J recombination. For the TCR-beta chain, in humans there are 48 V segments, 2 D segments, and 13 J segments. Several bases may be deleted and others added (called N and P nucleotides) at each of the two junctions. In a minority of T-cells, the TCRs consist of gamma and delta chains. The TCR gamma chain is generated by VJ recombination, and the TCR delta chain is generated by V(D)J recombination (Kenneth Murphy, Paul Travers, and Mark Walport, Janeway's Immunology 7th edition, Garland Science, 2007, which is herein incorporated by reference in its entirety).

[00234] The DNA and RNA analyzed in the methods can correspond to sequences encoding heavy chain immunoglobulins (IgH) with constant regions (alpha, delta, gamma, epsilon, or mu) or light chain immunoglobulins (IgK or IgL) with constant regions lambda or kappa. Each antibody can have two identical light chains and two identical heavy chains. Each chain is composed of a constant (C) and a variable region. For the heavy chain, the variable region is composed of a variable (V), diversity (D), and joining (J) segments. Several distinct sequences coding for each type of these segments are present in the genome. A specific VDJ recombination event occurs during the development of a B-cell, marking that cell to generate a specific heavy chain. Diversity in the light chain is generated in a similar fashion except that there is no D region so there is only VJ recombination. Somatic mutation often occurs close to the site of the recombination, causing the addition or deletion of several nucleotides, further increasing the diversity of heavy and light chains generated by B-cells. The possible diversity of the antibodies generated by a B-cell is then the product of the different heavy and light chains. The variable regions of the heavy and light chains contribute to form the antigen recognition (or binding) region or site. Added to this diversity is a process of somatic hypermutation which can occur after a specific response is mounted against some epitope. In this process mutations occur in those B-cells that are able to recognize the specific epitope leading to greater diversity in antibodies that may be able to bind the specific epitope more strongly. All these factors contribute to great diversity of antibodies generated by the B-cells. Many billions and maybe more than a trillion distinct antibodies may be generated. The basic premise for generating T-cell diversity is similar to that for generating antibodies by B-cells. An element of T-cell and B-cell activation is their binding to epitopes. The activation of a specific cell leads to the production of more of the same type of cells leading to a clonal expansion.

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[00235] Complementarity determining regions (CDR), or hypervariable regions, are sequences in the variable domains of antigen receptors (e.g., T cell receptor and immunoglobulin) that can bind an antigen. The chain of each antigen receptor contains three CDRs (CDR1, CDR2, and CDR3). The two polypeptides making T cells (alpha and beta) and immunoglobulin (IgH and IgK or IgL) contribute to the formation of the three CDRs.

[00236] The part of CDR1 and CDR2 that is coded for by TCR-beta lies within one of 47 functional V segments. Most of the diversity of CDRs is found in CDR3, with the diversity being generated by somatic recombination events during the development of T lymphocytes.

[00237] A great diversity of BCR is present inter and intra-individuals. The BCR is composed of two genes IgH and IgK (or IgL) coding for antibody heavy and light chains. Three Complementarity Determining Region (CDR) sequences that bind antigens and MHC molecules have the most diversity in IgH and IgK (or IgL). The part of CDR1 and CDR2 coded for by IgH lies within one of 44 functional V segments. Most of the diversity in naive B cells emerges in the generation of CDR3 through somatic recombination events during the development of B lymphocytes. The recombination can generate a molecule with one of each of the V, D, and J segments. In humans, there are 44 V, 27 D, and 6 J segments; thus, there is a theoretical possibility of more than 7,000 combinations. In a small fraction of BCRs (about 5%) two D segments are found. Furthermore, several bases may be deleted and others added (called N and P nucleotides) at each of the two junctions generating a great degree of diversity. After B cell activation a process of affinity maturation through somatic hypermutation occurs. In this process progeny cells of the activated B cells accumulate distinct somatic mutations throughout the gene with higher mutation concentration in the CDR regions leading to generating antibodies with higher affinity to the antigens. In addition to somatic hypermutation activated B cells undergo the process of isotype switching. Antibodies with the same variable segments can have different forms (isotypes) depending on the constant segment. Whereas all naive B cells express IgM (or IgD), activated B cells mostly express IgG but also IgM, IgA and IgE. This expression switching from IgM (and/or IgD) to IgG, IgA, or IgE occurs through a recombination event causing one cell to specialize in producing a specific isotype. There is one segment for each IgM, IgD, and IgE, two segments for IgA, and four segments for IgG.

Methods

Application to health care and biotechnology uses

[00238] Use of the compositions and methods described herein to identify antibodies and TCRs and to group antibody and TCR sequences into clonal families has many useful and novel

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applications to health care and biotechnology research. Antibody clonal families can comprise affinity-matured non-identical clones and TCR clonal families can comprise identical clones. These applications include, but are not limited to: 1) the discovery and development of antibody or antibody-derived therapeutics; 2) the discovery and development of diagnostics; 3) the discovery and development of research tools useful in health and biotechnology research; and 4) the development and assessment of candidate vaccines and identification of antigens useful as vaccine components.

[00239] As the invention can be used with any type of B or T cell, the cell source and specific B or T cell subtype(s) are chosen based on the profile of the desired ultimate product. Examples of specific subclasses of B or T cells and their use are described in the subsection, "Isolation and enrichment of cells and cell subpopulations" in the General Materials and Methods section. In general, the cells can be from a particular human or animal subject having a particular clinical state or course of disease, or having received a particular treatment regimen, or having been exposed to a particular challenge, immunization, or set of conditions that induces an immune response.

Application to discovery and development of therapeutics, diagnostics, and research tools

[00240] To develop an antibody or molecule derived from an antibody for use as a therapeutic, diagnostic, or research tool, the antibody and/or derivatives of the antibody's antigen-binding regions can first be identified or discovered as binding a/the desired antigen(s) or epitope(s) and/or having a desired functional consequence in an in vivo or in vitro system. These candidate antibodies are then further screened for other desired properties specific to the intended product. These target product properties will be different for different types of therapeutic, diagnostic, and research tool antibodies, and the invention provides a useful means of identifying candidates for further development toward any of these product paths.

[00241] Based on the desired profile of the properties of the ultimate product, the source of relevant B cells can be, but is not limited to, a patient with disease, such as an infectious disease, cancer, or an autoimmune condition; a patient receiving a treatment, such as cancer therapy or a vaccine; or an animal with disease or treated in a manner to induce an immune response, such as immunization or induction/establishment of a disease model.

[00242] In general, candidate antibodies, or candidate macromolecules derived from the antigen-binding regions, that are intended for development as therapeutics, diagnostics, or research tools are discovered via multiple technologies that fall into one of two general approaches: 1) isolation of antibodies of interest from B cells of a human's or an animal's

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immune response; and 2) isolation of antibodies derived from expression libraries of immunoglobulin molecules, or derivatives thereof, expressed heterologously and screened using one or more display technologies (reviewed in Hoogenboom HR, Trends Biotechnol., 1997,15:62-70; Hammond PW, MAbs, 2010, 2:157-64; Nissim A, Chernajovsky Y, Handb Exp Pharmacol., 2008, (181):3-18; Steinitz M, Hum Antibodies, 2009;18:1-10; Bradbury AR, Sidhu S, Dübel S, and McCafferty, Nat Biotechnol., 2011, 29:245-54; *Antibody Engineering* (Kontermann RE and Dübel S eds., Springer, 2nd edition)).

[00243] For the former approach (#1), candidate antibodies are selected from particular clonal families identified from relevant donors as described in, e.g., the General Materials and Methods section. The invention can be applied as described to the appropriate B-cells (e.g. blasting B-cells) from the appropriate human donor or animal to discover or identify candidate antibodies. For example, for a cancer therapeutic antibody candidate, the appropriate human donor can be a patient who has successfully suppressed cancer progression via an immune response; or for a particular diagnostic antibody candidate, the appropriate donor can be a patient who has autoantibodies against the diagnostic marker or a mouse immunized against the marker; or for an antibody reagent tool candidate, the appropriate donor can be a mouse, a rabbit, a goat, , a rat, a horse, a chicken, a dog, or other animal immunized with the target molecule and/or epitope that the antibody reagent is meant to recognize. Sequences and selection of antibodies for expression and testing can be performed as described in the General Materials and Methods section. Such applications of the technology can provide candidate antibodies often obtained via more laborious and time-consuming methods (e.g. hybridoma technology, virus-induced immortalization of B cells, etc).

[00244] For the latter approach (#2), a subset of, or the entire set of paired heavy and light chain sequences from a one or more human or animal antibody repertoires, obtained as in #1, are used to seed expression libraries containing identification regions to track sample origin and original cognate pairs from the sample when a library and/or a selected/enriched subset of a library is sequenced using a next generation sequencing platform. Variable regions and framework region information can be incorporated into one or more antibody display library formats to discover candidate antibodies. Variable regions of Ig genes can be cloned and incorporated into expression vectors using methods described in the subsection, "Cloning and expression of cloned light and heavy chain immunoglobulin pairs" in the General Materials and Methods section. For example, fragments and/or domains from cognate pair heavy and light chains obtained as in #1 can be used to seed Fab yeast (Weaver-Feldhaus JM, Lou J, Coleman JR, et al., FEBS Lett, 2004, 564:24-34) or phagemid (Kashyap AK, Steel J, Oner AF, et al., Proc

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Natl Acad Sci U S A, 2008, 105:5986-91) libraries with identification region tracking of each chain to the proper, original B cell of origin regardless of combinatorial matching of different heavy and light chains into non-endogenous (non-cognate) pairings. The cognate pair heavy and light chains obtained as in #1 can also be used with other display platforms, beyond phagemid or yeast, and can be used with other antibody derivative expression constructs beyond Fab fragment expression constructs [*Antibody Engineering* (Kontermann, RE and Dübel S eds., Springer, 2nd edition)]. In an alternate application of the identification regions, identification regions can be added to already existing display libraries to provide the benefits of identification region tracking and error correction of next generation sequencing data. Depending on the library type, format, and expression/display system, identification regions can be incorporated using PCR reactions or reverse transcriptase followed by PCR reactions (see, e.g., the subsection, "Sequencing of paired light and heavy chain immunoglobulin genes from single B-cells" in the General Materials and Methods section).

[00245] Candidate antibodies, whether from B cell repertoires (see, e.g., General Materials and Methods section) or display expression library "repertoires" (Kashyap AK, Steel J, Oner AF, et al., Proc Natl Acad Sci U S A, 2008, 105:5986-91; Weaver-Feldhaus JM, Lou J, Coleman JR, et al., FEBS Lett, 2004, 564:24-34; Ravn U, Gueneau F, Baerlocher L, et al., Nucleic Acids Res, 2010, 38:e193; *Antibody Engineering* (Kontermann, RE and Dübel S eds., Springer, 2nd edition), are identified by expressing and testing the antibody or antibody-derivative molecules, or libraries of molecules, in assays for binding against desired antigen/target(s) and/or epitope(s) or in assays for testing of functional consequence in an in vivo or in vitro (including ex vivo samples/preparations) setting. Published reports have described the use of identification regions to track the donor source of antibody sequences obtained from a B-cell repertoire for use in an expression library (e.g. Kashyap AK, Steel J, Oner AF, et al., Proc Natl Acad Sci U S A, 2008, 105:5986-91). The identification region technology described herein, uniquely provides useful improvements upon such identification region usage. The invention: 1) provides a means to track, not only each donor, but each donor's B cells for cognate pairing of heavy and light chains; 2) provides a means to index back to the original B cell sample for retrieval of more sample for cloning and/or testing; 3) provides a means of tracking heavy and light chain origin despite non-cognate combinatorial pairings within the expression library; 4) provides a means of tracking heavy and light chain origin across rounds of selection-enrichment (e.g. when monitoring sequence evolution during pool selection in vitro, such as in Ravn U, Gueneau F, Baerlocher L, et al., Nucleic Acids Res, 2010, 38:e193).

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[00246] Identification of the most frequently represented heavy or light chain sequences in a B cell immune response repertoire, and combining heavy and light chain pairs based on rank order frequency of the individual chains, has been shown to be a viable way to identify some candidate antibodies, despite the fact that the cognate pair information is not retained in the next generation sequence analyses when performed in this manner (Reddy ST, Ge X, Miklos AE, et al., Nat Biotechnol, 2010, 28:965-9). The invention also allows for this type of frequency analysis methodology, but can further provide a means to use next generation sequencing to assess the frequency of actual antibodies in the repertoire, not simply isolated, independent heavy or light chains.

[00247] Furthermore, the invention provides at least three improvements of significant utility beyond frequency analysis: 1) because the cognate pairing of heavy and light chains can be tracked, the discovery of actual antibodies from the immune response and the actual antibody clonal families produced by the B cells in the immune response can be identified (a clone involves a specific, cognate pair of heavy and light chains that co-evolved from the same cell progenitors and information about natural pairings within the affinity maturation process would improve upon approaches described in the literature to analyze immune responses using next generation sequencing [e.g. Wu X, Yang ZY, Li Y, Hogerkorp CM, et al., Science, 2010, 329:856-61]); 2) identification regions provide the means to minimize, or even eliminate, the effect on sequence analyses of sequencing errors common to next generation sequencing platforms (see, e.g., subsection, "Other sequencing data analysis options" in the General Materials and Methods section); and 3) identification regions provide the ability to link and track ≥2 sequences co-expressed at the single cell level.

[00248] For those candidate antibodies that have been identified as having desirable binding properties to an antigen, target, or epitope, or that have a desired functional effect, more antibodies from the respective clonal family can be cloned and expressed (see, e.g., General Materials and Methods section) to test for the presence of similar, but potentially more optimal, antibodies or antibodies that are the same in binding or functional properties but contain other difference of import to the final product profile.

[00249] For cases in which candidates are identified from display expression libraries, identification regions can provide a means to identify antibodies of potentially similar sequence to candidates by identifying sequences that were not selected in screening enrichment but which contain identification regions of the identified candidates and thus are derived from the same original heavy and/or light chains that seeded the library. Antibodies that are lost in rounds of in vitro selection, but are similar to selected, candidate antibodies can be recovered or "rescued"

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for further analysis as potential candidates. Such rescue can obviate the effect of bias in expression or assays systems that may miss useful and functional antibodies (Ravn U, Gueneau F, Baerlocher L, et al., Nucleic Acids Res, 2010, 38:e193).

[00250] Once candidates with desired binding and/or functional properties are identified, they can then be advanced to relevant assays and other assessments based on the desired, downstream, product profile. For therapeutic antibodies intended for use in passive immunization, candidates are advanced to assays and preclinical testing models to determine the best candidates for clinical testing in humans or for use in animal health, including, but not limited, assessments of properties such as stability and aggregation, formulation and dosing ease, protein expression and manufacturing, species selectivity, pharmacology, pharmacokinetics, safety and toxicology, absorption, metabolism and target-antibody turnover, as well as immunogenicity [*See, e.g.,* Lynch CM, Hart BW, and Grewal IS, MAbs, 2009, 1: 2--11; Chapman K, Pullen N, Coney L, et al., mAbs, 2009, 1, 505-516; S. Dübel, *Handbook of Therapeutic Antibodies: Technologies, Emerging Developments and Approved Therapeutics* (John Wiley & Sons, 2010); Therapeutic *Monoclonal Antibodies: From Bench to Clinic*, (Z. An ed., John Wiley & Sons, 2009) *Antibody Engineering* (Kontermann RE and Dübel S eds., Springer, 2nd edition)]. Thus many candidates are selected because the majority will be insufficient for therapeutic testing in humans with respect to at least one of the many properties that need to be assessed prior to human testing (i.e. attrition). Clonal families can be mined, e.g., as described above, for candidates similar to ones already characterized, but possibly harboring differences regarding one or more of the properties that are assessed in preclinical work. Specific antibody engineering strategies may need to be employed to optimize for certain properties [*Antibody Engineering* (Kontermann RE and Dübel S eds., Springer, 2nd edition)].

[00251] For diagnostics, the invention can be used to identify antibodies, TCRs, and clonal families produced by infection or vaccination for use in the detection of infectious agents (Selvarajah S, Chatterji U, Kuhn R, et al., 2012, 6:29-37; Berry JD, Vet J, 2005, 170:193-211), as well as for any non-infectious disease, pathological condition, or medical treatment or therapy. Such antibodies, TCRs, and/or clonal families can provide useful diagnostic probes for biomarkers or provide immune system information about the disease state of, or effect of treatment on, a human or animal. As such, specific antibodies or TCRs, or specific clonal families of either immune receptor class can provide utility for diagnostic tools and personalized medicine. In another application to diagnostics, known disease or treatment response biomarkers can be used as immunogens to immunize mice or other animals from which B cells are harvested to identify antibodies (see, e.g., General Materials and Methods section) against

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the biomarker which could subsequently be used in diagnostic tests, such as ELISAs or other immunoassays (Selvarajah S, Chatterji U, Kuhn R, et al., 2012, 6:29-37). Once identified as having potential diagnostic utility, candidate antibodies, TCRs, and/or clonal families can be advanced to assays, models, and possibly trials relevant to the desired profile of the diagnostic product [Berry JD, Vet J, 2005, 170:193-211; *Diagnostic and Therapeutic Antibodies* in Methods in Molecular Medicine, Vol. 40 (George AJT and Urch CE eds., Humana Press); *Antibody Engineering* (Kontermann RE and Dübel S eds., Springer, 2nd edition) ; Colwill K, Renewable Protein Binder Working Group, and Gräslund S, Nat Methods, 2011, 8:551-8; Pershad K, Pavlovic JD, Gräslund S, et al., Protein Eng Des Sel, 2010, 23:279-88.]. Specific antibody engineering strategies may need to be employed to optimize for certain properties [*Antibody Engineering* (Kontermann RE and Dübel S eds., Springer, 2nd edition)].

[00252] For research tool antibodies, candidates identified, e.g., as described above, can be advanced to test how they perform in the research application for which the research tool antibody is intended (e.g. immunoprecipitation; immunoblotting; immunostaining and histology; immunoaffinity purification; capture-, and sandwich-, and detection immunoassays; for example as described in *Antibodies: A Laboratory Manual*, E Harlow and D Lane (Cold Spring Harbor Laboratory Press, 1988). Validation criteria will be based on the final intended research use (Colwill K, Renewable Protein Binder Working Group, and Gräslund S, Nat Methods, 2011, 8:551-8; Pershad K, Pavlovic JD, Gräslund S, et al., Protein Eng Des Sel, 2010, 23:279-88). Specific antibody engineering strategies may need to be employed to optimize for certain properties [*Antibody Engineering* (Kontermann RE and Dübel S eds., Springer, 2nd edition)].

Application to vaccine discovery and development

[00253] The invention can be used to identify antibodies, TCRs, and clonal families of each of these immune receptor classes to a vaccine challenge in a human or animal. Specific antibodies can be used as probes to identify the vaccine component(s) recognized by the antibody and the clonal family to which the antibody that was used as a probe belongs. This information about antibody and clonal families can be used to make assessments about the proportions or strength of the immune response targeting particular antigens and/or epitopes of the vaccine. The assessment of antibody immune responses to different vaccine components can be complemented with information collected about the concomitant TCR repertoire response to the vaccine, (see, e.g., subsections, "For other cell types" and "PCR of other immunoglobulin heavy chains and T-cell receptor (TCR) chains" in the General Materials and Methods section). This information can be subsequently used to understand which components of the vaccine, or

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which variants of a vaccine, or what adjuvants produce effective or more optimal responses from an immune system of a human or animal (Haynes BF, Gilbert PB, McElrath MJ, et al., N Engl J Med, 2012, 366:1275-86). The approach can also be used to compare individuals or populations in their response to a vaccine.

[00254] Similar analyses can be performed to identify and assess the antibodies, TCRs, and the clonal families produced in response to an actual pathogen and which may correlate with clinical outcomes of interest, such as protective responses to infection (for example, identification of antibodies from survivors of a severe influenza pandemic, Yu X, Tsibane T, McGraw PA, et al., Nature, 2008, 455:532-6; or identification of specific antibodies from HIV-infected individuals with broadly HIV-neutralizing sera, Wu X, Yang ZY, Li Y, Hogerkorp CM, et al., Science, 2010, 329:856-61 and Walker LM, Phogat SK, Chan-Hui PY, et al., Science, 2009, 326:285-9). Identification of such correlates of protection can be compared to the response produced by the vaccine and/or specific vaccine components as described above and the two datasets can be compared to assess the ability of the vaccine to produce immune responses that correlate to desired outcomes seen in cases of actual infection.

[00255] Thus, the invention provides a useful means of obtaining a surrogate readout of disease protection and vaccine response, via antibody and/or TCR repertoire sequence analysis, before a human or animal is challenged with actual infection. Once a clonal family has been identified as binding a particular antigen or epitope, the identification of antigens or epitopes targeted by other immune response repertoires is possible without doing assays in cases where the same or similar clonal families are found across repertoires. Thus, in those cases where enough information about a clonal family and it antigen/epitope binding is known (see, e.g., subsection, "Screening of expressed human antibodies" in the General Materials and Methods section), sequence analysis alone of newly analyzed repertoires can provide a readout of the antigens of that repertoire for the known clonal families that it contains. This application can provide a useful means to monitor responses across one, a few, or many subjects in vaccine clinical trials and to monitor immunity and infectious disease relationships for one, a few, or many people on a population level.

[00256] Furthermore, antibody, TCR, and clonal families that correlate with protection from a pathogen can be used to identify the specific antigens and sets of antigens (including both known and novel antigens) that mediate protective and/or effective immune responses against the pathogen. Identification of the antigens targeted in effective immune responses can be used to guide the selection of antigens to be included in vaccines that are expected to produce protective antibody- and TCR-mediated responses in immunized humans or animals.

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[00257] Antibodies, TCR, and clonal families that do not bind known antigens in assays become candidates for identifying potentially novel antigens and/or epitopes of the pathogen against which the antibodies and/or TCRs provide protection. Antibodies known to not bind already known antigens can be used as probes in combination with immunoseparation and mass spectroscopy to identify the previously unidentified antigen or epitope (Zhu YZ, Cai CS, Zhang W, et al., PLoS One, 2010, 5:e13915; and see, e.g., subsections, "Immunoprecipitation of staph antigens with antibodies derived from staph-infected patients" and "Mass spectrometry identification of peptides" in the General Materials and Methods section). Such novel antigens or epitopes can be used as vaccine components that are expected to produce or contribute to the production of protective antibody- and TCR-mediated responses in immunized humans or animals.

[00258] In addition to facilitating development of vaccines for microbial pathogens, the antibody, TCR and clonal families can also be used to develop tumor vaccines. Humans or animals that mount an immune response against a cancer or pre-cancerous cells can yield antibodies, TCR, and clonal families that can be used to identify individual and combinations of antigens that can be incorporated into preventative or therapeutic vaccines for cancer.

Methods for producing one or more polynucleotides of interest

[00259] In some aspects, a method includes obtaining a cDNA library comprising a plurality of cDNAs associated with a plurality of samples obtained from one or more subjects, wherein each cDNA is associated with a single sample in the plurality of samples, and wherein each cDNA associated with each sample is present in a separate container; adding an adapter molecule to the cDNA associated with each sample, wherein the adapter molecule comprises a sample identification region and an adapter region, wherein the sample identification region is coupled to the adapter region, and wherein the sequence of the sample identification region of each adapter molecule is distinct from the sequence of the sample identification region of the other adapter molecules added to each cDNA in the library; and allowing the adapter region to attach to each cDNA in the library to produce the one or more polynucleotides of interest.

[00260] In some aspects, obtaining the cDNA library comprises obtaining the plurality of samples and processing the samples to prepare the cDNA library. In some aspects, obtaining the cDNA library comprises receiving the cDNA library directly or indirectly from a third party that has processed the plurality of samples to prepare the cDNA library.

[00261] In some aspects, the adapter molecule further comprises a universal primer region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample

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identification region. In some aspects, each cDNA region comprises an mRNA polynucleotide hybridized to a cDNA polynucleotide.

[00262] In some aspects, each sample comprises a cell. In some aspects, the cell is a B cell. In some aspects, the B cell is a plasmablast, memory B cell, or a plasma cell. In some aspects, each sample comprises a plurality of cells.

[00263] In some aspects, each adapter region is attached to each cDNA via binding, e.g., G:C binding.

[00264] In some aspects, the adapter molecule is single-stranded, and further comprising incorporating the adapter molecule into each cDNA by allowing an enzyme to make the adapter molecule double-stranded. In some aspects, the adapter molecule is incorporated into each cDNA to produce the polynucleotide of interest by an MMLV H$^-$ reverse transcriptase.

[00265] In some aspects, methods can include amplification steps such as PCR and other amplification reactions generally known in the art.

Methods for linking and barcoding polynucleotides of interest

[00266] In some aspects, the method includes the linking of two polynucleotide sequences of interest, e.g., an antibody light chain (LC) and heavy chain (HC) from a single sample, and providing one or more barcode or sequence identification sequences. In this aspect, there is provided a physical linkage between the polynucleotide sequences of interest as well as one or more barcodes to provide an identifier to allow polynucleotide sequences derived from a particular source or sample to be determined, e.g., single cell, sample well, single sample, etc. Single samples can comprise one or more B-lineage cells or other cell types. Examples of methods to link two polynucleotide sequences of interest are known in the art, for example, WO 99/16904, WO 93/03151, and US 7,749,697, which are hereby incorporated by reference. Among other advantages associated with the use of barcodes on linked polynucleotide sequences include facilitation of high-throughput sequencing and mapping of a sequence back to an original sample so that it can be re-sequenced and PCR cloned to express the polynucleotide sequences, e.g., HC and LC immunoglobulin polynucleotides. Some of the high-throughput sequencing technologies exhibit sequencing error rates of 1-10+%, and the use of barcodes enables repeat sequencing of templates to facilitate bioinformatic error correction. This is particularly important for distinguishing sequencing errors from gene variations, such as those in immunoglobulin polynucleotides. Specifically, it can be difficult to ascertain if closely related sequences are in fact distinct sequences or if they instead represent artifacts produced by sequencing errors. Barcodes, by enabling analysis of repeat sequencing of individual templates thereby enable sequencing error correction, thus providing determination of whether sequences

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are distinct vs. artifacts from sequencing error(s). In one embodiment, the polynucleotide sequences are immunoglobulin HC and LC sequences that have diverged due to somatic hypermutation, and differ by only 1 nucleotide.

[00267] In this aspect, physically linked and barcoded structures as shown in Figure 15 are generally obtained. Figure 15 illustrates the physical linkage of two nucleic acid segments, A and B (e.g., two cDNAs). A barcode (BC) is appended to any one of ends or in the linker connecting A and B. This physical linkage of A and B, as well as, addition of the barcode is accomplished through any of a number of means, including by ligation, recombination, amplification, or overlap-extension, or a combination of these methods, as described in greater detail below. Optionally, additional barcodes can be added to the structure shown in Figure 15, to provide compound barcoding to enable sequencing of a large number of linked polynucleotides using a lesser number of barcodes. Also, it will be appreciated that depending on the particular strategy used to link the two nucleic acid segments, any relative orientation of the segments can be obtained, with respect to sense and antisense orientations, i.e., the segments, such as cDNAs, can be joined head to tail, head to head, or tail to tail.

[00268] Barcodes can be added to the polynucleotide sequences before, during or after physical linkage using methods known in the art. These methods include, but are not limited to, for example, ligation methods, such as blunt end ligation of barcode adaptors, and by the annealing and ligation of compatible ends, such as those generated by homopolymeric tailing, restriction enzyme digestion of a linker, or 3' tailing of cDNA by reverse transcriptase. Barcodes can also be added in amplification reactions using suitable primers carrying barcode sequences. Barcodes can also be added in a reverse transcription reaction using suitable primers containing the barcode sequence. Barcodes can also be added by incorporation into oligonucleotides used to link the genes of interest together, through overlap-extension tails or other methods, such that they are located between the two genes of interest. Accordingly, using these methods, barcodes can be incorporated onto the ends of physically linked polynucleotide sequences or into the linker sequences joining the two polynucleotide sequences of interest.

[00269] In one embodiment, the linkage is accomplished through the use of overlap-extension (see Figure 16). In general, overlap extension tails are complementary sequences that are added to polynucleotide sequences of interest to be joined. Annealing of overlap-extension tails appended to the polynucleotide molecules of interest allow them to be linked (see Figures 17, 18, 19, and 20). As described below, overlap-extension tails can be added through a number of well known methods including, but not limited to, polynucleotide synthesis reactions, such as nucleic acid amplification and reverse transcription, ligation, and recombination. Because of the

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variety of methods available to effect linkage, it will be recognized that different relative orientations of the polynucleotide segments can be obtained, with respect to sense and antisense orientations, i.e., the segments, e.g., antibody heavy and light chains, can be joined head to tail, head to head, or tail to tail.

[00270] In one embodiment, overlap-extension tails enable the linkage of polynucleotide sequences generated during a polynucleotide synthesis reaction. For example, overlap extension tails can be introduced during the course of polynucleotide synthesis reactions, such as amplification or reverse transcription reactions, by using primers carrying an overlap-extension tail. Alternatively, ligation reactions can be used. As shown in Figures 17, 18, 19, and 20, after annealing of complementary overlap extension tails, the DNA is filled-in in a 5' to 3' direction during the extension phase of a polynucleotide synthesis reaction, such as PCR, to generate a double stranded polynucleotide with the two polynucleotides of interest physically joined.

[00271] In some embodiments, an overlap-extension RT-PCR method allows the sequences to be linked simultaneously as the reaction proceeds in a single tube, thus eliminating the need for intermediate purification. In some embodiments, an overlap extension tail comprises a barcode sequence.

[00272] Figure 17 illustrates generally one example of the use of overlap-extension tails to join polynucleotide sequences encoding antibody light and heavy chains and to provide at least one barcode. Other methods useful for linking two polynucleotide sequences of interest are discussed below. In this example, after polynucleotide synthesis, e.g., reverse transcription, has occurred, the use of a LC gene specific PCR primer containing a barcode, optional sequencing primer site, and optional restriction site (RE1) allows these elements to be added to the end of the resulting PCR product. Primers specific for LC (in one embodiment the V_L region) and HC (in one embodiment the V_H region) with extension overlaps and encoding an optional restriction site (RE3) are indicated. In one embodiment, the LC comprises the rearranged VJ with or without a short segment of the constant region, and the HC comprises the rearranged V(D)J with or without a short segment of constant region of the heavy chain. In one embodiment, the overlap-extension primers also contain a barcode sequence. A reverse primer specific for HC containing an optional RE2 is also used. As amplification with these primers proceeds, a nucleic acid with the linked structure shown is generated with a barcode at one end. Products from reactions conducted in single samples can be easily integrated into the other work flows disclosed herein. For example, an optional second barcode can be added and used in conjunction with the first barcode to further enable multiplexing to identify large numbers of

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sequences using a relative minimum number of barcodes. For sequencing, a single barcode may be sufficient.

[00273] Variations of the general scheme shown in Figure 17, examples of which are illustrated herein, will be apparent. For example, the barcode can be placed at the other end of the final product, or at both ends, or between the polynucleotides. Furthermore, a barcode can be included as part of the extension overlap region (e.g., on either side of RE3 or the barcode can be split by the RE3 sequence).

[00274] LC (including V_L sequences) and HC (including V_H sequences) sequences can be derived through a variety of means. For example, they can be generated through reverse transcription of mRNA with either oligo dT or gene specific primers. In one embodiment, a reverse transcription and subsequent amplification reactions are performed simultaneously, i.e., an RT-PCR reaction, to arrive at the final product. When reverse transcription is used, the extension overlap region, as well as other elements, such as restriction sites, sequencing primer sites, or universal sequences, can be added via the annealing of an adaptor comprising one or more G residues to the one, two, three, or more C residues generated by the 3' tailing of cDNA generated in the reverse transcription reaction as shown, for example, in Figures 18, 19, and 20. Template switching by the reverse transcriptase allows an extension overlap region (and other sequence elements) to be added to the cDNA. For example, as shown in Figure 18, when taking advantage of the 3' tailing and template switching activities of reverse transcriptase, a first adaptor can be used to add an extension overlap sequence and a barcode to a first polynucleotide of interest, while a second adaptor with a sequence complementary to the overlap-extension of the first adaptor can be added to a second cDNA of interest. The complementary extension overlap sequences anneal during a subsequent nucleic acid synthesis reaction, such as PCR, to join the two polynucleotides of interest. Extension from the point of overlap results in a double stranded DNA molecule in which the two polynucleotides of interest are linked with a barcode between them. Variations that allow the generation of two internally located barcodes between two linked polynucleotide sequences are shown in Figures 19 and 20.

[00275] Other methods for joining or linking the polynucleotide sequences of interest include by ligation. In this embodiment, the primer mix used for the amplification is designed such that the amplified target sequences can be cleaved with appropriate restriction enzymes, and covalent linkage by DNA ligation can be performed. Following amplification with such a primer mix, the restriction enzymes needed to form compatible ends of the target sequences, are added to the mixture. Target sequences are then ligated together with a ligase. No purification of the PCR

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products is needed prior to either the restriction enzyme digest or ligation steps, although purification may be performed.

[00276] In another embodiment, the polynucleotide sequences of interest can be linked by recombination. In this approach, the amplified polynucleotide sequences of interest can be joined using identical recombination sites. Linkage is performed by adding the appropriate recombinase to facilitate recombination. Suitable recombinase systems include Flp recombinase with a variety of FRT sites, Cre recombinase with a variety of lox sites, integrase ΦC31 which carries out recombination between the attP site and the attB site, the β-recombinase-six system as well as the Gin-gix system. Linkage by recombination has been exemplified for two nucleotide sequences (V_H linked with V_L) (Chapal, N. et al. 1997 BioTechniques 23, 518-524), hereby incorporated by reference.

[00277] Accordingly, in one aspect, the method comprises amplifying by PCR or RT-PCR amplification, nucleotide sequences of interest using a template derived from an isolated single cell or a population of isogenic cells and (1) effecting linkage of the amplified nucleotide sequences of interest and (2) adding one more barcodes to the linked polynucleotide sequences. The method comprises an optional step of performing an additional amplification of the linked products to, for example, add additional barcodes, restriction sites, sequencing primer sites, and the like.

[00278] In another aspect, a method of producing a library of barcoded linked pairs comprising antibody heavy and light chains from single cells from a donor is provided. This aspect comprises providing a lymphocyte-containing cell fraction from a donor, which is optionally enriched for a particular lymphocyte population from said cell fraction. Further, a population of isolated single cells is obtained by distributing cells from the lymphocyte-containing cell fraction, or the enriched cell fraction, individually among a plurality of vessels, containers, or wells. Multiplex molecular amplification (e.g., multiplex RT-PCR amplification) of the variable region encoding sequences contained in the population of isolated single cells is performed and linkage of pairs of heavy and light chains and barcode addition is affected, wherein an individual pair is derived from a single cell. Further, in different embodiments, the method can comprise two optional steps: in the first step, the individual isolated single cell in the population of single cells can be expanded to a population of isogenic cells prior to performing multiplex RT-PCR amplification, thereby providing a plurality of vessels, containers, or wells with a population of isogenic cells (one population of isogenic cells in one vessel, container, or well). Another optional step encompasses performing an additional amplification of the linked light and heavy chain encoding sequences. This additional amplification step can be used to

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simply increase the amount of the linked nucleic acid, or to add a first or second barcode sequence or other sequence elements to the linked nucleic acid.

[00279] In some aspects, the multiplex RT-PCR amplification can be performed either as a two-step process, where reverse transcription (RT) is performed separate from the multiplex PCR amplification (or alternative multiplex molecular amplification), or as a single-step process, where the RT and multiplex PCR amplification steps are performed with the same primers in one tube.

[00280] The reverse transcription (RT) is performed with an enzyme containing reverse transcriptase activity resulting in the generation of cDNA from total RNA, mRNA or target specific RNA from an isolated single cell. Primers which can be utilized for the reverse transcription include oligo-dT primers, random hexamers, random decamers, other random primers, or primers that are specific for the nucleotide sequences of interest. In some embodiments, such primers can contain elements such as barcodes, universal priming sites, restriction sites, sequencing primer sites, and the like.

[00281] The two-step multiplex RT-PCR amplification procedure allows for the cDNA generated in the RT step to be distributed to more than one vessel allowing for the storage of a template fraction before proceeding with the amplification, if desired. Additionally, the distribution of cDNA to more than one tube, allows for the performance of more than one multiplex PCR amplification of nucleic acid derived from the same template. This two-step approach can for example be used to amplify and link heavy chain variable region and kappa light chain variable region encoding sequences in one tube and heavy chain variable region and lambda light chain variable region encoding sequences in a different tube utilizing the same template. A single cell usually only expresses one of the light chains. However, it will often be easier to perform the reactions simultaneously instead of awaiting the result of one of the reactions before performing the other. Further, the amplification of both kappa and lambda serves as an internal negative control, since it would be expected that only kappa or lambda would amplify from a single cell.

[00282] In the single-step multiplex RT-PCR procedure, reverse transcription and multiplex PCR amplification is carried out in the same vessel, container, or well. All the components necessary to perform both the reverse transcription and the multiplex PCR in a single step are initially added into the vessels, containers, or wells and the reaction is performed. Generally, there is no need to add additional components once the reaction has been started. The advantage of single-step multiplex RT-PCR amplification is that it reduces the number of steps necessary to generate the barcode linked nucleotide sequences of the present invention even further. This is

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particularly useful when performing multiplex RT-PCR on an array of single cells, where the same reaction is carried out in a plurality of vessels. Generally, the composition needed for the single-step multiplex RT-PCR comprises a nucleic acid template, an enzyme with reverse transcriptase activity, an enzyme with DNA polymerase activity, deoxynucleoside triphosphate mix (dNTP mix comprising dATP, dCTP, dGTP and dTTP) and a multiplex primer mix. The nucleic acid template is preferably total RNA or mRNA derived from an isolated single cell either in a purified form, as a lysate of the cell, or as contained in the intact cell.

[00283] In one aspect, the methods generate libraries of linked and barcoded polynucleotides of interest. In some aspects, the plurality of polynucleotide compositions in a polynucleotide library can comprise at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, at least 100,000, at least 300, 000, at least 1,000,000, at least 3,000,000, at least 10,000,000, at least 30,000,000, or more members. In other aspects, the plurality of polynucleotide compositions in a polynucleotide library can comprise at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, or more genes of a cell sample's whole transcriptome. In other aspects, the plurality of polynucleotide compositions in a polynucleotide library comprises at least 1, at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 10,000, at least 100,000, at least 1,000,000, at least 10,000,000, at least 1,000,000,000 or more of the different antibody species present in the blood of an individual. These the antibody species can be expressed by plasmablasts, plasma cells, memory B cells, long-lived plasma cells, naïve B cells, other B lineage cells, or combinations thereof.

[00284] The linked and barcoded polynucleotide compositions generated by the methods disclosed above can advantageously be subjected to high throughout, multiplexed sequencing, preferably, using NextGen sequencing platforms as described herein.

[00285] The linked and barcoded polynucleotide compositions generated by the methods disclosed above can also used for cloning, producing polypeptides of interest, and screening as disclosed hercin.

Methods of producing one or more polynucleotides of interest for sequencing

[00286] In some aspects, the method includes obtaining a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region derived from a single sample, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample

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identification region of each polynucleotide derived from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library derived from one or more samples distinct from the first single sample; and amplifying the polynucleotide library with a set of primers to produce the one or more polynucleotides of interest for sequencing, wherein the one or more polynucleotides of interest for sequencing comprises a first sequencing region, a first plate identification region, a universal primer region, a sample identification region, an adapter region, an amplicon region derived from a single sample, and a second sequencing region.

[00287] In some aspects, a method further includes sequencing the one or more polynucleotides of interest. In some aspects, the sequencing is 454 sequencing.

[00288] In some aspects, sequencing includes longer sequencing reads such that the forward and reverse sequencing reads overlap enough to enable reconstruction of the entire, approximately 600 base pair (bp) sequence of, e.g., antibody light chains (LCs) (where exact sequence length can depend on the length of the 5' untranslated region (UTR)), and approximately 700 bp sequence of the heavy chains (HCs). Therefore, in some aspects, any sequencing technology that can yield sequencing reads of at least 350-400bp and thereby achieve the overlap included for sequence assembly can be used, and sequencing technologies that enable 600-700+ bp reads would allow one to sequence using just a forward primer (sequencing from the 5' end).

[00289] Any technique for sequencing nucleic acid known to those skilled in the art can be used. DNA sequencing techniques include classic dideoxy sequencing reactions (Sanger method) using labeled terminators or primers and gel separation in slab or capillary electrophoresis. In a preferred embodiment, next generation (NextGen) sequencing platforms are advantageously used in the practice of the invention. NextGen sequencing refers to any of a number of post-classic Sanger type sequencing methods which are capable of high throughput, multiplex sequencing of large numbers of samples simultaneously. Current NextGen sequencing platforms, such as those described in greater detail below, are capable of generating reads from multiple distinct nucleic acids in the same sequencing run. Throughput is varied, with 100 million bases to 600 giga bases per run, and throughput is rapidly increasing due to improvements in technology. The principle of operation of different NextGen sequencing platforms is also varied and can include: sequencing by synthesis using reversibly terminated labeled nucleotides, pyrosequencing, 454 sequencing, allele specific hybridization to a library of labeled oligonucleotide probes, sequencing by synthesis using allele specific hybridization to a library of labeled clones that is followed by ligation, real time monitoring of the incorporation of

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labeled nucleotides during a polymerization step, polony sequencing, single molecule real time sequencing, and SOLiD sequencing. Sequencing has been demonstrated by sequential or single extension reactions using polymerases or ligases as well as by single or sequential differential hybridizations with libraries of probes. These reactions have been performed on many clonal sequences in parallel including demonstrations in current commercial applications of over 100 million sequences in parallel. These sequencing approaches can thus be used to study the repertoire of T-cell receptor (TCR) and/or B-cell receptor (BCR) and other sequences of interest.

[00290] The sequencing techniques can generate at least 1000 reads per run, at least 10,000 reads per run, at least 100,000 reads per run, at least 500,000 reads per run, or at least 1,000,000 reads per run.

[00291] The sequencing techniques can generate about 30 bp, about 40 bp, about 50 bp, about 60 bp, about 70 bp, about 80 bp, about 90 bp, about 100 bp, about 110, about 120 bp per read, about 150 bp, about 200 bp, about 250 bp, about 300 bp, about 350 bp, about 400 bp, about 450 bp, about 500 bp, about 550 bp, about 600 bp, about 650 bp, or about 700 bp or more bp per read.

[00292] The sequencing techniques can generate at least 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700 or more nucleotides per read.

[00293] A sequencing technique that can be used, for example, Helicos True Single Molecule Sequencing (tSMS) (Harris T. D. et al. (2008) Science 320:106-109). In the tSMS technique, a DNA sample is cleaved into strands of approximately 100 to 200 nucleotides, and a polyA sequence is added to the 3' end of each DNA strand. Each strand is labeled by the addition of a fluorescently labeled adenosine nucleotide. The DNA strands are then hybridized to a flow cell, which contains millions of oligo-T capture sites that are immobilized to the flow cell surface. The templates can be at a density of about 100 million templates/cm^2. The flow cell is then loaded into an instrument, e.g., HeliScope^{TM} sequencer, and a laser illuminates the surface of the flow cell, revealing the position of each template. A CCD camera can map the position of the templates on the flow cell surface. The template fluorescent label is then cleaved and washed away. The sequencing reaction begins by introducing a DNA polymerase and a fluorescently labeled nucleotide. The oligo-T nucleic acid serves as a primer. The polymerase incorporates the labeled nucleotides to the primer in a template directed manner. The polymerase and unincorporated nucleotides are removed. The templates that have directed incorporation of the fluorescently labeled nucleotide are detected by imaging the flow cell surface. After imaging, a cleavage step removes the fluorescent label, and the process is repeated with other fluorescently

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labeled nucleotides until the desired read length is achieved. Sequence information is collected with each nucleotide addition step.

[00294] Another example of a DNA sequencing technique that can be used is 454 sequencing (Roche) (Margulies, M et al. 2005, Nature, 437, 376-380). 454 sequencing involves two steps. In the first step, DNA is sheared into fragments of approximately 300-800 base pairs, and the fragments are blunt ended. Oligonucleotide adaptors are then ligated to the ends of the fragments. The adaptors serve as primers for amplification and sequencing of the fragments. The fragments can be attached to DNA capture beads, e.g., streptavidin-coated beads using, e.g., Adaptor B, which contains 5'-biotin tag. The fragments attached to the beads are PCR amplified within droplets of an oil-water emulsion. The result is multiple copies of clonally amplified DNA fragments on each bead. In the second step, the beads are captured in wells (pico-liter sized). Pyrosequencing is performed on each DNA fragment in parallel. Addition of one or more nucleotides generates a light signal that is recorded by a CCD camera in a sequencing instrument. The signal strength is proportional to the number of nucleotides incorporated.

[00295] Pyrosequencing makes use of pyrophosphate (PPi) which is released upon nucleotide addition. PPi is converted to ATP by ATP sulfurylase in the presence of adenosine 5' phosphosulfate. Luciferase uses ATP to convert luciferin to oxyluciferin, and this reaction generates light that is detected and analyzed.

[00296] Another example of a DNA sequencing technique that can be used is SOLiD technology (Applied Biosystems). In SOLiD sequencing, genomic DNA is sheared into fragments, and adaptors are attached to the 5' and 3' ends of the fragments to generate a fragment library. Alternatively, internal adaptors can be introduced by ligating adaptors to the 5' and 3' ends of the fragments, circularizing the fragments, digesting the circularized fragment to generate an internal adaptor, and attaching adaptors to the 5' and 3' ends of the resulting fragments to generate a mate-paired library. Next, clonal bead populations are prepared in microreactors containing beads, primers, template, and PCR components. Following PCR, the templates are denatured and beads are enriched to separate the beads with extended templates. Templates on the selected beads are subjected to a 3' modification that permits bonding to a glass slide.

[00297] The sequence can be determined by sequential hybridization and ligation of partially random oligonucleotides with a central determined base (or pair of bases) that is identified by a specific fluorophore. After a color is recorded, the ligated oligonucleotide is cleaved and removed and the process is then repeated.

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[00298] Another example of a sequencing technology that can be used is SOLEXA sequencing (Illumina). SOLEXA sequencing is based on the amplification of DNA on a solid surface using fold-back PCR and anchored primers. Genomic DNA is fragmented, and adapters are added to the 5' and 3' ends of the fragments. DNA fragments that are attached to the surface of flow cell channels are extended and bridge amplified. The fragments become double stranded, and the double stranded molecules are denatured. Multiple cycles of the solid-phase amplification followed by denaturation can create several million clusters of approximately 1,000 copies of single-stranded DNA molecules of the same template in each channel of the flow cell. Primers, DNA polymerase and four fluorophore-labeled, reversibly terminating nucleotides are used to perform sequential sequencing. After nucleotide incorporation, a laser is used to excite the fluorophores, and an image is captured and the identity of the first base is recorded. The 3' terminators and fluorophores from each incorporated base are removed and the incorporation, detection and identification steps are repeated.

[00299] Another example of a sequencing technology that can be used includes the single molecule, real-time (SMRT™) technology of Pacific Biosciences. In SMRT, each of the four DNA bases is attached to one of four different fluorescent dyes. These dyes are phospholinked. A single DNA polymerase is immobilized with a single molecule of template single stranded DNA at the bottom of a zero-mode waveguide (ZMW). A ZMW is a confinement structure which enables observation of incorporation of a single nucleotide by DNA polymerase against the background of fluorescent nucleotides that rapidly diffuse in an out of the ZMW (in microseconds). It takes several milliseconds to incorporate a nucleotide into a growing strand. During this time, the fluorescent label is excited and produces a fluorescent signal, and the fluorescent tag is cleaved off. Detection of the corresponding fluorescence of the dye indicates which base was incorporated. The process is repeated.

[00300] Another example of a sequencing technique that can be used is nanopore sequencing (Soni G V and Meller A. (2007) Clin Chem 53: 1996-2001). A nanopore is a small hole, of the order of 1 nanometer in diameter. Immersion of a nanopore in a conducting fluid and application of a potential across it results in a slight electrical current due to conduction of ions through the nanopore. The amount of current which flows is sensitive to the size of the nanopore. As a DNA molecule passes through a nanopore, each nucleotide on the DNA molecule obstructs the nanopore to a different degree. Thus, the change in the current passing through the nanopore as the DNA molecule passes through the nanopore represents a reading of the DNA sequence.

[00301] Another example of a sequencing technique that can be used involves using a chemical-sensitive field effect transistor (chemFET) array to sequence DNA (for example, as

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described in US Patent Application Publication No. 20090026082). In one example of the technique, DNA molecules can be placed into reaction chambers, and the template molecules can be hybridized to a sequencing primer bound to a polymerase. Incorporation of one or more triphosphates into a new nucleic acid strand at the 3' end of the sequencing primer can be detected by a change in current by a chemFET. An array can have multiple chemFET sensors. In another example, single nucleic acids can be attached to beads, and the nucleic acids can be amplified on the bead, and the individual beads can be transferred to individual reaction chambers on a chemFET array, with each chamber having a chemFET sensor, and the nucleic acids can be sequenced.

[00302] Another example of a sequencing technique that can be used involves using an electron microscope (Moudrianakis E. N. and Beer M. Proc Natl Acad Sci USA. 1965 March; 53:564-71). In one example of the technique, individual DNA molecules are labeled using metallic labels that are distinguishable using an electron microscope. These molecules are then stretched on a flat surface and imaged using an electron microscope to measure sequences.

[00303] In some aspects, obtaining the polynucleotide library comprises preparing the polynucleotide library in a laboratory. In some aspects, obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has prepared the polynucleotide library.

Methods for analyzing sequencing data

[00304] In some aspects, the method includes obtaining a dataset associated with a plurality of polynucleotides, wherein the dataset comprises sequencing data for the plurality of polynucleotides, wherein each polynucleotide in the plurality of polynucleotides comprises a sample identification region, and wherein each sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the sample identification region of each polynucleotide derived from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the plurality of polynucleotides derived from one or more samples distinct from the first single sample; and analyzing the dataset to match together polynucleotides with identical sample identification regions, wherein a match indicates that the polynucleotides originated from the same sample.

[00305] In some aspects each polynucleotide in the plurality of polynucleotides further comprises a first plate identification region, wherein each combination of each first plate identification region and sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the first plate identification region of each

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polynucleotide derived from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the plurality of polynucleotides derived from one or more single sample sets distinct from the first set of single samples, and further comprising analyzing the dataset to match together polynucleotides with identical first plate identification regions and identical sample identification regions, wherein a match between both regions indicates that the polynucleotides originated from the same sample.

[00306] In some aspects, both polynucleotides include a variable region. In some aspects, one polynucleotide includes a variable region. In some aspects, neither polynucleotide includes a variable region.

[00307] In some aspects, obtaining the dataset comprises obtaining the plurality of polynucleotides and sequencing the plurality of polynucleotides to experimentally determine the dataset. In some aspects, obtaining the dataset comprises receiving the dataset directly or indirectly from a third party that has sequenced the plurality of polynucleotides to experimentally determine the dataset. In some aspects, the dataset is stored on an electronic storage medium. In some aspects, the dataset is transferred over the Internet.

[00308] In some aspects, the method is implemented on a computer, e.g., it is a computer-implemented method.

[00309] In some aspects, the single sample is a single cell. In some aspects, the single sample comprises a single cell. In some aspects, the single sample comprises a single B cell. In some aspects, the single sample comprises a plurality of B cells. In some aspects, the single sample comprises a single B cell and one or more other cells.

[00310] In some aspects, data generated from sequencing (e.g., 454 sequencing) can be analyzed by 454 GS FLX data analysis software, and sequences with poor-quality scores can be filtered out. Good-quality sequences can then be subdivided according to their sample identification region (and in some embodiments the combination of their sample identification region and plate identification region) by using a script in Python before sequences are assembled using bioinformatics approaches, for example, by using Newbler. Because reverse reads can have only a second plate identification region in some aspects, it is possible that sequence assembly could occur between forward and reverse reads of sequences from different cells. For circumventing this potential problem, the heavy- and light-chain V(D)J usage of both forward and reverse reads can first be identified using HighV-QUEST. Sequences can then be further grouped according to their V(D)J usage before being assembled. In some aspects, sequence assembly can be intolerant of nucleotide mismatches, thereby preventing assembly of forward and reverse reads from different cells that share the same V(D)J usage. In some aspects,

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sequences can then be clustered together based on their V(D)J usage by using a computer program.

[00311] In some aspects, bioinformatics methods may be used to identify groups of sequences forming clonal families and subfamilies, and thereby immunoglobulin sequences of interest. Such bioinformatics methods involve measurements of sequence similarity. Such bioinformatics methods may be used to identify sequences of interest derived from an individual human, derived from one or more humans, derived from one or more humans with a condition, or derived from one or more humans with different conditions.

[00312] In some aspects, related immunoglobulin heavy and/or light chain sequences can be identified through computational phylogenetic analysis of the homology between the immunoglobulin heavy chain and/or light chain V(D)J sequences. In some aspects, standard classification methods (i.e. clustering) of the sequences representing the individual or combinations of the V, D, and/or J gene segments and/or other sequences derived from the immunoglobulin heavy chain and/or light chain can be used to identify clonal families or subfamilies (for example, by using ClustalX).

[00313] As used herein "clonal family" refers to a plurality of immunoglobulin sequences each having V, D, and/or J regions, wherein each sequence is a mutated version of the same germline immunoglobulin sequence having a V, D, and/or J region or the germline immunoglobulin sequence having the V, D, and/or J region. In some aspects, the plurality is a plurality of heavy chain sequences. In some aspects, the plurality is a plurality of light chain sequences. In some aspects, the plurality is a plurality of paired heavy and light chain sequences. In some aspects, each sequence has V, D, and J regions. In some aspects, each sequence has V and D regions. In some aspects, each sequence has D and J regions. In some aspects, each sequence has V and J regions. In some aspects, each sequence has a V region. In some aspects, each sequence has a D region. In some aspects, each sequence has a J region. In some aspects, the one or more mutations are located within the V, D, and/or J regions. In some aspects, the one or more mutations are located between the V, D, and/or J regions.

[00314] In some aspects, a set of antibodies whose heavy chains all use the same V and J gene segments are a clonal family. In some aspects, a set of antibodies whose heavy chains all use the same V and J gene segments and whose sum of the length of P/N nucleotides and D nucleotides are of the same length are a clonal family. In some aspects, a set of antibodies whose heavy chains all use the same V, D and J gene segments are a clonal family. In some aspects, a set of antibodies whose heavy chains all use the same V, D and J gene segments, and whose P/N nucleotides between the V and D gene segments are the same length, and whose P/N

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nucleotides between the D and J gene segments are the same length, are a clonal family. In some aspects, a set of antibodies whose heavy chains all use the same V and J gene segments and whose light chains all use the same V and J gene segments are a clonal family. In some aspects, a set of antibodies whose heavy chains all use the same V and J gene segments and whose sum of the length of P/N nucleotides and D nucleotides are of the same length, and whose light chains all use the same V and J gene segments and whose P/N nucleotides are of the same length, are a clonal family. In some aspects, a set of antibodies whose heavy chains all use the same V, D and J gene segments and whose light chains all use the same V and J gene segments are a clonal family. In some aspects, a set of antibodies whose heavy chains all use the same V, D and J gene segments, and whose P/N nucleotides between the V and D gene segments are the same length, and whose P/N nucleotides between the D and J gene segments are the same length, and whose light chains all use the same V and J gene segments, and whose P/N nucleotides between the V and J gene segments are the same length, are a clonal family.

[00315] Methods for constructing clonal families

[00316] The V, D and J usage for a T cell receptor (TCR) or an immunoglobulin variable gene query sequence can be determined by identifying the germline V, D (if applicable) and J gene segments most likely to have given rise to the sequence. D segments are present in some TCR and immunoglobulin sequences (e.g. TCRβ, TCRδ and antibody heavy chain sequences) but not others (e.g TCRα, TCRγ and antibody light chain sequences). The following description includes D segments but the same approaches can be applied to variable region sequences that lack D segments. In all cases the determination of V(D)J usage uses a reference database of germline V, D and J gene segment sequences such as IMGT/GENE-DB (Giudicelli V, Chaume D, Lefranc MP. IMGT/GENE-DB: a comprehensive database for human and mouse immunoglobulin and T cell receptor genes. Nucleic Acids Res. 2005 Jan 1;33(Database issue):D256-61.).

[00317] In one approach to determination of V(D)J usage, the query sequence is compared serially to each V, D and J germline gene segment separately and the most similar gene segment of each type (V, D or J) is selected as the most likely to have given rise to the query sequence. V-QUEST and High V-QUEST are examples of this approach (Giudicelli V, Chaume D, Lefranc MP. IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. Nucleic Acids Res. 2004 Jul 1;32(Web Server issue):W435-40.; Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 2008 Jul 1;36(Web Server issue):W503-8.). V-QUEST first generates

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pairwise alignments for the query sequence and each V gene segment sequence. Then it generates pairwise alignments for the query sequence region downstream of the deduced 3' end of the V segment and each J gene segment sequence. If a D segment is present, V-QUEST then generates pairwise alignments for the query sequence region found between the regions matching V and J segments and each D gene segment sequence. V-QUEST can also infer the boundaries of the V-D, V-J and/or D-J junction regions.

[00318] In another approach to determination of V(D)J usage, the combination of germline V, D and J segments most likely to have given rise to the query sequence is identified in a single step rather than in three separate steps for V, D and J respectively. This approach has the advantage that the identification of one type of segment (V, D or J) can take into account information about the potential matches to the other two types of segments. For example, the best matching D segment might depend upon which V segment match is being considered. SoDA is an example of this approach (Volpe JM, Cowell LG, Kepler TB. SoDA: implementation of a 3D alignment algorithm for inference of antigen receptor recombinations. Bioinformatics. 2006 Feb 15;22(4):438-44.). SoDA first selects candidate V, D and J segments. It generates pairwise local alignments for the query sequence and each V gene segment sequence and then keeps only the V segments with alignments meeting a score threshold. It repeats these steps for the J segments and D segments. Then an optimal alignment is generated for each possible combination of candidate V, D and J segments. The alignments are generated using the same general dynamic programming approach widely used in sequence alignment (Needleman,S.B. and Wunsch,C.D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol., 48,443–453.), but allowing for the insertion of additional nucleotides at the V-D, V-J and/or D-J junctions. Such insertion commonly takes place during the biological process of V(D)J recombination. In sequence alignment by dynamic programming there are typically penalty scores associated with insertions, deletions and mismatches. However, in this approach to determining V(D)J usage, no penalties are applied for the insertion of nucleotides at the junctions between segments. TheV(D)J combination yielding the highest-scoring alignment is selected to indicate the V(D)J usage for the query sequence. This approach can also identify the boundaries of junction sequence regions.

[00319] From the clonal families and subfamilies, a variety of approaches can be used to select specific clones for expression of their encoded paired heavy and light chain immunoglobulin genes and characterization of their binding properties. In some aspects, the highest frequency clones from clonal families and/or clonal subfamilies as well as other

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representative clones from clonal families and subfamilies are expressed and screened for their binding properties. Clones may also be randomly selected, from all clones, from all or select clonal families, and/or from all or select clonal subfamilies for expression and characterization of their binding characteristics. Clones may also be selected based on possessing larger numbers of variations in the variable region of the antibody. A phylogenetic tree may be constructed, and clones may be selected based on features of the phylogenetic tree, for example by descending the tree always choosing the branch with the largest number of leaf nodes underneath.

[00320] In some aspects, a method further includes selecting one or more polynucleotides for cloning.

Methods for identifying a second polynucleotide of interest based on selection of a first polynucleotide of interest

[00321] In some aspects, the method includes obtaining a dataset associated with a plurality of polynucleotides, wherein the dataset comprises sequencing data for the plurality of polynucleotides, wherein each polynucleotide in the plurality of polynucleotides comprises a sample identification region, and wherein each sample identification region on each polynucleotide is unique to a single sample thereby associating each polynucleotide in the plurality of polynucleotides with a distinct single sample, wherein the sequence of the sample identification region of each polynucleotide derived from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the plurality of polynucleotides derived from one or more samples distinct from the first single sample; and selecting a first polynucleotide of interest associated with a first single sample from the dataset and identifying a second polynucleotide of interest in the first single sample based on the sample identification region of the first polynucleotide of interest.

[00322] In some aspects, each polynucleotide in the plurality of polynucleotides further comprises a first plate identification region, wherein each combination of each first plate identification region and sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the first plate identification region of each polynucleotide derived from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the plurality of polynucleotides derived from one or more single sample sets distinct from the first set of single samples, and further comprising identifying a second polynucleotide of interest in the first single sample based on the sample identification region and first plate identification region of the first polynucleotide of interest.

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[00323] In some aspects, both polynucleotides include a variable region. In some aspects, one polynucleotide includes a variable region. In some aspects, neither polynucleotide includes a variable region.

[00324] In some aspects, the method is implemented on a computer, e.g., it is a computer-implemented method.

[00325] In some aspects, the first single sample comprises a B cell. In some aspects, the first single sample comprises a single B cell and one or more other cells. In some aspects, the first single sample comprises a plurality of B cells. In some aspects, the first single sample comprises a B cell, wherein the first polynucleotide of interest comprises an antibody heavy chain nucleotide sequence, and wherein the second polynucleotide of interest comprises an antibody light chain nucleotide sequence. In some aspects, the first single sample comprises a B cell, wherein the first polynucleotide of interest comprises an antibody light chain nucleotide sequence, and wherein the second polynucleotide of interest comprises an antibody heavy chain nucleotide sequence.

[00326] In some aspects, obtaining the dataset comprises obtaining the plurality of polynucleotides and sequencing the plurality of polynucleotides to experimentally determine the dataset. In some aspects, obtaining the dataset comprises receiving the dataset directly or indirectly from a third party that has sequenced the plurality of polynucleotides to experimentally determine the dataset. In some aspects, the dataset is stored on an electronic storage medium.

Methods of producing one or more polynucleotides of interest for cloning

[00327] In some aspects, the method includes obtaining a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region derived from a single sample, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide derived from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library derived from one or more samples distinct from the first single sample; and amplifying the polynucleotide library with a set of primers to produce the one or more polynucleotides of interest for cloning, wherein the one or more polynucleotides of interest for cloning comprises a first restriction site region, a universal primer region, a sample identification region, an adapter region, an amplicon region derived from a single sample, and a second restriction site region.

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[00328] In some aspects, obtaining the polynucleotide library comprises preparing the polynucleotide library in a laboratory. In some aspects, obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has prepared the polynucleotide library.

[00329] In some aspects, a methods further include cloning one or more polynucleotides, e.g., into a vector disclosed herein.

Methods of producing a molecule of interest

[00330] In some aspects, the method includes obtaining a host cell comprising a polynucleotide of interest; and culturing the host cell under conditions sufficient to produce the molecule of interest.

[00331] In some aspects, obtaining the host cell comprises preparing the host cell comprising the polynucleotide in a laboratory. In some aspects, obtaining the host cell comprises receiving the host cell comprising the polynucleotide directly or indirectly from a third party that has prepared the host cell.

[00332] In some aspects, the molecule of interest is a polypeptide. In some aspects, the molecule of interest is an antibody. In some aspects, the molecule of interest is a human monoclonal antibody.

[00333] In some aspects, the method further includes collecting the molecule of interest.

[00334] In some aspects, it is desirable to "refold" certain polypeptides, e.g., polypeptides comprising one or more ABP components or the ABP itself. In certain embodiments, such polypeptides are produced using expression systems discussed herein. In certain embodiments, polypeptides are "refolded" and/or oxidized to form desired tertiary structure and/or to generate disulfide linkages. In certain embodiments, such structure and/or linkages are related to certain biological activity of a polypeptide. In certain embodiments, refolding is accomplished using any of a number of procedures known in the art. Exemplary methods include, but are not limited to, exposing the solubilized polypeptide agent to a pH typically above 7 in the presence of a chaotropic agent. An exemplary chaotropic agent is guanidine. In certain embodiments, the refolding/oxidation solution also contains a reducing agent and the oxidized form of that reducing agent. In certain embodiments, the reducing agent and its oxidized form are present in a ratio that will generate a particular redox potential that allows disulfide shuffling to occur. In certain embodiments, such shuffling allows the formation of cysteine bridges. Exemplary redox couples include, but are not limited to, cysteine/cystamine, glutathione/dithiobisGSH, cupric chloride, dithiothreitol DTT/dithiane DTT, and 2-mercaptoethanol (bME)/dithio-bME. In certain

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embodiments, a co-solvent is used to increase the efficiency of refolding. Exemplary cosolvents include, but are not limited to, glycerol, polyethylene glycol of various molecular weights, and arginine.

[00335] In certain embodiments, one substantially purifies a polypeptide, e.g., a polypeptide comprising one or more ABP components or the ABP itself. Certain protein purification techniques are known to those of skill in the art. In certain embodiments, protein purification involves crude fractionation of polypeptide fractionations from non-polypeptide fractions. In certain embodiments, polypeptides are purified using chromatographic and/or electrophoretic techniques. Exemplary purification methods include, but are not limited to, precipitation with ammonium sulphate; precipitation with PEG; immunoprecipitation; heat denaturation followed by centrifugation; chromatography, including, but not limited to, affinity chromatography (e.g., Protein-A-Sepharose), ion exchange chromatography, exclusion chromatography, and reverse phase chromatography; gel filtration; hydroxyapatite chromatography; isoelectric focusing; polyacrylamide gel electrophoresis; and combinations of such and other techniques. In certain embodiments, a polypeptide is purified by fast protein liquid chromatography or by high pressure liquid chromotography (HPLC). In certain embodiments, purification steps can be changed or certain steps can be omitted and still result in a suitable method for the preparation of a substantially purified polypeptide.

[00336] In certain embodiments, one quantitates the degree of purification of a polypeptide preparation. Certain methods for quantifying the degree of purification are known to those of skill in the art. Certain exemplary methods include, but are not limited to, determining the specific binding activity of the preparation and assessing the amount of a polypeptide within a preparation by SDS/PAGE analysis. Certain exemplary methods for assessing the amount of purification of a polypeptide preparation comprise calculating the binding activity of a preparation and comparing it to the binding activity of an initial extract. In certain embodiments, the results of such a calculation are expressed as "fold purification." The units used to represent the amount of binding activity depend upon the particular assay performed.

[00337] In certain embodiments, a polypeptide comprising one or more ABP components or the ABP itself is partially purified. In certain embodiments, partial purification can be accomplished by using fewer purification steps or by utilizing different forms of the same general purification scheme. For example, in certain embodiments, cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "fold purification" than the same technique utilizing a low-pressure chromatography system. In certain

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embodiments, methods resulting in a lower degree of purification can have advantages in total recovery of polypeptide, or in maintaining binding activity of a polypeptide.

[00338] In certain instances, the electrophoretic migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE. See, e.g., Capaldi et al, Biochem. Biophys. Res. Comm., 76: 425 (1977). It will be appreciated that under different electrophoresis conditions, the apparent molecular weights of purified or partially purified polypeptide can be different.

Methods of screening

[00339] In some aspects, a molecule of interest is screened for activity. In some aspects, the molecule of interest is an ABP. In some aspects, the molecule of interest is an antibody.

[00340] In some aspects, methods of screening the libraries disclosed herein are used to identify ABPs capable of binding to a desired target. Any *in vitro* or *in vivo* screening method that allows for selection of an ABP from a library, based upon the ABP binding to a target molecule, is contemplated.

[00341] In one embodiment, a library may be screened using an art recognized in vitro cell-free phenotype-genotype linked display. Such methods are well known in the art and are described, for example, in U.S. Pat. Nos. 7,195,880; 6,951,725; 7,078,197; 7,022,479; 6,518,018; 7,125,669; 6,846,655; 6,281,344; 6,207,446; 6,214,553; 6,258,558; 6,261,804; 6,429,300; 6,489,116; 6,436,665; 6,537,749; 6,602,685; 6,623,926; 6,416,950; 6,660,473; 6,312,927; 5,922,545; and 6,348,315. These methods involve transcription of protein in vitro from a nucleic acid in such a way that the protein is physically associated or bound to the nucleic acid from which it originated. By selecting for an expressed protein with a target molecule, the nucleic acid that codes for the protein may also be selected.

[00342] To improve the expression of scFv proteins, the above referenced in vitro screening assays may include the addition or removal of certain reagents. In one embodiment, protein disulphide isomerase enzymes may be added to the in vitro expression system to improve the production of functional scFv molecules. In another embodiment, a mild oxidizing agent (for example, GSSG (oxidized glutathione)/GSH (reduced glutathione), for example 100 mM GSSG/10 mM GSH) may be added to in vitro translation reaction mixture of the scFv proteins to allow intra-chain disulphide bond formation in the VH and VL regions of the scFv molecule. In another embodiment, reducing agents (for example, dithiothreitol (DTT)) may be removed from the in vitro translation reaction mixture of the scFv.

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[00343] In another embodiment, one or more labeled amino acids, or derivatives thereof, may be added to the in vitro translation system such that the labeled amino acid(s) becomes incorporated into the resultant antibody. Any art recognized labeled amino acid is contemplated, for example, a radiolabelled amino acid, for example, ^{35}S-labelled methionine or cysteine.

[00344] In one embodiment, the in vitro screening assays may include that after in vitro selection of an antibody or plurality of antibodies the mRNA that is physically associated with the antibody or plurality of antibodies may be reverse transcribed to generate cDNA encoding said antibody or plurality of antibodies. Any suitable method for reverse transcription is contemplated, for example, enzyme mediated, for example, Moloney murine leukemia virus reverse transcriptase.

[00345] The screening methods may include amplification of the nucleic acid that encodes antibodies that bind specifically to a desired target. In one embodiment, mRNA that is physically associated with an antibody or plurality of antibodies may be amplified to produce more mRNA. Any art recognized method of RNA replication is contemplated, for example, using an RNA replicase enzyme. In another embodiment, mRNA that is physically associated with an antibody or plurality of antibodies is first reverse transcribed into cDNA before being amplified by PCR. In one embodiment, PCR amplification is accomplished using a high fidelity, proof--reading polymerase, for example, the KOD1 thermostable DNA polymerase from Thermococcus kodakaraensis or Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, Calif.). In another embodiment, PCR amplification may be performed under conditions that result in the introduction of mutations into amplified DNA, i.e., error-prone PCR.

[00346] Screening methods may also include that the stringency of the target-binding screening assay be increased to select for antibodies with improved affinity for target. Any art recognized methods of increasing the stringency of an antibody-target interaction assay are contemplated. In one embodiment, one or more of the assay conditions may be varied (for example, the salt concentration of the assay buffer) to reduce the affinity of the antibody molecules for the desired target. In another embodiment, the length of time permitted for the antibodies to bind to the desired target may be reduced. In another embodiment, a competitive binding step may be added to the antibody-target interaction assay. For example, the antibodies may first be allowed to bind to a desired immobilized target. A specific concentration of non-immobilized target may then be added, which serves to compete for binding with the immobilized target such that antibodies with the lowest affinity for antigen are eluted from the immobilized target, resulting in enrichment for antibodies with improved antigen binding

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affinity. In an embodiment, the stringency of the assay conditions may further be increased by increasing the concentration of non-immobilized target that is added to the assay.

[00347] Screening methods may also include multiple rounds of selection to enrich for one or more antibodies with improved target binding. In one embodiment, at each round of selection further amino acid mutations may be introduced into the antibodies using art recognized methods. In another embodiment, at each round of selection the stringency of binding to the desired target may be increased to select for antibodies with increased affinity for a desired target.

[00348] Screening methods may include purification of RNA-antibody fusion proteins from the components of an in vitro translation system. This may be accomplished using any art recognized method of separation. In one embodiment, the RNA-antibody fusion proteins may be separated by chromatography using a polydeoxythimidine (polydT) resin. In another embodiment, the RNA-antibody fusion proteins may be separated by chromatography using an antibody specific for an epitope present in the antibody component of the RNA-antibody fusion protein. In an embodiment, the epitope may be an amino acid sequence tag, for example, FLAG or HA tags, incorporated into the amino acid sequence of the antibody component of the RNA-antibody fusion protein, for example, at the N-terminal, C-terminal or in the inter variable region linker.

[00349] Selection of antibodies from a library may include the use of immobilized target molecules. In one embodiment, the target molecule may be directly linked to a solid substrate for example, agarose beads. In another embodiment, the target molecule may first be modified, for example, biotinylated and the modified target molecule may be bound via the modification to a solid support, for example, streptavidin-M280, neutravidin-M280, SA-M270, NA-M270, SA-MyOne, NA-MyOne, SA-agarose, and NA-agarose.

[00350] In some aspects, fluorescently-labeled antigens are used to single cell sort only plasmablasts or other B lineage cells with reactivity against specific, labeled antigens. In other aspects, fluorescently-labeled antigens are used to enrich for plasmablasts or other B lineage cells with reactivity against specific, labeled antigens, before single cell sorting occurs. In some aspects, fluorogenic or chromogenic molecules may be used to identify and sort B lineage cells. In some aspects, desired plasmablasts or other B lineage cells may be isolated by magnetic-activated cell sorting (MACS) or even by panning. Products resulting are generally monoclonal antibodies, against a variety of targets, including but not restricted to: cancer antigens, cytokines, chemokines, growth factors, secreted proteins, cell surface and other antigens to deplete cell

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types of specific interest, microbes, bacteria, mycobacteria, parasites, and viruses. Other screening methods are described in the Examples section below.

Computer implementation

[00351] In some aspects, one or more methods described herein can be implemented on a computer. In one embodiment, a computer comprises at least one processor coupled to a chipset. Also coupled to the chipset are a memory, a storage device, a keyboard, a graphics adapter, a pointing device, and a network adapter. A display is coupled to the graphics adapter. In one embodiment, the functionality of the chipset is provided by a memory controller hub and an I/O controller hub. In another embodiment, the memory is coupled directly to the processor instead of the chipset.

[00352] The storage device is any device capable of holding data, like a hard drive, compact disk read-only memory (CD-ROM), DVD, or a solid-state memory device. The memory holds instructions and data used by the processor. The pointing device may be a mouse, track ball, or other type of pointing device, and is used in combination with the keyboard to input data into the computer system. The graphics adapter displays images and other information on the display. The network adapter couples the computer system to a local or wide area network.

[00353] As is known in the art, a computer can have different and/or other components than those described previously. In addition, the computer can lack certain components. Moreover, the storage device can be local and/or remote from the computer (such as embodied within a storage area network (SAN)).

[00354] As is known in the art, the computer is adapted to execute computer program modules for providing functionality described herein. As used herein, the term "module" refers to computer program logic utilized to provide the specified functionality. Thus, a module can be implemented in hardware, firmware, and/or software. In one embodiment, program modules are stored on the storage device, loaded into the memory, and executed by the processor.

[00355] Embodiments of the entities described herein can include other and/or different modules than the ones described here. In addition, the functionality attributed to the modules can be performed by other or different modules in other embodiments. Moreover, this description occasionally omits the term "module" for purposes of clarity and convenience.

Kits

[00356] A kit can include a polynucleotide, a polynucleotide library, a vector, and/or a host cell disclosed herein and instructions for use. The kits may comprise, in a suitable container, a polynucleotide, a polynucleotide library, a vector, and/or a host cell disclosed herein, one or

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more controls, and various buffers, reagents, enzymes and other standard ingredients well known in the art.

[00357] The container can include at least one well on a plate comprising one or more wells. The container can include at least one vial, test tube, flask, bottle, syringe, or other container means, into which a polynucleotide, a polynucleotide library, a vector, and/or a host cell may be placed, and in some instances, suitably aliquoted. Where an additional component is provided, the kit can contain additional containers into which this component may be placed. The kits can also include a means for containing the polynucleotide, a polynucleotide library, a vector, and/or a host cell and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained. Containers can include labeling with instructions for use and/or warnings.

EXAMPLES

[00358] The examples are offered for illustrative purposes only, and are not intended to limit the scope of any embodiment of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

[00359] Various methods can employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.*, T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry 3rd Ed.* (Plenum Press) Vols A and B(1992); *Current Protocols in Molecular Biology* (2002- ; Wiley; Online ISBN: 9780471142720; DOI: 10.1002/04711142727); *Current Protocols in Immunology* (2001- ; Wiley; Online ISBN: 9780471142737; DOI: 10.1002/0471142735).

General Materials and Methods

[00360] Blood collection and isolation of PBMCs

[00361] All human samples were collected after informed consent and under Investigational Review Board (IRB)-approved human subject protocols. Blood was collected in heparin tubes (Beckton Dickinson and Company, catalog #BD366664) or in CPT tubes (Beckton Dickinson

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and Company, catalog BD362761) tubes. For processing of the heparin tubes, one milliliter of blood was transferred into a microfuge tube and spun down at 12,000 rpm for 3 minutes, plasma was collected and frozen at -80°C (for later testing for antibody reactivities), the remainder of the blood was layered over Ficoll and centrifuged in a Beckman Coulter Allegra X-15R benchtop centrifuge with a SX4750 Swinging Bucket Rotor at 800 g for heparin tubes for 20 min at room temperature, with minimal acceleration and without use of the brake, and the peripheral blood mononuclear cell (PBMC) layer was collected. Alternatively, CPT tubes were directly centrifuged at 1,500g for 20 min at room temperature, with minimal acceleration and without use of the brake, and the PMBC layer was collected. The collected PBMCs were then washed twice with PBS before use.

[00362] PBMCs may also be frozen for future use and isolation of B-cells, memory B-cells, plasmablasts, plasma cells, or other B-cell populations. One method for freezing PBMCs involves resuspending the PBMCs in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) in cryovials, and then slowly freezing the cells contained in the vials overnight at -80°C in a Mr. Frosty (Sigma C1562-1EA). The vials of frozen cells were then transferred for long-term storage in liquid nitrogen, and can be thawed at a later date for the isolation of individual B-cells and for the high-throughput sequencing of paired immunoglobulin genes. Thawed cells were incubated in a media containing an excess of DNase I, usually 25ug/ml (Sigma D4513) till the end of the 1st sort to prevent cell clumping.

Isolation and enrichment of cells and cell subpopulations

[00363] ***Plasmablasts.*** For some samples, PBMCs were first enriched for plasmablasts by using a modified Plasma Cells Isolation Kit II (Miltenyi 130-093-628). This is an optional step. This yielded fewer total cells for subsequent sorting, resulting in shorter sort times. This was used primarily when multiple samples needed to be single-cell sorted on the same day. It is also possible to use different kits to enrich for different B-cell populations (*see* below). For every $5x10^7$ PBMCs, cells were suspended in 200 µL of ice-cold MACS buffer (PBS with 0.5% FBS). 50 uL of non-plasma cell biotin-antibody cocktail was added, and cells were incubated in the fridge (4°C) for 10 minutes. 100 µL of MACS buffer, 100 µL of non-plasma cell microbead cocktail, and 50 µL of CD56 microbeads were added and incubated in the fridge for an additional 10 minutes. Cells were then washed with 7 mL of MACS buffer, centrifuged at 300g for 5 minutes at 4°C, resuspended in 500 µL of MACS buffer, and run on an equilibrated LS column in a magnetic field. The column was washed with 4 x 3 mL of MACS buffer and enriched cells were in the negative fraction.

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[00364] ***Memory B-cells.*** CD19+ microbeads (Miltenyi 130-050-301) and CD27+ microbeads (130-051-601) may be used to enrich for memory B-cells before cell sorting, to shorten sort times. Other enrichment methods, such as Memory B-cell isolation kit (Miltenyi 130-093-546), may also be used, provided that they enrich for $CD19^+CD27^+$ cells. For every $5x10^7$ PBMCs, 300 µL of ice-cold MACS buffer is used for resuspension. 100 µL of CD19 microbeads and 100 µL of CD27 microbeads are then added, and the sample is incubated at 4°C for 15 minutes. Cells are then washed with 7 mL of MACS buffer, centrifuged at 300g for 5 minutes at 4°C, and resuspended in 500 µL of MACS buffer. Cells are then run through an equilibrated LS column in a magnetic field, and washed with 2x 3 mL of MACS buffer. The LS column is then removed from the magnetic field, and the cells are washed out with 5 mL of MACS buffer to elute the enriched cells.

[00365] ***Total B-cells.*** CD19+ microbeads (Miltenyi 130-050-301) may be used to enrich for total B-cells before cell sorting, e.g., to shorten sort times. Other enrichment methods may also be used, provided that they enrich for $CD19^+$ cells. For every $5x10^7$ PBMCs, resuspend cells in 400 µL of ice-cold MACS buffer. Add 100 µL of CD19+ microbeads and incubate in the fridge (4°C) for 15 minutes. Cells are then washed with 7 mL of MACS buffer, centrifuged at 300g for 5 minutes at 4°C, and resuspended in 500 µL of MACS buffer. Cells are then run through an equilibrated LS column in a magnetic field and washed with 2x 3 mL of MACS buffer. The LS column is then removed from the magnetic field, and the cells are eluted with 5 mL of MACS buffer, yielding the enriched cells.

[00366] ***Other cell types.*** Although not necessary, MACS enrichment of the desired cell population can shorten sort times. Other cell populations, including plasma cells, other B-cell populations and non-B-cell populations may also be enriched using MACS or other systems using the appropriate reagents. For example, total T-cells may be enriched using CD3+ microbeads, and effector T-cells and helper T-cells isolated using CD8+ and CD4+ microbeads, respectively. CD45RO microbeads may be used to isolate memory T-cells and, in conjunction with CD8+ or CD4+ beads, used to isolate memory effector or memory helper T-cells, respectively.

Single-Cell Sorting

[00367] MACS enrichment is not required for sorting, but MACS enrichment for plasmablasts may be performed to shorten sort times. If PBMCs have undergone MACS enrichment, an aliquot of unenriched PBMCs (~1 million cells) is also analyzed in tandem, allowing the baseline plasmablast percentage in the sample to be determined. For sorting

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plasmablasts, cells were stained with manufacturer-recommended volumes of CD3-V450 (BD 560365), IgA-FITC (AbD Serotec STAR142F), IgM-FITC (AbD Serotec STAR146F) or IgM-PE (AbD Serotec STAR146PE), CD20-PerCP-Cy5.5 (BD 340955), CD38-PE-Cy7 (BD 335808), CD19-APC (BD 340437) and CD27-APC-H7 (BD 560222) in 50 µL of FACS buffer (PBS or HBSS with 2% FBS) on ice for 20 minutes in the dark. Some cells may also be stained with IgG-PE (BD 555787), CD138-PE (eBioscience 12-1389-42), or HLA-DR-PE (BD 555812) together with IgM-FITC instead. For simultaneous sorting of plasmablasts, memory and naive B-cells, the following staining scheme was used: IgD-FITC (Biolegend 348205), IgG-PE (BD 555787), CD20-PerCP-Cy5.5, CD38-PECy7, IgM-APC (BD 551062), CD27-APC-H7, IgA-biotin (AbD Serotec 205008) followed by Strepavidin-eFluor710 (eBioscience 49-4317-82) and CD19-BV421 (Biolegend 302233). Memory B-cells have also been sorted either as $CD19^+CD27^+IgG^+$ or $CD19^+CD20^+IgG^+$, naive B-cells have been sorted as $CD19^+IgD^+IgM^+$. IgA^+ plasmablasts have also been sorted, and are defined as $CD19^+CD20^-$ $CD27^+CD38^{++}IgA^+IgM^-$. Other cell surface markers may also be used, as long as the B-cell or other cell population is phenotypically identifiable using cell surface markers, the population can be single-cell sorted. *See* below. Cells were then washed once with 2 mL of FACS buffer and resuspended at an appropriate volume for FACS. Cells were first sorted on a BD Aria II into a 5 mL round bottom tube. Typically, purities of >80% were achieved from the first sort. Single cells were sorted into the first 11 columns of a 96-well PCR plate containing 6.65 µL of a hypotonic buffer (10mM Tris-HCl pH 7.6) containing 2 mM dNTPs (NEB N0447L), 5 µM oligo$(dT)_{20}$VN, and 1 unit of Ribolock (Fermentas EO0384), an RNase inhibitor. As a negative control, the last column was left devoid of cells. For IgG^+ plasmablasts, the gating (selection of cells) strategy was $CD19^+CD20^-CD27^+CD38^{++}IgA^-IgM^-$. Sorted plates were sealed with aluminum plate sealers (Axygen PCR-AS-600) and immediately frozen on dry ice and stored at -80°C.

[00368] ***Single-cell sorting gating strategies***

[00369] *B-cells.* For B-cells, the gating approach comprises sorting for one or more of the following markers: IgM, IgG, IgA, IgD, CD19, or CD20. For total IgG^+ B-cells, the gating approach comprises sorting for IgG^+. For total IgA^+ B-cells, the gating approach comprises sorting for IgA^+. For total IgM^+ B-cells, the gating approach comprises sorting for IgM^+.

[00370] *Activated B cells.* Activated B cells include B cells that have been stimulated through binding of their membrane antigen receptor to its cognate antigen and/or have received T cell help from T cells recognizing epitopes derived from the same macromolecular antigen. Activated B cells can be identified by a variety of properties including increased cell size (e.g.

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"blasting B cells"; see below), expression of cell surface marker or markers, expression of intracellular marker or markers, expression of transcription factor or factors, exiting the gap 0 (G0) phase of the cell cycle, progressing through the cell cycle, production of cytokines or other factors, and/or the down regulation of certain cell surface marker or markers, intracellular marker or markers, transcription factor or other factor. One method of identifying an activated B cell is to combine detection of a B cell marker such as CD19 or immunoglobulin with a marker of activation such as increased cell size or volume, the cell surface activation marker CD69, or progression through the cell cycle based on cell-permeable acridine orange DNA stain or another cell cycle analysis.

[00371] *Blasting B cells.* "Blasting B cells" are B cells that are activated and increased in size relative to resting B cells. Blasting B cells include the plasmablast population as well as other populations of activated B cells, and blasting B cells are physically larger in size than resting B cells. Blasting B cells can be single-cell sorted using several different approaches, including gating (selection) of B cells based on their physically being larger based on cell diameter, cell volume, electrical impedance, FSC, the integral (area) of a FSC pulse (FSC-A), FSC height (FSC-H), forward scatter pulse width (FCS-W), side scatter (SSC), side scatter pulse area (SSC-A), side scatter height (SSC-H), side scatter width (SSC-W), autofluorescence and/or other measures of cell size.

[00372] In flow cytometry, forward scatter (FSC) is measured using a light beam in line with the stream of cells and provides information regarding the proportional size and diameter of each cell. Using FSC one can select B cells with FSC greater than the median FSC of resting B cell, for example an FSC-A or FSC-H 5% greater than resting B cells, 10% greater than resting B cells, 15% greater than resting B cells, 20% greater than resting B cells, 30% greater than resting B cells, 40% greater than resting B cells, 50% greater than resting B cells, 60% greater than resting B cells. By analyzing calibration beads of specific sizes, one can use FSC to determine the relative size of B cells relative to the calibration beads. By doing so, one can specifically gate on and thereby select B cells that possess diameters of about 8um, >8 um, >9 um, >10 um, >11 um, >12 um, >13 um, > 14 um, >15 um, > 16 um, >17 um, >18 um, >19 um, or >20 um.

[00373] Another measurement of cell size is cell volume. The "gold standard" for cell volume uses the Coulter principle which is based on an electronic measurement (Tzur et al, PLoS ONE, 6(1): e16053. doi:10.1371/journal.pone.0016053, 2011). Although the method of sorting by droplet charging and deflection was first used in a device that measured cell volume by impedance, the currently available flow cytometers make only optical measurements. FSC measurements, specifically the FSC-A (FSC integral area) are commonly used to assess cell

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size, although FSC measurements can be influenced by the refractive index differences between particles and fluid (Tzur et al, PLoS ONE, 6(1): e16053. doi:10.1371/journal.pone.0016053, 2011). Some have shown that volume estimation can be improved by combining optical parameters, including FSC-W, SSC and 450/50-A auto fluorescence (Tzur et al, PLoS ONE, 6(1): e16053. doi:10.1371/journal.pone.0016053, 2011).

[00374] For example, selection of activated B cells based on increased size can be achieved through identifying B cells using a marker such as CD19 and assessing size through FSC or FSC-A. Other B cell markers and/or parameters for assessment of size are described herein.

[00375] *Plasmablasts.* For isolation of plasmablasts, the gating approach comprises sorting for $CD19^{+}CD38^{++}$ B-cells. For isolation of IgG^{+} plasmablasts, the gating approach comprises sorting for $CD19^{+}$ $CD38^{++}IgA^{-}IgM^{-}$ B-cells. For isolation of IgA+ plasmablasts, the gating approach comprises sorting for $CD19^{+}CD38^{++}IgA^{+}$ B-cells. For isolation of IgM+ plasmablasts, the gating approach comprises sorting for $CD19^{+}CD38^{++}IgM^{+}$ B-cells. In addition, other gating strategies can be used to isolate a sufficient number of plasmablasts to carry out the methods described herein. Plasmablasts were also isolated using the following marker expression patterns $CD19^{low/+}$, $CD20^{low/-}$, $CD27^{+}$ and $CD38^{++}$. Although use of all these markers generally results in the purest plasmablast population from single cell sorting, not all of the above markers need to be used. For example, plasmablasts may also be isolated using the following gating strategies: forward scatter high (FSC^{hi}) for larger cells, $FSC^{hi}CD19^{lo}$ cells, FSC^{hi} and $CD27^{+}$, $CD38^{++}$, or $CD20^{-}$ cells. Combination of any of these markers or other markers found to be able to distinguish plasmablasts from other B-cells will generally increase the purity of sorted plasmablasts, however any one of the above markers alone (including FSC^{hi}) can distinguish plasmablasts from other B-cells, albeit with a lower purity.

[00376] *For memory B-cells.* For IgG^{+} memory B-cells, the gating approach comprises sorting for $CD19^{+}$ $CD27^{+}IgG^{+}$ or $CD19^{+}$ $CD20^{+}IgG^{+}$. For IgA^{+} memory B-cells, the gating strategy comprises $CD19^{+}CD27^{+}IgA^{+}$ or $CD19^{+}CD20^{+}IgA^{+}$. For IgM^{+} memory B-cells, the gating strategy comprises $CD19^{+}CD27^{+}IgM^{+}$or $CD19^{+}CD20^{+}IgM^{+}$.

[00377] *For other cell types.* As long as the B-cell, T-cell, or other cell population is phenotypically identifiable using cell markers, it can be single-cell sorted. For example, T-cells can be identified as $CD3^{+}$ or TCR^{+}, naïve T-cells identified as $CD3^{+}CD45RA^{+}$, memory T-cells identified as $CD3^{+}CD45RO^{+}$. Effector and helper T-cells can be identified as $CD3^{+}CD8^{+}$ and $CD3^{+}CD4^{+}$, respectively. Cell populations can be further subdivided by using combinations of markers, such as $CD3^{+}CD4^{+}CD45RO^{+}$ for memory helper T-cells.

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Sequencing of paired light and heavy chain immunoglobulin genes from single B-cells

[00378] Reverse transcription with adaptor molecules

[00379] Single-cell sorted plates were thawed on ice and briefly centrifuged before use. Plates were incubated in the thermal cycler at 55°C for 3 minutes, 42°C for 2 minutes, and indefinitely at 4°C. Plates were briefly centrifuged again and carefully opened to avoid the formation of aerosols. 1 µL of a 10 µM solution of the appropriate adapter molecule (each adapter molecule generally has a sample identification region (sample-ID)) was added to each well, with all negative control wells (containing RNA preservative buffer alone, or non-B-cells) receiving identical adapter molecules. 2.35 µL of a mix containing 0.75 µL H_2O, 1 µL of 10x M-MuLV RT buffer (NEB B0253S), 0.6 µL of 50mM $MgCl_2$, 0.25 µL of Ribolock (40 U/µL), and 0.125 µL of Superscript III (200 U/µL) (Invitrogen 18080-085) was added and mixed by pipetting. Plates were briefly centrifuged and incubated at 42°C for 120 minutes to 8 hours using a thermal plate shaker and then kept at -20°C. After the reaction, RT products from all wells were pooled in a microfuge tube. Pooled RT products were then extracted with phenol-chloroform-isopropyl alcohol with ~0.1% 8-hydroxychloroquine (Sigma 77617), and then extracted with chloroform extraction in gel-lock phase tubes (5 PRIME 2302820). RT products were then concentrated and desalted by 5-minute spins at 14 000g with Amicon Ultra-0.5 30kDa (Millipore UFC503096) or Ultra-0.5 100kDa (Millipore UFC510096), followed by a 5 min spin at 14 000g with TE (10mM Tris-HCl pH 7.6 with 1mM EDTA) and a final 5-minute spin at 14 000g with EB (Qiagen 19086). RT products were eluted by inverting the Amicon Ultra column in a new centrifuge tube and centrifuging at 1000g for 2 minutes. At this point, RT products were kept at -20°C or -80°C.

[00380] Touchdown PCR

[00381] For 454 sequencing runs 1 and 2, the Touchdown PCR method was used as follows. For some samples in PCR runs 3 and 4, the PCR method was changed, leading to increased numbers of paired heavy and light chains. This change is detailed under the sub-section "Non-touchdown PCR" below.

[00382] For both the 1st PCR and the nested 2nd PCR, Phusion Hot Start II DNA polymerase (NEB F-549L) was used in the provided GC buffer. For IgG, primers and adapter molecules are shown in Table 1. Sample-ID sequences are shown in Table 2. Plate-ID sequences are shown in Table 3. *See* also Figs. 3 and 9. Reaction conditions included a final $MgCl_2$ concentration of 1.8 mM, 200 µM dNTPs, 0.2 µM for all primers, 0.2 U of Phusion polymerase, varying amounts of DMSO as an additive and 2 µL of template in a final volume of 25 µL. For the 1st PCR, lambda and kappa light chains and the gamma heavy chain were amplified in different wells, and

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DMSO is used at a final concentration of 8%, 5% and 10%, respectively. Forward primers used in the 1st PCR were the FW long primer1 and the FW short primer1. Because the FW long primer1 added a plate identification region (plate-ID) to the 5' end of amplicon regions (amplicons), FW long primer1 containing different plate-IDs was added to different samples. Gene-specific reverse primers were used to amplify the kappa, lambda, and gamma chains were kappa GSP1, lambda GSP1, and gamma GSP1, respectively. Cycling conditions for the 1st PCR included an initial denaturation step at 98°C for 30'', followed by 2 cycles of 98°C for 10'', 72°C for 25''; 7 touchdown cycles of 98°C for 10'', 71.5°C to 68.5°C for 15'' and 72°C for 20'' with a drop of 0.5°C for each subsequent annealing step; 30 cycles of 98°C for 10'', 68°C for 15'' and 72°C for 20'', followed by a final extension at 72°C for 5' and hold at 4°C indefinitely. Products from 1st PCR were diluted 100x in TE and 2 μL used for the nested 2nd PCR. For the 2nd PCR, 5% DMSO was used as an additive in all samples. Forward primer is the FW primer2 and reverse primers were the RV primer2 and the GSP long primer2. Kappa GSP long primer2, lambda GSP long primer2 and gamma long primer2 were used to amplify their respective amplicons. Because the GSP long primer2 also added the plate-ID to the 3' end of the amplicons, a different GSP long primer2 with plate-specific plate-IDs was added to each pooled-plate sample. Cycling conditions for the nested 2nd PCR included an initial denaturation step of 98°C for 30'', 30-40 cycles of 98°C for 10'', 67°C for 15'', and 72°C for 20'', followed by a final extension of 72°C for 5' and hold at 4°C indefinitely.

[00383] Non-Touchdown PCR

[00384] For the non-touchdown PCR, conditions were identical to the touchdown PCR unless otherwise stated. The 1st PCR cycling parameters were an initial denaturation of 95°C for 5', 15-25 cycles of 98°C 30", 62°C 30", 72°C 30", a final extension of 72°C 5' and hold at 4°C indefinitely. 1st PCR was a multiplex PCR, where all 3 gene-specific reverse primers, the kappa, lambda, and gamma constant regions reverse primers were used in conjunction at 0.2, 0.2 and 0.24 μM, respectively. All other primers used were the same as in touchdown PCR. The gene-specific primers can be those used in touchdown PCR and also any one of those designated as suitable for 1st PCR (Table 6). DMSO was used at a final concentration of 5%; 0.1mg/ml of BSA (NEB B9001S), and ET-SSB (NEB M2401S) may also be added at 1:100 for the PCR reaction. During the 1st PCR, 4-6ul of cDNA template was used in a total 80 or 90ul reaction volume. Each PCR1 reaction was split into eight or nine 10ul reactions, each occurring in a different well. 1st PCR was pooled again after the PCR and diluted 100x in TE0.1, and 2ul used for 2nd PCR. 2nd PCR is a separate reaction for each gene-specific primer (not multiplex), and the reaction mix was identical to the touchdown 2nd PCR except for the following: any of the

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gene-specific constant region primers designated as working for the 2nd PCR may be used (Table 6), primers were used at either 0.2μM or 0.4μM throughout, or the gene-specific primers were used at 0.2μM and the rest used at 0.4μM. 0.1mg/ml BSA was added to the reaction and ET-SSB may also be used at 1:100. The 2nd semi-nested PCR cycling parameters were an initial denaturation of 95°C for 5', 20-35 cycles of 98°C 30", 67°C 30", 72°C 30", a final extension of 72°C 5' and hold at 4°C indefinitely. The total number of PCR cycles for 1st and 2nd PCR combined was typically between 50-60 cycles for the non-touchdown PCR. As different pooled-wells undergoing the PCR cycling tend to use different number of cycles to obtain a reasonable amount of DNA product (typically between 1-12ng/ul), 4 different PCR cycles were carried out for each 2nd PCR, e.g. 23, 26, 30 and 33 cycles, 5ul run on a 2% agarose gel and compared. Based on qualitative judgment of the amount of PCR product, only PCR product from one of the 2nd PCR cycle numbers was used for each pooled-well 2nd PCR in preparing for the 454 sequencing run.

[00385] For PCR of other immunoglobulin heavy chains in humans, immunoglobulin heavy and light chains in mice and TCR chains in humans and mice, PCR conditions are identical to the non-touchdown PCR section above except that 1st PCR is non-multiplex, with each cDNA being individually amplified. The following 3' primers in Tables 10 and 11 are used in PCR1 and 2.

[00386] Preparing for 454 XLR70 sequencing run

[00387] For the 1st and 2nd 454 runs, sequencing primers (Titanium Primers A and B, respectively) for a 454 Titanium sequencing run were added onto the amplicons during the 1st and nested 2nd PCRs. 5 μL of each amplicon were run on an agarose gel with a mass DNA ladder (Fermentas SM0383), an image was taken, and band intensities were analyzed and quantified with AlphaFC Imager software (Cell Biosciences). 5 ng of each of the kappa, lambda, and gamma amplicons were separately pooled, run on a 0.8% agarose gel, and visualized with GelGreen (Biotium 41005). Bands of the appropriate sizes (~600bp for kappa and lambda, and ~750bp for gamma) were cut and purified with MinElute Gel Extraction kit (Qiagen 28606), according to manufacturer's instructions with slight modifications. Briefly, agarose gel was melted in QG buffer without heating, and the additional QG wash step is done. PE wash buffer was allowed to sit for 5 minutes before spinning. An additional PE wash step was also performed. Samples were eluted with 25 uL of EB buffer. Samples were also cleaned once with SPRI beads using a ratio of 1:0.65 for DNA volume: bead volume for 454 2nd run. DNA concentration was determined with Picogreen DNA assay kit (Invitrogen P11496), and samples were pooled such that DNA concentration of gamma:kappa:lambda is 2:1:1. Pooled

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samples were at a concentration of >0.5ng/μL and were shipped to a 454 DNA sequencing facility for 454 sequencing.

[00388] For the 3rd and subsequent 454 sequencing runs, the protocol was changed. The amplicons were still separately pooled to normalize DNA quantities from each PCR reaction, but first underwent an SPRI bead cleanup to remove small DNA fragments according to manufacturer's instructions. The amplicons were run on a 3% agarose gel, and the appropriate bands cut and purified with MinElute Gel Extraction kit as before. Thereafter, the amplicons underwent another 2 rounds of SPRI bead cleanup to remove even more small DNA fragments, and quantitated with Picogreen, quality checked with Nanodrop to ensure the OD260/280 ratio was >1.8 and 1μl run on a gel to ensure there were no small DNA fragments. Lambda and kappa amplicons were pooled in a 1:1 ratio, gamma was used as-is. DNA was then diluted to 1 x 10^9 copies per 454's instructions, and sent to sequencing facility (Roche) for emPCR at 1cpb and sequenced; gamma heavy chain in one region and the pooled light chains in the other region of the picotiter plate.

[00389] **Preparing for 454 XL+ sequencing run**

[00390] Currently the 454 XL+ sequencing run does not support the Lib-A sequencing kit that was used for the XLR70 run. XL+ currently only supports the Lib-L kit, which is unidirectional sequencing. To adapt our protocol to do XL+ sequencing, the protocol for XLR70 run is followed, but after the gel cleanup step, each amplicon (kappa, lambda and gamma) underwent 2 separate PCRs, each 5 cycles long to add on the Lib-L A and B adaptors. PCR conditions are as follows: Phusion polymerase is used, with 5x GC buffer and a final concentration of 5% DMSO. Primers are used at 0.2uM. 0.1mg/ml BSA is added to the reaction. The PCR cycling parameters are an initial denaturation of 95°C for 5', 20-35 cycles of 98°C 30", 67°C 30", 72°C 30", a final extension of 72°C 5' and hold at 4°C indefinitely. Two PCRs are done for each amplicon: 5LIB-LA and 3LIB-LB in one PCR, and 5LIB-LB and 3LIB-LA in the other PCR. The adaptors are added such that each amplicon becomes either 5'-LibA-amplicon-LibB-3' or 5'-LibB-amplicon-LibA-3'. These amplicons have either the LibA "A" or "B" adaptors on the 5' end (and the corresponding "B" or "A" adaptor on the 3' end), which allows for bidirectional sequencing. Amplicons with the new Lib A adaptors then undergo 3 rounds of SPRI bead cleanup before following the protocol for XLR70 runs to quantitate and quality check the DNA before diluting it to 1 x 10^9 copies and sending to a 454 sequencing facility (Roche) for emPCR at 1cpb and sequencing.

[00391] **Preparing for PacBio sequencing run**

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[00392] For PacBio sequencing run, touchdown PCR was employed as above. DNA pooling and cleanup was done as the section above on "preparing for 454 XLR70 run." To obtain sufficient DNA (500ng) for sequencing requirements, a minimum of 1ug of DNA was pooled for gel and SPRI cleanup. Picogreen quantitation and $1x10^9$ dilutions were not done as it was not required for PacBio sequencing. If insufficient DNA was obtained from the 2nd PCR, the 2nd PCR and pooling steps were repeated until sufficient DNA was obtained. A minimum of 500ng of cleaned-up DNA was sent to PacBio sequencing facility for sequencing.

[00393] Other sequencing approaches

[00394] The methods disclosed herein are not dependent on 454 or PacBio sequencing. Lambda and kappa light chains are ~600bp and gamma heavy chain ~700bp. Thus, what is generally desired is the ability to have longer sequencing reads such that the forward and reverse sequencing reads overlap enough to enable reconstruction of the entire, approximately 600 bp sequence of the light chains (LCs) (exact sequence length depends on the length of the 5' untranslated region (UTR)), and approximately 700 bp sequence of the heavy chains (HCs). Therefore, any sequencing technology that can yield sequencing reads of at least about 350-400bp and thereby achieve the overlap used for sequence assembly can be utilized, and sequencing technologies that enable app. 600-700+ bp reads would allow one to sequence using just the forward (Fw) primer (sequencing from the 5' end).

Sequences

[00395] The sequence data for the above runs was received from the relevant facility and processed as described below.

[00396] Sequence Nomenclature

[00397] Each sequence in the sequence listing which corresponds to a sequencing read, sequence assembly or amino acid translation from a sequence has an identifier. Each such identifier has 9 fields separated by a period, ".". The fields are numbered from 1 to 9 and give the following information:

1. Read ID. A Read ID assigned by the software associated with the sequencing technology used to determine the read, or "NA" if the sequence is not a raw read.
2. Plate Number. A plate number that the sequence is associated with. See Table 12 (plate to sample mapping table) for corresponding biological sample information.
3. Sample ID. Sample ID indicating the well that the sequence is associated with. Sample ID numbers are between 1 and 89 inclusive. *See* Table 2 for correspondence between Sample ID and Well Name.
4. Well Name. Well name containing the well that the sequence is associated with. The well name corresponds to the usual 96 well plate name, e.g. D07. The well name and the Sample ID are equivalent ways of specifying a particular well on a plate.

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5. Contig ID. The contig ID distinguishes different sequences associated with a well from a given assembly and chain type.
6. Platform. The platform field indicates the sequencing technology that the sequence is derived from. The possible values for platform are 454, Sanger, and PacBio.
7. Chain Type. The chain type field indicates whether the sequence is associated with a set of heavy chain antibody sequences, light chain antibody sequences, or a set containing both heavy and light chain antibody sequences. Possible values are "heavy", "light" or "CMB".
8. Run ID. An identifier for a set of reads on a particular platform.
9. Sequence Type. The type of the sequence. Possible values are "raw" for raw sequencing technology reads, "nb", "urt", "multim50", "zerom50" or "pb" for assembled reads (see Assembly of Sequences section), or "nb-aa", "urt-aa", "multim50-aa", "zerom50-aa" or "sanger-aa" for amino acid sequences derived from the various nt assembly consensus sequences.

[00398] Preparation of sequences for analysis

[00399] Data generated from the 454 sequencing were analyzed by 454 GS FLX data analysis software, and filter-passed high quality sequences were returned. Due to the stringency of the default amplicon filter used by 454 GS FLX data analysis software, filter stringency may need to be relaxed to obtain sufficient long reads. One way is to follow the suggestions in 454 technical bulletin APP No. 001-2010. Changing <vfScanAllFlows> from "TiOnly" to "False" of the amplicon filter can lead to a large increase in filter-passed sequences of good quality. Another option is to change the <vfTrimBackScaleFactor> to a lower number. For 454 run 1, standard shotgun processing was used and for run 2, <vfScanAllFlows> was changed to "False", and standard amplicon pipeline processing was used for runs 3 and 4.

[00400] Data generated from Pacific Biosciences sequencing was received from Pacific Biosciences as Circular Consensus Sequence reads with associated quality scores.

[00401] Assignment of Sequences to Wells

[00402] cDNA from samples was sequenced with either 454 or Pacific Biosciences sequencing technology. The reads are those in the Sequence Listing whose Sequence Type is "raw". The sequencing reads were analyzed and either assigned to a source plate and well or discarded.

[00403] Plate and well assignments for reads were made by comparing the observed read sequence to the possible plate identification region, universal primer region, and sample identification region sequences using regular expressions. The comparison was done in three stages using the regular expressions listed in Tables 13, 14 and 15.

[00404] In stage 1, analysis of possible plate identification regions, a read was checked against all of the regular expressions listed in column "Plate Identification Region Regular Expression" in Table 13, requiring a match to begin with the first nucleotide of the sequence. If

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no match was found the read was discarded and plate/well assignment continued with the next available read to process, if any. If a match was found, the sequence was assigned the corresponding ID from the "Plate ID" column as its plate ID. The nucleotides of the read matching to the plate regular expression were recorded for use during later stages of matching and during assembly.

[00405] In stage 2, analysis of the universal primer region, a read was checked against the "universal primer regular expression" "CACGACCGGTGCTCGATT+AG", requiring a read match to begin with the first nucleotide following the last read nucleotide matching to the Plate Regular Expression. If the read did not match the universal primer regular expression, the read was discarded and plate/well assignment continued with the next available read to process, if any. Otherwise the nucleotides of the read matching to the universal primer regular expression were recorded for use during the last stage of matching and during assembly.

[00406] In stage 3, analysis of possible sample identification regions, a read was checked against all of the regular expressions listed in column "Sample Identification Region Regular Expression" in Table 14, requiring a match to begin with the first nucleotide following the last read nucleotide matching to the universal primer regular expression. If no match was found the read was discarded and plate/well assignment continued with the next available read to process, if any. If a match was found and the sample ID column contained only a single identifier, the sample ID of the read was assigned to be the ID found in the sample ID column. If the sample ID column contained more than a single identifier, those identifiers were considered "candidate sample IDs". The read was then checked sequentially against all of the regular expressions listed in column "Sample Identification Region Regular Expression" of Table 15 where at least one of the corresponding sample IDs in the "Sample ID" column of Table 15 matched with a candidate sample ID. If the read matched the regular expression, and the match began with the first nucleotide after the last read nucleotide matching to the universal primer regular expression, the right-most identifier from the candidate sample IDs was assigned as the read's sample ID. Otherwise, the right-most identifier was removed from the list of candidate sample IDs and the process repeated with the smaller list of candidate sample IDs until either a match was found, or, if no matching regular expression was found in the list of regular expressions in Table 15 then the last candidate sample ID (that is, the left-most in the original list of candidate sample IDs) was assigned as the sample ID for the read.

[00407] Reads that were discarded during the plate ID and sample ID assignment process were not included in the sequence listing.

[00408] **Assembly of Sequences**

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[00409] All sequence reads assigned to a sample ID associated with a well were assembled to produce consensus sequences. These consensus sequences correspond to the heavy and light chain mRNA sequences expressed in the sorted cells.

[00410] Sequences were assembled with Newbler 2.5 (Runs 1 and 2), and Newbler version 2.6 and/or Mira version 3.4.0 for other sequences.

[00411] Sequences in the listing with a Platform field of "454", a Chain Type field of "mixed", a Run ID of "1" or "2" and a Sequence Type of "nb" are contigs resulting from an assembly using newbler. To assemble these sequences, sff output files from 454 sequencing, which contain both sequences and quality scores for each nucleotide, were read into Python using the Biopython package and sequences subdivided according to their compound barcodes (sample-ID + plate-ID) as described above and output into separate sff files. These files were then reparsed by sfffile (provided by GS FLX data analysis software) into sff files with file headers understood by Newbler, a sequence assembler provided in the GS FLX data analysis software suite, using the "-force", "-cdna" and "-urt" options. Newbler then assembled forward reads with shared compound barcodes. Because reverse reads have only a 3' plate-ID, it is possible that sequence assembly could occur between forward and reverse reads of sequences from different cells. For circumventing this potential problem, the heavy- and light-chain V(D)J usage of both assembled forward and unassembled reverse reads can be first identified using HighV-QUEST (http://imgt.cines.fr/HighV-QUEST/index.action). Sequences can then further grouped according to their V(D)J usage before being assembled again with Newbler using one assembled forward read and the reverse reads that share the same V(D)J usage. This can be repeated for all assembled forward reads. Sequence assembly can also be done to be intolerant of nucleotide mismatches, thereby preventing assembly of forward and reverse reads from different cells that share the same V(D)J usage. This way, inappropriate sequence assembly of reverse reads between highly similar sequences from different cells can be largely avoided.

[00412] Sequences in the listing with a Platform field of "454", a Chain Type field of "heavy" or "light", a Run ID of "3" or "4" and a Sequence Type of "nb" are contigs resulting from assemblies of 454 reads, executing Newbler with this command line: runAssembly -cdna -o output seqs.fastaq where seqs.fastq contained a single well's trimmed reads in FastQ format.

[00413] Any wells for Run ID 3 or Run ID 4 reads which Newbler did not create exactly one heavy chain contig or exactly one light chain contig were reanalyzed by assembling with mira. Sequences in the listing with a Platform field of "454", a Chain Type field of "heavy" or "light", a Run ID of "3" or "4" and a Sequence Type of "multim50" or "zerom50" are contigs resulting from these assemblies, executing mira with this command line: mira --project=seqs --

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job=denovo,est,accurate,454 454_SETTINGS -ED:ace=yes -AL:egp=no -CL:pvlc=yes --fastq –notraceinfo

[00414] A file named seqs_in.454.fastq contained a single well's trimmed reads in FastQ format.

[00415] For wells from Run ID 3 or Run ID 4 reads where neither Newbler nor mira created contigs using the above assembly commands, a different Newbler command was executed. Sequences in the listing with a Platform field of "454", a Chain Type field of "heavy" or "light", a Run ID of "3" or "4" and a Sequence Type of "urt" are contigs resulting from these assemblies, where Newbler was executed with this command line: runAssembly -cdna -ud -urt -o output seqs.fastaq where seqs.fastq contained a single well's trimmed reads in FastQ format.

[00416] Sequences in the listing with a Platform field of "PacBio", a Chain Type of "heavy" and a Sequence Type of "pb" are contigs resulting from assemblies of reads from the PacBio platform, executing mira with the command line: mira --project=seqs --job=denovo,est,accurate,454 454_SETTINGS -ED:ace=yes -AL:egp=no -CL:pvlc=yes --fastq –notraceinfo

[00417] A file named seqs_in.454.fastq contained a single well's trimmed reads in FastQ format.

[00418] **Amino Acid Sequences**

[00419] Sequences in the listing with a Platform Field of "454" and a Sequence Type of "nb-aa", "urt-aa", "multim50-aa" or "zerom50-aa" are amino acid sequences determined by translating the nucleotide sequences of assemblies of 454 reads as described under "Assembly of Sequences".

[00420] Sequences in the listing with a Platform Field of "PacBio" and a Sequence Type of "pb-aa" are amino acid sequences determined by translating the nucleotide sequences of assemblies of Pacifc Biosciences reads as described under "Assembly of Sequences".

[00421] Sequences in the listing with a Sequence Type of "sanger-aa" are amino acid sequences determined by directly translating reads determined by Sanger sequencing.

[00422] **Other sequencing data analysis options**

[00423] The workflow of data analysis described above can be used to accurately determine the heavy- and light-chain sequences of each cell. However, this information is not absolutely necessary for our "selection screening" approach (*see* "screening of expressed human antibodies"). For the selection screen to work, we first cluster paired antibody sequences into clonal families, on the basis of their heavy chain V(D)J usage and light chain VJ usage. Therefore, we do not require the full sequence of the immunoglobulin heavy and light chains,

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and can use sufficient sequence information to determine V(D)J usage. Therefore, we can tolerate sequencing errors and can use lower-quality reads generated by 454 or any other sequencing technology. Sequence assembly of the forward read is not generally an issue because all sequences can be first grouped according to their compound barcode before being assembled. Because each compound barcode comes from one sample/cell, there is only one 'correct' sequence for each immunoglobulin chain with the same compound barcode. Sequencing errors in different strands can then be averaged out because it is unlikely that all sequencing errors occur at the same bases, meaning that taking the consensus base sequence will give the most accurate sequence. In cases of ambiguity, bases with high Phred quality scores will generally be chosen instead. Because 454 trims sequence reads from the 3' end until only higher quality reads remain, this can result in very short reads. With our method, we can tolerate lower-quality reads and thereby use much longer reads generated by 454 (400-500bp). With these longer reads, we can identify V(D)J usage without requiring assembly of the forward read with the reverse read, thereby making the 3' plate-ID non-essential in some aspects. Furthermore, the latest generation of 454 sequencing can sequence up to a mean of 746bp and a mode of 800bp. Thus, sequencing from just the forward reads can be sufficient to cover the entire heavy- and light-chain immunoglobulin amplicons, also making the 3' plate-ID non-essential, in some aspects, because assembling forward with reverse reads is no longer required.

Selection and cloning of antibodies

[00424] After assembly, heavy and light chain sequences were analyzed to select antibodies for characterization. Antibodies were selected based on predicted V(D)J germline usage and inspection of evolutionary trees derived from the antibody sequences. The selected antibodies were cloned, expressed, and characterized in different assays.

[00425] V(D)J Assignment

[00426] Heavy and light chain sequences from Runs 1 and 2 were analyzed with V-QUEST (Brochet, X. et al., *Nucl. Acids Res.* 36, W503-508 (2008)), software that compares an antibody chain sequence to a database of known alleles of germline sequences and predicts the germline alleles used in the antibody, how the germline sequences were recombined, and mutations in the antibody relative to the germline. Table 18 shows results of a V-QUEST V(D)J assignment for the antibodies which were chosen for further characterization; the same data were obtained for all other sequence assemblies from Run 1 and Run 2 as well. Some sequences from Run 3 and Run 4 assemblies were analyzed with SoDA (Volpe, Cowell and Kepler, *Bioinformatics* (2006) 22 (4): 438-444), software similar to V-QUEST.

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[00427] If the patient's genome has been sequenced, the genome sequence data can be used as the germline sequences for the VDJ assignment analysis, further improving the ability to reliably identify somatic hypermutations in the patient's antibody sequences.

[00428] Evolutionary Trees and Clonal Families

[00429] The nucleotide sequences corresponding to the mature peptides of heavy chains from Runs 1 and 2 were separated into sets that corresponded to the patient from which they were derived. From these individual sets the software clustalx2 (Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. (2007) *Bioinformatics*, 23, 2947-2948) was used to generate an alignment and tree using default settings for all parameters.

[00430] Evolutionary trees of sequences from a patient can also be constructed from the set of sequences from individual clonal families The putative progenitor antibody heavy and light chain sequences for the family can be inferred and added to the set of sequences if they are not already in the set. An evolutionary tree for the set can be constructed using, for example, Maximum Parsimony, Maximum Likelihood, or any suitable algorithm, and the tree can be rooted at the sequence of the progenitor antibody. The tree can be constructed on the basis of the heavy chains alone, or the light chains alone, or preferably by constructing the tree based on the individual heavy and light chains simultaneously, so that the tree represents the co-evolution of the heavy and light chains.

[00431] Antibody Selection

[00432] For each patient, the table of V-QUEST results was reviewed in conjunction with the trees built from Run 1 or Run 2 sequences (viewed in TreeViewX: http://darwin.zoology.gla.a.c.uk/~rpage/treeviewx). Representative sequences were selected to cover the different families of VDJ present based on the V-QUEST data, inspecting the corresponding sequences on the tree to choose sequences that appeared to be representative of the clade. Typically one sequence was selected from each clade. Some of the selected sequences came from families with many members, but some were also selected from families with few members or one member. The selected sequences are described in Table 18. For each antibody in Table 18, the column "Antibody" is the same as the text following the "-" in the Contig ID field of the name associated with the sequence in the Sequence Listing.

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Cloning and expression of cloned light and heavy chain immunoglobulin pairs

[00433] Vectors

[00434] One system is a Neomycin and dihydrofolate reductase (DHFR) selectable vector system modified from Invitrogen vectors pcDNA3.3 and pOptivec. An alternative system is the Lonza GS system, in which the amplifiable, selectable marker is glutamine synthetase (GS) (*see* below). Sequences encoding the immunoglobulin kappa light chain, lambda light chain, and gamma heavy chain are inserted into vectors. Kozak consensus and leader sequences are already present in the clones, and thus do not need to be engineered into the vectors. The constant regions are synthesized to contain 5'-flanking restriction sites and one or more other internal restriction sites. For facilitating the cloning of varied immunoglobulin heavy and light chains, inserts are engineered with multiple restriction sites that increase the possibility that the clone itself will not contain the restriction site and therefore not be cut internally. The inserts have two different 8-cutter restriction sites at the 5' end of the insert region and at two different restriction sites engineered into constant regions. The 5' restriction sites are FseI and PacI for both light chains, and AscI and AsiSI for the gamma heavy chain. Restriction sites engineered into the constant region themselves are NheI and XhoI for both light chains, and EcoRI and SacII for the gamma heavy chain. See Table 16 for the sequence of the constant region inserts containing the restriction sites. Heavy or light chain clones from the 1st PCR reaction are then subjected to a 2nd round of PCR with cloning primers, which have 5' flanking restriction sites that are incorporated into the clones. Appropriate restriction enzymes are used for cutting the expression vectors and the clones, which now have complementary ends and are ligated together using T4 DNA ligase. Both the Invitrogen and Lonza GS vector systems contain an amplifiable selection marker. This marker is DHFR in the Invitrogen system and GS in the Lonza GS system. Under selection pressure from the appropriate selector (methotrexate for DHFR and L-methionine sulfoximine for the glucose synthetase (GS)), genes linked to the selection marker are amplified together with it. With more copies of the immunoglobulin genes, there is greater secretion of antibodies. This is useful when large amounts of antibody need to be purified for subsequent *in vivo* screening for neutralizing antibodies.

[00435] Cloning and expression

[00436] Assuming that the highest-affinity plasmablasts are selected for during germinal center maturation, we expect that the highest-affinity clonal family also has the highest number of clones. Furthermore, the highest-affinity clone within each clonal family will also be the most frequent clone within that family. On the basis of these assumptions, we choose to express the highest-frequency clone from the 5 largest clonal families from each patient sample in some

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aspects. Clones are amplified from the 1st PCR cDNA, where all samples from the same plate, each containing a single cell, have been pooled together. The forward primer contains the sample-ID and therefore amplifies only DNA barcoded with that particular sample-ID. This is highly specific because sample-IDs contain nucleotide differences between one another. Some sample-IDs can have identical cloning forward primers and clones amplified by these primers must subsequently be distinguished from each other by bacterial colony selection too. Both forward and reverse primers contain flanking restriction sites (first and second restriction site regions) that allow the clone to integrate (with coding frame aligned) into a vector that already contains a kappa, lambda, or gamma constant region. See Tables 4 and 5 for cloning primer sequences. Light chains are cloned into modified pcDNA3.3, and heavy chains into modified pOptivec, or both chains are cloned into the Lonza GS dual-expression vector. Mammalian cells are either doubly transfected with separate expression vectors encoding the immunoglobulin heavy and light chain genes, or singly transfected with a dual-expression vector containing both the heavy and light chain genes. Supernatants containing the secreted antibodies are then collected and screened for the desired properties.

[00437] In some instances, variable regions of Ig genes may be cloned by DNA synthesis, and incorporating the synthesized DNA into the vector containing the appropriate constant region using restriction enzymes and standard molecular biology. During synthesis, the exact nucleotide sequence need not be followed as long as long as the amino acid sequence is unchanged, unless mutagenesis is desired. This allows for codon optimization that may result in higher expression levels. This also allows for adding in restriction sites for the purpose of cloning. Non-translated sequences such as 5' UTR and barcode sequences need not be synthesized and leader sequences can also be swapped for other signal peptide sequences known for higher expression levels. These result in an Ig nucleotide sequence that can be very different from the high-throughput reads but give identical amino acid sequenced when expressed.

[00438] In some instances, the sample-ID barcode adaptor added on during reverse transcription may already incorporate a restriction enzyme site. This results in an adaptor with a restriction site 3' of the sample-ID barcode in the PCR amplicon pool. During cloning with cloning primers, desired amplicons are amplified from a plate-specific amplicon pool using 5' primers that are complementary to the sample-ID barcode sequences, and chain specific 3' primers (for the kappa, lambda and gamma chains). 3' primers will add on 3' restriction sites. 5' primers do not need to add restriction sites as the 5' primer already contains a restriction site 3' of the well-ID barcode. Following this amplification, restriction enzymes are used to cut the amplicon for ligation into the vector containing the constant region insert. During the restriction

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enzyme digest, sequences added on to the 5' end of the Ig gene sequences, such as barcodes and universal sequences are cut as they are 5' of the 5' restriction site.

[00439] Alternative methods for cloning and expression

[00440] In another aspect, variable regions of Ig genes may be cloned by DNA synthesis, and incorporating the synthesized DNA into the vector containing the appropriate constant region using restriction enzymes and standard molecular biology. During synthesis, the exact nucleotide sequence need not be followed as long as long as the amino acid sequence is unchanged, unless mutagenesis is desired. This allows for codon optimization that may result in higher expression levels. This also allows for adding in restriction sites for the purpose of cloning. Non-translated sequences such as 5' UTR and barcode sequences need not be synthesized, leader sequences can also be swapped for other signal peptide sequences known for higher expression levels. These result in an Ig nucleotide sequence that can be very different from the high-throughput reads but give identical amino acid sequenced when expressed.

[00441] In another aspect, the well-ID barcode adaptor added on during reverse transcription may already incorporate a restriction enzyme site. This results in an adaptor with a restriction site 3' of the well-ID barcode in the PCR amplicon pool. During cloning with cloning primers, desired amplicons are amplified from a plate-specific amplicon pool using 5' primers that are complementary to the well-ID barcode sequences, and chain specific 3' primers (for the kappa, lambda and gamma chains). 3' primers will add on 3' restriction sites. 5' primers do not need to add restriction sites as the 5' primer already contains a restriction site 3' of the well-ID barcode. Following this amplification, restriction enzymes are used to cut the amplicon for ligation into the vector containing the constant region insert. During the restriction enzyme digest, sequences added on to the 5' end of the Ig gene sequences, such as barcodes and universal sequences are removed as they are 5' of the 5' restriction site.

[00442] Cloning of heavy and light chains into Lonza vectors

[00443] *Cloning immunoglobulin constant regions*

[00444] Lonza vectors were obtained through Stanford University's academic licensing agreement with Lonza. Kappa and lambda light chains were inserted into vector pEE12.4 and the gamma heavy chain was inserted into vector pEE6.4. Heavy and light chain sequences were cloned in two steps: first, the constant regions were cloned in, followed by the 5' end of the immunoglobulin chains (the leader and V(D)J sequences). Constant regions inserts were gene-synthesized by Intergrated DNA Technologies (IDT), and contained appropriate silent mutations for gene optimization and incorporation of restriction sites. Inserts were obtained from IDT in their proprietary pIDTSmart vector. Insert sequences are in Table 17. IgG1 was used as the

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gamma heavy chain constant region. The alleles used were Km3, Mcg˙Ke˙Oz˙ and G1m3 for kappa, lambda and gamma chains respectively. To incorporate the constant regions into the Lonza vectors, the Lonza vectors and the pIDTSmart-constant region inserts were all individually transformed into competent dam˙dcm˙ *E. coli* and plasmids were purified using Qiagen miniprep kit following manufacturer's instructions. Plasmids were then digested using HindIII and BclI at 37°C for 1 hour and run on a 1.2% agarose gel at 150V for 1 hour. Digested Lonza vectors and the constant region inserts were gel purified and ligated (pEE12.4 with Km3 or Mcg˙Ke˙Oz˙ light chains and pEE6.4 with G1m3 gamma1 heavy chain) using T4 DNA ligase for 10 minutes at RT in a ratio of 3:1 insert:vector. T4 DNA ligase was then inactivated at 70°C for 8 minutes and 5ul of the ligation mix was transformed into heat shock competent TOP10 cells using standard molecular biology techniques. Colonies were picked and insertion was verified via Sanger sequencing.

[00445] *Cloning immunoglobulin variable regions*

[00446] Next, pEE12.4 containing either lambda or kappa light chain was digested using AscI and XmaI for 5 hours at 37°C and gel purified. pEE6.4 containing the gamma 1 heavy chain was digested using AscI and AgeI for 5 hours at 37°C and gel purified. Selected amplicons were selectively amplified using well-ID specific forward primers and constant region-specific reverse primers (Tables 4 and 5) from the specific plate-ID 100x dilution of the 1st PCR. The forward primers had the restriction site AscI on the 5' end of the primer, and the reverse primers contained the XmaI or AgeI restriction site for the light and heavy chain primers constant region primers respectively. PCR cycling was done using an initial denaturation at 98°C for 30 seconds, and 35 to 45 cycles at 98°C for 10 seconds, 68°C for 15 seconds and 72°C for 20 seconds. The final extension was 72°C at 5 minutes and on hold at 4°C indefinitely. The PCR products were purified using PCR_{u96} ultrafiltration plates from Millipore, following manufacturer's instructions. Following this, PCR products were double digested for 3 hours at 37°C using AscI and XmaI for the light chains and AscI and AgeI for the gamma 1 heavy chain. Digested products were then run on a 2.5% low melt agarose with gelgeen (Biotium) and visualized under blue light. Gel slices containing bands at the appropriate sizes were excised. In gel ligation was performed by melting the gel slices at 65°C and adding the appropriate digested and Antarctic phosphatase (NEB)-treated Lonza vectors containing the constant region insert, and incubating with T4 DNA ligase for 1-3 hours at RT. Heat-shock competent bacteria were then transformed and plated on ampicillin agar. 6 clones per construct were picked and grown in 2xLB (2x concentrated Luria-Bertani broth). Miniprep was performed using Millipore's Multiscreen 96-well filter plates according to manufacturer's instructions

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(www.millipore.com/techpublications/tecg1/tn004) to obtain plasmid DNA. Colony PCR was performed using an initial denaturation of 95°C for 5 minutes, and 40 cycles of 95°C for 1 minute, 50°C for 2 minutes, 72°C for 1 minute, and a final extension at 72°C for 5 minutes and holding t 4°C indefinitely. Clones with the appropriate insert were sent for Sanger sequencing at Sequetech, Mountain View, CA, USA. Clone VDJ identification was done using IMGT HIGHV-Quest and the correct clones were kept as both bacterial stock (stored at -80°C with 15% glycerol) and plasmids.

[00447] Expression of monoclonal antibodies in 293T

[00448] Transient dual transfections of paired pEE12.4-lightchain and pEE6.4-heavychain constructs were done using Lipofectamine 2000 following manufacturer's protocol. Transfections have been done in 48-well, 24-well, 6-well, 60mm and 100mm dishes. In brief, 293T cells were cultured in DMEM+10% ultralow IgG FBS (Invitrogen) to prevent bovine IgG from competing with secreted human IgG at the downstream protein A purification step. 293T cells were cultured for 20 passages before new aliquots liquid N_2 were thawed and used. For 48-well plate transfections, each well was seeded the day before with $8x10^4$ cells, and allowed to grow to ~90% confluency the next day. 50ng each of heavy and light chain constructs were incubated in Optimem media for a final volume of 50ul, and Lipofectamine 2000 was also separately incubated with 50ul of Optimem media. Both incubations were from 5-25 minutes. Lipofectamine 2000 and the constructs were then mixed by gentle pipetting and incubated for 20 minutes before adding to 293T cells and gently mixed. Media was changed the next day and culture supernatants were collected every other day (e.g., Monday, Wednesday, and Friday) for 2 weeks. For transfections of other sizes, the following amounts of constructs and Lipofectamine 2000 were used: for 24-well plate transfections, 100ng of each construct were used with 1.25ul of Lipofectamine 2000. For 60mm dishes, 625ng of each construct were used with 12.5ul of Lipofectamine 2000. For 100mm dish transfections, 3ug of each construct were used in 37.5ul of Lipofectamine 2000.

[00449] Anti-human IgG ELISA

[00450] In some instances, human IgG ELISA was done on the sample to quantitate the amount of expressed IgG in the culture supernatant, and the culture supernatant was used directly in downstream applications after normalizing the amount of antibody. Anti-human IgG ELISA quantitation kit was purchased from Bethyl Laboratories and performed according to manufacturer's instructions. In brief, 100ul of capture antibody was coated on Nunc Maxisorp plates overnight at 4°C and washed 5x with PBST (PBS with 0.05% Tween20). Wells were blocked with 1% BSA in PBS for an hour at RT, then washed 5x with PBST. Wells were then

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incubated with the appropriate standard dilutions from the kit or diluted culture supernatants for 1 hr at RT, then washed 5x with PBST. 100ul of diluted HRP detection antibody was added to each well and incubated at RT for an hour, then washed 5x with PBST. 50ul of TMB substrate solution was added and the reaction stopped with 50ul of stop solution. Absorbance was read on a SpectraMax M5 spectrophotometer at 450nm and standard curves generated with a 4-parameter curve. Antibodies were kept at 4°C in PBS with 0.1% sodium azide as a preservative.

[00451] Protein A-IgG purification of expressed monoclonal antibodies

[00452] In other instances, antibodies were first purified from culture supernatant and quantitated using BCA before use. In brief, culture supernatants were collected 3x a week for 2 weeks into 50ml tubes and stored at 4°C with 0.1% sodium azide as an additive until protein A-IgG purification. Culture supernatants were spun down and decanted to remove any cellular aggregates. 1M pH 9.0 Tris was added to culture supernatants to ensure pH is between 7.5-8.0 as determined by pH indicator strips. Protein A plus agarose beads (Pierce) were washed 2x with PBS before 400ul of a 50% slurry was added to culture supernatants and incubated at 4°C overnight on a rotator to ensure even mixing of the beads. Beads were recovered by spinning culture supernatant at 1000g for 5 minutes and pipetting out the beads from the bottom of the tubes into 5ml gravity flow columns. Beads were washed with 4x2ml of PBS before elution with 2x1.5ml of IgG elution buffer (Pierce), which is a low pH elution buffer, into an Amicon-4 100kDa concentrator column. Eluted antibody was immediately neutralized with 400ul of 1M Tris pH8.0. Antibodies were then concentrated by spinning at 1000g in Amicon-4 100kDa concentrators for 10 minutes, followed by a 2mL wash of PBS, and a 2ml wash of PBS with 0.1% sodium azide. Antibody concentrations were determined by BCA assay, and adjusted to 0.5mg/ml. Protein A Plus Agarose was regenerated by washing with 1x2ml of PBS, 3x2ml of IgG elution buffer and a wash with 3ml of PBS with 0.1% sodium azide and kept at 4°C. Columns can be regenerated up to 5 times.

Screening of expressed human antibodies

[00453] Screens for antibody-antigen binding

[00454] Selected antibodies (see cloning and expression section above) are first screened for their ability to bind to the antigen of interest, and then antibodies of the entire clonal family are expressed and screened for their ability that block or neutralize the antigen (see "functional screens" below). The IgG concentration in supernatants containing antibodies of interest is first determined by IgG ELISA, so that the same amount of IgG can be used for each sample in the antibody-antigen binding screen. In other cases, IgG was purified from the supernatant using

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Protein A agarose beads, and quantified with BCA assay using bovine immunoglobulins as the standard. Purified IgGs were then normalized to the same concentration before use in screening antibodies. To screen antibodies that bind antigen, we perform an indirect ELISA. A 96-well plate is coated overnight with the antigen of interest, and excess antigen is then washed off. Supernatants containing antibodies of interest are then added to the wells and incubated for four hours, after which the wells are washed. As a positive control, known amounts of commercially available antibodies (from non-human species) that are specific for the antigen are added to separate wells containing the antigen. As a negative control, commercially available antibodies specific for an irrelevant antigen are added to separate wells containing the antigen of interest. An HRP-conjugated secondary antibody is then added to the wells, incubated for 30 minutes, and excess washed off. Tetramethylbenzidine (TMB) is then added to the plate, and the reaction allowed to proceed until color is observed in the positive-control wells. The reaction is then stopped with acid, and absorbance is measured. Specific well supernatants are deemed to contain antibodies that bind the antigen of interest if the absorbance readout they yield is significantly higher than that in the negative control.

[00455] Fluzone ELISA

[00456] Volunteers were administered the 2010/2011 season flu vaccine from Fluzone, which consists of 3 strains of inactivated virus, the A/California/7/2009, A/Perth/16/2009, B/Brisbane/60/2008 strains. Fluzone ELISAs were done to determine if monoclonal antibodies derived from vaccinated volunteers bind to the flu vaccine itself as an initial screen for expressed antibodies with binding activity. Fluzone vaccine was diluted 100x in a pH9 carbonate buffer and coated on Nunc Maxisorp plates either at RT for 1 hour or overnight at 4°C. Plates were then washed 5x with PBST (PBS w/ 0.05% Tween20) and blocked with PBS w/ 1% BSA for 1 hour at RT. 100ul of 100ng/ml of expressed flu antibodies were then added to wells at RT for 1 hour, before washing 5x with PBST and adding diluted HRP detection antibody (from Bethyl Labs human IgG ELISA quantitation kit) for 1 hour at RT. Plates were washed 5x with PBST and 50ul of TMB substrate were added. Color was allowed to develop for up to 30min before stopping the reaction with 50ul of stop solution. Plates were read on a SpectroMax M5 spectrophotometer at 450nm absorbance. Antibodies used in this assay are described in Table 19. Sequences of antibodies can be referred to in the master table, Table 18.

[00457] Surface plasmon resonance determination of flu antibody affinities

[00458] Binding of monoclonal antibodies (mAbs) to HA molecules was analyzed at 25°C using a ProteOn surface plasmon resonance biosensor (BioRad Labs). Expressed flu monoclonal antibodies derived from the plasmablasts of a flu-vaccinated donor (25 nM in pH

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4.5 acetate buffer) were coupled to a GLC sensor chip with amine coupling at a target density of 800 resonance units (RU) in the test flow cells using EDAC-NHS chemistry. Unreacted active ester groups were quenched with ethanolamine. Purified recombinant hemagglutinin H3 (HA(ΔTM)(H3N2/Perth/16/2009) and H1 (HA(ΔTM)(A/California/07/2009)(H1N1)) were purchased from Immune Technology Corp. (New York, NY) and were diluted to 100, 50, 25, 12.5, 6.25, nM, along with a blank buffer control were injected at a flow rate of 30 μL/min with 120 seconds contact time and 2000 seconds dissociation time. Binding kinetics and data analyses were performed with BioRad ProteON manager software. Affinity measurements were calculated using the bivalent analyte algorithm, as HA consisted of several repeating units. The accuracy of the fitted curves was verified by checking that the $\chi 2$ values of the goodness of each fit was below 10% of the peak binding value (Rmax). Antibodies used in this assay are described in Table 20. Sequences of antibodies can be referred to in the master table, Table 18.

[00459] RA antibody reactivities on RA antigen microarrays

[00460] To print the antigen microarrays, antigens were diluted in phosphate buffered saline to 0.2 mg/mL and attached to ArrayIt SuperEpoxy slides using an ArrayIt NanoPrint Protein LM210 system. Slides were marked with a hydrophobic marker pap pen and blocked overnight in PBS with 3% fetal bovine serum and 0.05% Tween 20 at 4°C, gently rocked at 30 rpm. Arrays were probed with 400uL of 40ug/mL monoclonal antibody for one hour at 4°C, gently rocked at 30 rpm. Arrays were then washed and incubated in Cy3 conjugated anti-human IgG/IgM secondary antibody diluted at 1:2500 for 45 minutes at 4°C, gently rocked at 30 rpm. Following another wash, slides were scanned using a GenePix 4300A Microarray Scanner. GenePix 7 software was used to find the median florescence intensity of each feature and background.

[00461] To analyze the data, background fluorescence intensities were subtracted from each feature and expressed as the median value of the four antigen features on each array. Median intensities were log transformed with a cut-off value of 10. These values were subjected to hierarchical clustering using Cluster software to arrange antigens based on similarities to each other. The relationships were displayed as a heatmap using Java TreeView software. Antibodies used in this assay are described in Table 21. Sequences of antibodies can be referred to in the master table, Table 18.

[00462] Anti-histone 2A ELISA

[00463] A direct ELISA was used for detection of antibodies to histone 2A. Microtiter plates (Nunc Maxisorp) were coated with 100 μl of recombinant H2A in carbonate buffer, at a concentration of 20 μg/ml, and incubated at 4°C overnight. After blocking in PBS containing 1%

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bovine serum albumin (BSA), RA patient-derived antibodies were used in a titration from 15ug/ml to 250 ug/ml in dilution buffer (PBS containing 0.1% BSA and 0.1% Tween-20), added to the plate in duplicate at 100 µl/well, and incubated for 2 hours at room temperature. The samples were then incubated for 1 hour at room temperature with a 1:5,000 dilution of a monoclonal, horseradish peroxidase–labeled goat anti-human antibody. The reaction was developed by application of 3,3',5,5'-tetramethylbenzidine substrate (TMB) (Sigma-Aldrich) for 15 minutes and stopped by addition of 50 µl of 2N H2SO4. Relative quantification of antibodies against was performed by optical densitometry at 450nm using known seropositive RA serum as a positive control. Antibodies used in this assay are described in Table 22. Sequences of antibodies can be referred to in the master table, Table 18.

[00464] Anti-CCP2 ELISA

[00465] Anti-CCP2 ELISA was performed according the manufacturer's instructions (Eurodiagnostica, Malmo, Sweden). Briefly, antibodies derived from RA patients are diluted to approximately 125 ug/ml in dilution buffer (PBS containing 0.1% BSA and 0.1% Tween-20), added to the pre-blocked commercial CCP2 ELISA plate at 100 µl/well, and incubated for 2 hours at room temperature. The samples were then incubated for 1 hour at room temperature with a 1:5,000 dilution of a monoclonal, horseradish peroxidase–labeled goat anti-human antibody. The reaction was developed by application of 3,3',5,5'-tetramethylbenzidine substrate (TMB) (Sigma-Aldrich) for 15 minutes and stopped by addition of 50 µl of 2N H2SO4. Relative quantification of antibodies against was performed by optical densitometry using standards provided by the vendor and known positive RA serum. Antibodies used in this assay are described in Table 22. Sequences of antibodies can be referred to in the master table, Table 18.

[00466] Anti-Rheumatoid Factor ELISA

[00467] For detection of antibodies to rheumatoid factor (RF), microtiter plates (Nunc Maxisorp) were coated with 10ug/ml of rabbit IgG in carbonate buffer, and incubated at 4°C overnight. After blocking in PBS containing 1% bovine serum albumin (BSA), RA patient derived antibodies were at 5ug/ml dilution buffer (PBS containing 0.1% BSA and 0.1% Tween-20), added to the plate in duplicate at 100 µl/well, and incubated for 2 hours at room temperature. The samples were then incubated for 1 hour at room temperature with a 1:5,000 dilution of a monoclonal, horseradish peroxidase–labeled goat anti-human antibody. The reaction was developed by application of 3,3',5,5'-tetramethylbenzidine substrate (TMB) (Sigma-Aldrich) for 15 minutes and stopped by addition of 50 µl of 2N H2SO4. Relative quantification of antibodies against was performed by optical densitometry at 450nm using two

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known RF+ control serum as a positive controls. Antibodies used in this assay are described in Table 23. Sequences of antibodies can be referred to in the master table, Table 18.

[00468] Immunohistochemistry of antibodies from lung adenocarcinoma patient on lung cancer tissue arrays

[00469] Two different types of tissue microarray slides were purchased from US Biomax. They were VLC 12 and BS0481. A variety of lung carcinomas tissue cores are included on the slides, including lung adenocarcinomas, and also normal lung tissue controls. Slides were heated in a citrate pH6.0 antigen retrieval buffer at 95-99oC for 40 minutes before allowing to cool to RT. Slides were pre-treated with 0.02% Triton-X and 0.6% H2O2 for 20 minutes. Slides were then blocked with 10% normal goat serum in TBST (TBS with 0.05% Tween20) for 2 hours before further blocking in 100ug/ml of F(ab) goat-anti-human IgG (Jackson Immunoresearch) overnight at 4oC. Slides then underwent an avidin/biotin block (Vector Laboratories) according to manufacturer's instructions. Slides were then incubated in 5 or 10ug/ml of expressed lung antibody for 1 hour at RT, washed 3x5 minutes with TBST, and then incubated with a biotinylated goat-anti-human secondary antibody for 20 minutes at RT. Slides were then washed 3x5 minutes with TBST and incubated with prepared Vectastain ABC reagent for 30 minutes at RT. Slides were then washed 3x5 minutes of TBST and stained with Vector Red (Vector Laboratories) and the color development tracked with a light microscope. After the appropriate staining time, the reaction was stopped by washing with distilled water and counterstained with hematoxylin. Slides were aqueous-mounted and photographed with a BX-51 microscope. Antibodies used in this assay are described in Table 24. Sequences of antibodies can be referred to in the master table, Table 18.

[00470] Flow cytometry determination of binding of antibodies expressed from lung adenocarcinoma patient to lung cancer cell lines

[00471] Lung cancer cell lines used were A549, H226, H441, H23, H1975, H1437, H2126, H1650 and H2009. HEK 293T cells were also used as a negative control. Cells were detached by incubating cells in PBS without Ca^{2+} and Mg^{2+} with 2mM EDTA for 1 hour at 37°C. This is to prevent damaging cell surface antigens that may be done with trypsinizing or any other proteolytic digest to detach cells. Cells were washed once with FACS buffer (HBSS with 2% FCS) before suspended in 50ul of FACS buffer and incubated with 10ug/ml, 3ug/ml, 1ug/ml, 0.2ug/ml of expressed lung antibodies to titrate the dose. The optimal concentration was found to be from the 0.2-1ug/ml range. Therefore, 1ug/ml, 0.5ug/ml, 0.25ug/ml of lung antibodies were used subsequently. Lung antibodies were incubated for 30 minutes at 4°C before washing with 2x 200ul of FACS buffer in 96-well plates. Anti-human IgG-PE was then added and

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incubated for 15 minutes at 4°C in the dark. Samples were then washed 2x200ul with FACS buffer and resuspended in 200ul of FACS buffer and analyzed on a BD LSR II or LSR Fortessa. Sytox blue was used as a live/dead staining. Antibodies used in this assay are described in Table 24. Sequences of antibodies can be referred to in the master table, Table 18.

[00472] Staph Flow Cytometry

[00473] Fixed *S. aureus* particles (Wood strain) were obtained from Invitrogen. Wood strain is a strain that expresses minimal protein A by some of the bacteria. The particles were suspended in 50ul of FACS buffer at $10x10^6$ cells/50ul, and incubated with a titration of 10ug/ml, 5ug/ml or 1ug/ml of expressed antibodies derived from staph individuals for 1 hour minutes at 4°C. Fixed staph particles were then washed with twice with FACS buffer before incubating for 15min in the dark at 4°C with anti-human IgG-FITC antibody. Particles were then washed with 1ml of FACS buffer and resuspended in 200ul of FACS buffer for analysis on a BD LSR II or LSR Fortessa. Antibodies used in this assay are described in Table 25. Sequences of antibodies can be referred to in the master table, Table 18.

[00474] **Functional screens**

[00475] Blocking antibody to receptor-ligand interactions

[00476] To screen antibodies for their ability to block ligand-receptor interactions (e.g. cytokine-receptor interactions), we transfect 293T cells with a vector encoding the appropriate receptor. These 293T cells are also stably transfected with an NF-κB-dependent luciferase reporter—such that these stably transfected 293T cells express luciferase when NF-κB is activated. We then culture the transfected 293T cells with the appropriate ligand in the presence or absence of the candidate antibodies. 293T cells are finally assayed for luciferase expression by measuring luciferase-dependent light emission. Interaction between the ligand and its receptor, e.g. interaction between IL-17A and IL-17R, activates NF-κB. Blocking antibodies prevent NF-κB signaling by ligand-receptor binding and therefore abrogate the expression of luciferase. In cases where the ligand-receptor interaction does not activate NF-κB, other transcriptional response elements are used to drive the promoter of the luciferase gene, e.g. AP-1 response elements, etc.

[00477] Screening antibodies for their ability to inhibit cytokine function or inhibit a functional assay

[00478] Functional assays can also be used to screen for anti-cytokine antibodies in patient sera or in cloned and expressed antibodies. In this approach, the expressed human antibodies are tested for their ability to inhibit cytokine or other immune mediator induction of a cellular response.

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[00479] Antibodies targeting bacteria, virally-infected cells, parasites or cancer cells

[00480] To screen for antibodies that kill or neutralize bacteria, virally-infected cells, parasites, or cancer cells, we culture the appropriate cell type either in the presence or absence of antibody, together with non-heat-inactivated serum (which contains complement factors). If the antibody is a neutralizing antibody, it will opsonize the bacteria, other microbes, or cancer cells and activate complement components that form the membrane attack complex (MAC), which induces cell death. To test neutralization, we run a fluorescent live/dead assay (Invitrogen), in which live and dead cells are stained with different fluorophores. Cells can be assayed for percentages of live and dead cells by using flow cytometry. The antibody that results in the highest percentage of dead cells will be a good candidate neutralizing antibody that will be further analyzed in *in vivo* screens.

[00481] Antibodies that neutralize viruses

[00482] To screen for antibodies that neutralize viruses, we perform a standard plaque-reduction assay or other *in vitro* cellular infection assays. Neutralizing antibodies are expected to decrease viral infection of cells. Candidate antibodies are then tested in an *in vivo* model.

[00483] Influenza microneutralization assay

[00484] Some expressed flu antibodies that showed binding activity to the Fluzone ELISA were sent to an external CRO, Virapur, LLC for microneutralization assays. In brief, two-fold dilutions of each antibody, starting from 100ug/ml, were mixed with an equal volume of approximately 100 $TCID_{50}$ infectious units of titered stock virus in quadruplicates in wells of a 96-well plate. Virus/antibody solutions were incubated for 2 hours and then the mixture was transferred to a 96-well plate containing 80% confluent MDCK cells. Cells, antibody and virus were incubated for an additional 2 hours at 37°C, after which virus was removed, monolayers rinsed and viral growth media added to each well. Wells were observed microscopically after 72 hours for the presence of influenza virus infection. Antibodies used in this assay are described in Table 26. Sequences of antibodies can be referred to in the master table, Table 18.

[00485] Staph Inhibition Assay

[00486] *S. aureus* were used when in log-phase growth. They were added to 96-well polypropylene plates, and anti-staph antibody from staph patients was added at 10ug/ml. Baby rabbit complement (Cedarlane) was added at manufacturer's recommended amount and mixed thoroughly. Plates were incubated at 37°C for 45 minutes before being diluted 1:10, 1:100 and 1:1000 and plated on 5% TSA blood agar plates and grown overnight. Bacterial CFUs were counted and tabulated the next day. Antibodies used in this assay are described in Table 27. Sequences of antibodies can be referred to in the master table, Table 18.

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[00487] Immunoprecipitation of staph antigens with antibodies derived from staph-infected patients

[00488] Staph protein lysate was made by lysing S. aureus using B-Per Bacterial Protein Extraction Reagent (Pierce) with 100ng/ml of lysostaphin for 30 minutes at RT along with 1x Halt protease inhibitor, and separated from the insoluble fraction by centrifuging at 15000rpm in a microcentrifuge. Lysate was precleaned by incubating with protein G Dynabeads for 1 hour at RT. 5ug of antibody derived from Staph patient was bound onto protein G Dynabeads by incubating for 1 hour at RT. Protein G-bound antibodies were then incubated with precleaned staph lysate overnight at 4°C. Beads were then washed 3x with PBST (PBS with 0.1% Tween20) and heated with 5x reducing lane sample buffer (Thermo Scientific) at 95°C for 5 minutes before running an SDS-PAGE on a 4-12% Criterion Bis-Tris gel. Proteins were visualized with RAPIDStain Reagent (Calbiochem). Antibodies used in this assay are described in Table 28. Sequences of antibodies can be referred to in the master table, Table 18.

[00489] Mass spectrometry identification of peptides

[00490] Stained protein bands of interest were cut out of the gels, immersed in 10 m*M* ammonium bicarbonate containing 10 m*M* DTT and 100 m*M* iodoacetamide, treated with 100% acetonitrile, and then digested overnight at 37°C with 0.1 mg trypsin (Sigma-Aldrich) in 10 m*M* ammonium acetate containing 10% acetonitrile. The trypsinized proteins were identified with LCMS by using the Agilent 1100 LC system and the Agilent XCT Ultra Ion Trap (Agilent Technologies, Santa Clara, CA) as previously described (Lopez-Avila V, Sharpe O, Robinson WH: Determination of ceruloplasmin in human serum by SEC-ICPMS. Anal Bioanal Chem 2006, 386:180-7.). LCMS data was scanned against the SwissProt or NCBInr databases by using the SpectrumMill software (Agilent) for the detection of peptides used to identify proteins. Antibodies used in this assay are described in Table 29.

Example 1: High-throughput sequencing of paired heavy- and light-chain sequences from individual B cells.

[00491] We developed a method of adding compound barcodes (sample-ID + plate-ID) to sequences in order to unambiguously identify which sequences originated from the same well in a plate. We used this approach to sequence paired heavy chain and light chain immunoglobulin genes from individual B cells. Individual B cells can be sorted by flow cytometry from blood, bulk peripheral blood mononuclear cells (PBMCs), bulk B cells, plasmablasts, plasma cells, memory B cells, or other B cell populations (Figure 1).

[00492] First, B cells were single-cell-sorted into 96-well PCR plates, leaving one column of wells empty, as a negative control. Oligonucleotides containing different sample-ID barcodes

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were added into different wells during reverse transcription (RT). After reverse transcribing the mRNA, the MMLV H⁻ reverse transcriptase switches templates and transcribes the oligonucleotide, incorporating it and the sample-ID barcode into the 3' end of the 1st strand cDNA (Fig 2a). All cDNAs (barcoded with a sample-ID) from one plate were then pooled and subjected to two rounds of PCR. During the PCR, 454 sequencing primers (first and second sequencing regions) and plate-ID barcodes were added onto the 5' and 3' ends of the amplicon by using PCR primers with 5'-flanking barcode sequences. Amplicons (amplicon regions) from different plates now have n different plate-IDs, and the compound barcode comprising a plate-ID and a sample-ID unambiguously identifies sequences as coming from a particular cell, allowing pairing up of sequenced heavy- and light-chain genes (Fig 2b-c).

[00493] Figure 3 describes the general methodology used and the associated sequences. Primers and adapter molecules are shown in Table 1. Sample-ID sequences are shown in Table 2. Plate-ID sequences are shown in Table 3. Cloning primers are shown in Table 4, with the 3' sequence of cloning forward primers shown in Table 5.

[00494] We obtained PCR products at the expected sizes: ~600bp for the kappa and lambda light chains, and ~700bp for the gamma heavy chain (Figure 4a). Next, we sent the material for Sanger sequencing. We obtained sequences that were identified by NCBI BLAST as kappa, lambda, and gamma chains (data not shown). Further investigation of the DNA chromatogram showed a mix of several peaks starting at the sample-ID barcodes, showing that we successfully added sample-ID barcodes to cDNA from cells in different wells during RT and successfully amplified them in two subsequent rounds of PCR (Figure 4b). The Sanger sequencing chain from the 3' end also showed a mix of several peaks starting at the VJ junction, owing to amplification of genes from different cells, which differed after the VJ junction as a result of insertions and deletions and random recombination of different V and J genes. Furthermore, when we performed PCR with cloning primers specific for the well A1 sample-ID, we obtained a single peak rather than a mix of several peaks, showing that we indeed can amplify sequences from a specific cell within the pool (Figure 4c-d).

Example 2: Gating scheme for single cell sorting of plasmablasts.

[00495] Plasmablasts were defined as $CD19^{+}CD20^{-}CD27^{+}CD38^{++}$ for this experiment. Figure 22 shows a gating scheme for flow cytometry sorting of single plasmablast cells into 96-well plates.

[00496] Single PBMCs were prepared and stained as described above. Cells were first gated on based on their FSC and SSC profile (data not shown). Live $CD19^{+}$ B cells were then gated on (left panel), and further narrowed down to $CD20^{-}$ B cells (2nd panel from left), and refined to

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$CD27^{+}CD38^{++}$ cells. From this, IgG^{+} plasmablasts were determined as IgA^{-} and IgM^{-}, as IgG^{+} plasmablasts do not express cell surface IgG. This population was single cell sorted into 96-well plates.

Example 3: Plasmablasts are present in subjects undergoing immunological challenge.

[00497] Plasmablasts generally represent about 0.15% of B cells in healthy donors, but can range from about 3.3%-16.4% in subjects undergoing a variety of immunological challenges including infections (e.g., S. aureus and C. diff infections), cancer associated with non-progression (e.g., metastatic melanoma and metastatic adenocarcinoma of the lung in which patients following an intervention (chemotherapy in the case of the lung adenocarcinoma patient and ipilimumab therapy in the case of the metastatic melanoma patient) became long-term non-progressors associated with an active B cell response), and vaccinations (e.g., influenza).

[00498] Figure 23 shows that plasmablasts were present in and obtainable from a range of subjects for high-throughput sequencing of the paired antibody repertoire and characterization of the active humoral response antibody repertoire. This demonstrates that the methodologies disclosed herein can be used to obtain evolutionary trees of heavy and light (H&L) chains and use this information to clone and/or express antibodies for, e.g.: a) novel antigen discovery; b) to inform vaccine design – for example, using the immune system to inform us as to which are the known and novel antigens are likely useful for opsonization and phagocytosis and/or killing/inhibition of a pathogen or target of interest and, optionally, to put that into vaccine design; c) making neutralizing monoclonal antibodies, e.g., from vaccines; d) making binding monoclonal antibodies; e) making antibodies against microbial pathogens; and f) making antibodies against cancers. Examples of these are described in more detail below.

Example 4: Disease activity in CCP+ RA is correlated with circulating plasmablasts.

[00499] Having shown that our method can be used for sequencing of immunoglobulin genes in identifiable pairs, we used our method to investigate the antibody repertoire of plasmablasts in CCP+ RA patients . We obtained blood samples from consented RA patients and stained for plasmablasts by flow cytometry (Figure 5a). Circulating plasmablasts were expressed as a percentage of total PBMCs. We found that CCP+ RA patients have significantly higher peripheral blood plasmablast percentages than CCP- RA patients (Figure 5b). Furthermore, plasmablast percentages in CCP+ patients, but not in CCP- patients, correlated with disease activity ($r = 0.35$ and $p = 0.028$) (Figure 5c).

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Example 5: Plasmablasts produced anti-CCP antibodies.

[00500] Although CCP+ patients have plasmablast percentages that correlate with disease activity, these patients could have an ongoing infection or other factors that elevated their circulating plasmablast percentages. To determine the specificity of circulating plasmablasts in CCP+ patients, RosetteSep-enriched B cells from patients were cultured in RPMI supplemented with 10% FBS. Other media supplements, such as anti-IgM, IL-6, BAFF etc., were not used so that plasmablasts would be the only cells secreting antibodies (with other B cells remaining inactivate). To confirm that only plasmablasts produce antibodies, we depleted some of the samples of plasmablasts (Figure 5d). B cells were then cultured for seven days before collecting the supernatant and running it on a Luminex peptide array. The array assays antibody reactivity to citrullinated peptides. Antibody reactivity was absent in supernatants of plasmablast-depleted samples compared to supernatants of mock-depleted B cells, suggesting that plasmablasts secrete significant amounts of anti-citrulline peptide autoantibodies (Figure 5e). Furthermore, when peptides with a mean fluorescent intensity (MFI) above 60 for each sample were counted, a strong correlation was found between circulating plasmablast percentages and the number of peptides to which antibodies reacted ($r = 0.90$ and $p = 0.0139$). An MFI of 60 was chosen as this was the threshold below which >99% of peptide reactivity falls in supernatants of plasmablast-depleted samples.

Example 6: 454 sequencing and analysis of sequences.

[00501] Plasmablasts from patients were single-cell sorted into 96-well plates as described above, and their RNA reverse transcribed and PCR amplified according to "Touchdown PCR" in materials and methods section such that they contained sample-ID (sample identification region) and plate-ID (plate identification region) barcodes, as described above. *See* Figure 3. Sequences of the cDNA were then obtained from a 454-sequencing facility (DNA Sequencing Center, Brigham Young University and 454 sequencing center, Roche).

[00502] Sequences were obtained from a first 454 sequencing run using the shotgun pipeline. Sequences of acceptable quality were obtained from a 2nd 454 sequencing run through a modified amplicon filter of the 454 GS FLX data analysis suite. The amplicon filter was modified to have <vfScanAllFlows> set to "false", and <vfBadFlowThreshold> changed to "6". Sequences from a third and fourth run were obtained using a standard 454 amplicon filter. Filter-passed sequences were then processed as described in "Assignment of sequences to wells" section in the materials and methods, and sequences in each well were individually assembled as described in "Assembly of sequences" in the materials and methods. Assembled sequences were then parsed with IMGT HighV-Quest to obtain identification of VDJ regions used.

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[00503] After sequence assembly and V(D)J usage identification, ClustalX was used to cluster sequences into clonal families (Figure 6). Alternatively, sequences can be assembled using both forward and reverse reads. In this case, subdivided forward sequences are first assembled as above. Forward and reverse sequences V(D)J usage are then identified using HighV-QUEST and forward and reverse sequences subset according to plate-ID and V(D)J usage, and forward and reverse sequences assembled using Newbler. Because immunoglobulin sequences are largely similar, assembly from a smaller subset of sequences avoids potential problems of sequences from different cells being incorrectly paired.

Example 7: Clustering of sequences into evolutionary trees

[00504] Peripheral blood mononuclear cells (PBMCs) were isolated from human subjects with the indicated diagnoses or after vaccination. Plasmablasts were single-cell sorted into individual wells in 96-well plates, creating single-cell samples in each well, the mRNA in each well was then reverse transcribed, and then well contents were pooled and subjected to two rounds of PCR to amplify the immunoglobulin heavy and light chain cDNAs. The reverse transcription added an identifying sample-ID to all cDNAs generated from each single sample, and the first round and second rounds of PCR added plate-IDs and then 454 Titanium Primers A and B to every amplicon, respectively, as described in "Touchdown PCR and non-touchdown PCR" in the materials and methods section. The general methodology is outlined in Figure 3. Pooled amplicons were sequenced with 454 sequencing technology, reads of acceptable quality obtained as described above. Reads were assigned to wells and assembled as described in "Assignment of reads to well" and "Assembly of sequences" sections in the materials and methods. V(D)J segments in assembled sequences were then identified using HighV-QUEST. Identified heavy and light chain sequences with shared compound barcodes can then paired simply by putting assembled sequences with matching compound barcodes together.

[00505] Amplicons from individual human subjects were clustered based on these V(D)J segments, and sequences expressing the same V(D)J segments were classified as being from the same clonal family (Figure 7). Each pie chart represents the percentage of clones derived from individual plasmablasts from an individual human subject expressing identical V(D)J gene segments (*i.e.*, percentage of clones in each clonal family). Human subjects included those with sepsis (2 subjects), rheumatoid arthritis (3 subjects), lung cancer (1 subject), and after vaccination for influenza (1 subject). These subjects were chosen to show that clonal families can be isolated from plasmablasts of subjects undergoing both acute (sepsis and flu vaccine) and chronic conditions (rheumatoid arthritis and lung cancer).

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[00506] The immunoglobulin heavy chain V(D)J sequences from the individual human subjects of Figure 7 were clustered using ClustalX and displayed using Treeview as unrooted radial trees (Figure 8). Each radial tree represents the heavy chain sequences derived from an individual human subject. For each radial tree, the terminal ends represent a unique sequence. The major branches represent clonal families, and the smaller branches represent clonal subfamilies that differ from one another by mutations that arose via junctional diversity (addition of P-nucleotides or N-nucleotides, or nucleotide deletion), somatic hypermutation and affinity maturation.

Example 8: Cloning, expression, and purification of antibodies.

[00507] All selected antibodies were cloned, expressed, and isolated as described above in the Materials and Methods section (*See* sections: Cloning of heavy and light chains into Lonza vectors; Expression of monoclonal antibodies in 293T; Anti-human IgG ELISA; and Protein A-IgG purification of expressed monoclonal antibodies). Purified antibodies were then used for further study, as discussed below.

Example 9: Characterization of antibodies from subjects following influenza vaccination.

[00508] Antibodies from humans administered an influenza vaccine were selected and isolated as described above. The antibodies selected for further characterization below are indicated in the appropriate sections.

Fluzone ELISA

[00509] Volunteers were administered the 2010/2011 season flu vaccine from Fluzone, which consists of 3 strains of inactivated virus, the A/California/7/2009, A/Perth/16/2009, B/Brisbane/60/2008 strains. Fluzone ELISAs were performed as described above to determine if monoclonal antibodies derived from vaccinated volunteers bind to the flu vaccine itself as an initial screen for expressed antibodies with binding activity. 14 of 31 antibodies bound to Fluzone ELISA (Figure 26) and a subset of these were subsequently selected and tested for binding activity to hemagglutinin using surface plasmon resonance. Antibodies characterized by Fluzone ELISA were: Flu14 - Flu23, Flu25 - Flu27, Flu29, Flu30, Flu34, Flu35, Flu37, Flu39 - Flu41, Flu43 - Flu46. S1 and S2 were used as negative controls.

Surface plasmon resonance determination of flu antibody affinities

[00510] Binding of monoclonal antibodies (mAbs) to HA molecules was analyzed at 25°C using a ProteOn surface plasmon resonance biosensor (BioRad Labs) as described above. Of the 14 antibodies that bound to Fluzone ELISA, 10 bound to H3, and 1 bound to H1, while 3 did not

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bind (Figure 27). One of the non-binders, the H1 binder, and 4 other randomly chosen H3 binders were selected and sent to a contract research organization (CRO) to test neutralization activity in a microneutralization assay. Antibodies characterized by SPR were: Flu14 - Flu22, Flu26, Flu29, Flu34, Flu35, Flu46.

Influenza microneutralization assay

[00511] Some of the expressed flu antibodies that showed binding activity in the Fluzone ELISA were sent to an external CRO, Virapur LLC, for microneutralization assays as described above. The results of the assays showed that the antibody that bound to H1 in previous assays neutralized H1, while the antibodies that bound to H3 in previous assays neutralized H3. The non-binder did not neutralize influenza virus (Figure 28). Antibodies characterized by microneutralization assay were Flu15, Flu16, Flu18, Flu19, Flu20, Flu21.

CDR Variation

[00512] Flu antibodies were obtained as described above. Figure 25 shows a partial dendrogram blown-up for clarity (a). Clonal families are clearly visible and the shaded clonal family has the assigned V(D)J as shown in the grey box. Amino acid sequence across the CDRs (boxed region) for the heavy and light chains are shown in Figure 25 (b) and (c) respectively, showing some residue differences between the chains.

[00513] These above results demonstrate that evolutionary trees can be obtained using the compositions and methods described herein. Fully human monoclonal antibodies can be isolated from activated B cells, such as plasmablasts, of subjects undergoing acute conditions using the compositions and methods described herein. These fully human monoclonal antibodies can also be neutralizing antibodies using the compositions and methods described herein. The results also demonstrate that the compositions and methods disclosed herein can be used to isolate mAbs targeted against foreign antigens.

Example 10: Characterization of antibodies from subjects with RA.

[00514] Antibodies from humans suffering from rheumatoid arthritis (RA) were selected and isolated as described above. The antibodies selected for further characterization below are indicated in the appropriate sections.

RA antibody reactivities on RA antigen microarrays

[00515] Antibodies derived from RA patients were probed on an RA antigen array and florescence scanned with a GenePix machine as described in "RA antibody reactivities on RA antigen arrays" section in materials and methods. The identified relationships were displayed as

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a heatmap using Java TreeView software (Figure 37). The antibodies characterized by this assay were: RA1, RA2, RA3, RA4, RA8 - RA13, RA16, RA19, RA22 and RA23. Flu14 and Flu26 were used as negative controls.

Anti-histone 2A ELISA

[00516] For detection of antibodies to H2A, a direct ELISA was performed as described in "Anti-histone 2A ELISA" in materials and methods section. Figure 35a shows the absorbance values detected for each antibody tested. The antibodies characterized in Figure 35a and in anti-CCP2 ELISA below were: RA1, RA2, RA4-RA16, RA19, RA23-RA24. Figure 36 shows the selected antibodies (RA1, RA2, RA8, RA9) on another independent ELISA using 30ug/ml of antibodies.

Anti-CCP2 ELISA

[00517] Anti-CCP2 ELISA was performed as described in "Anti-CCP2 ELISA" in materials and methods section. Figure 35b shows the absorbance values detected for each antibody tested.

Anti-Rheumatoid Factor ELISA

[00518] Antibodies derived from RA patients were used as the primary antibody in a direct ELISA and anti-human IgG-HRP was used as the secondary antibody, and visualized with TMB substrate. For detection of antibodies to rheumatoid factor (RF), Anti-RF ELISA was performed as described in "anti-rheumatoid factor" ELISA in the materials and methods section. Figure 34 shows that antibodies RA2 and RA3 showed reactivity. Antibodies characterized here were: RA1-RA6, RA8-RA12, RA14.

[00519] These above results demonstrate that antibodies can be isolated from activated B cells, such as plasmablasts, of subjects undergoing chronic conditions using the compositions and methods described herein. The results also demonstrate that the compositions and methods disclosed herein can be used to isolate mAbs targeted against self antigens.

Example 11: Characterization of antibodies from subjects with lung cancer.

[00520] Antibodies from a long-term non-progressor human suffering from metastatic lung adenocarcinoma were selected and isolated as described above. This human developed metastatic lung adenocarcinoma and was expected to succumb to cancer, however following chemotherapy this patient entered a state of long-term non-progression for over 4 years that was associated with plasmablasts constituting 3.1% of all peripheral blood B cells. The elevated peripheral blood plasmablast levels in this patient indicated that an ongoing immune response could be contributing to her long-term non-progression. The following antibodies were selected

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for further characterization below: LC1, LC5-LC7, LC9-LC18. Flu16 was used as the negative control.

Immunohistochemistry of antibodies from lung adenocarcinoma patient on lung cancer tissue arrays

[00521] Immunohistochemistry using two different types of tissue microarray slides was performed as described in "Immunohistochemistry of antibodies from lung adenocarcinoma patient on lung cancer tissue arrays" in the materials and methods section. Our results demonstrated one of the expressed antibody bound to lung adenocarcinoma (Figure 32).

Flow cytometry determination of binding of antibodies expressed from lung adenocarcinoma patient to lung adenocarcinoma cell lines

[00522] Binding of antibodies to various lung cancer cell lines was performed as described in "Flow cytometry determination of binding of antibodies expressed from lung adenocarcinoma patient to lung cancer cell lines" in the materials and methods section. Our results showed that one antibody bound to lung adenocarcinoma cell lines and may be specific for lung adenocarcinomas (Figure 33).

[00523] These above results demonstrate that antibodies can be isolated from activated B cells, such as plasmablasts, of subjects undergoing chronic conditions such as cancer using the compositions and methods described herein. The results also demonstrate that the compositions and methods disclosed herein can be used to isolate mAbs targeted against self antigens.

Example 12: Characterization of antibodies from subjects with *Staphylococcus aureus* infection.

[00524] Humans with *S. aureus* infections, including a human with chronic *S. aureus* osteomyelitis with immune-mediated control of the infection in the absence of antibiotics, were used as sources for peripheral blood from which peripheral blood plasmablasts were stained and sorted. cDNA processing with barcoding, 454 sequencing, and bioinformatics analysis generated evolutionary trees of antibody repertoires in humans mounting effective immune responses against *Staph. aureus*. Antibodies from humans mounting effective immune responses against *S. aureus* infection were selected and isolated as described above. The antibodies selected for further characterization below are indicated in the appropriate sections.

Staph Flow Cytometry

[00525] Anti-staph antibodies were used to stain fixed *S. aureus* as described in "Staph Flow Cytometry" in the materials and methods. Our results showed that antibodies S6 and S11 bind to the surface of *S. aureus* and may be candidates for opsonization, resulting in phagocytosis and

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killing/inhibition of *S. aureus* (Figure 29). The antibodies characterized in this assay were: S1 - S4, S6 - S13, with F26 as a negative control.

Staph Inhibition Assay

[00526] *S. aureus* in log-phase growth were combined with anti-staph antibody to determined the inhibitory activity of the antibodies as described in "Staph Inhibition Assay" in the materials and methods. Our results demonstrate that several of the antibodies cloned and expressed exhibited potent killing/inhibition activity on *S. aureus* (Figure 30). Antibodies characterized by this assay were S6 and S9, with LC1 as the negative control.

Immunoprecipitation of staph antigens with antibodies derived from staph-infected patients

[00527] Antibodies were used to immunoprecipitate various candidate staph antigens as described in "Immunoprecipitation of staph antigens with antibodies derived from staph-infected patients" of the materials and methods. Immunoprecipitated proteins were then identified with mass spectrometry as described below. Antibodies characterized by this assay were S1 - S13.

Mass spectrometry identification of peptides

[00528] Stained protein bands of interest were selected and subjected to mass spectrometry as described in "Mass spectrometry identification of peptides" of the materials and methods. The results identified either phenol-soluble modulin alpha 1 peptide or delta-hemolysin as the likely binding target for antibody S4. This demonstrates that the methods disclosed herein can be used to perform novel antigen discovery (Figure 31). Antibody characterized by this assay was S4.

[00529] These above results demonstrate that antibodies can be isolated from activated B cells, such as plasmablasts of subjects undergoing acute conditions such as a bacterial infection using the compositions and methods described herein. The results also demonstrate that the compositions and methods disclosed herein can be used to isolate mAbs targeted against foreign antigens and to determine the identity of antigens bound by selected antibodies.

Example 13: Blasting Cells and Plasmablast characterization.

[00530] Immunoglobulin sequences from B cells that are activated by an ongoing immune response can be used to produce an evolutionary tree of an ongoing immune response, as described above. This evolutionary tree is typically characterized by multiple clonal families representing activated B cells from multiple lines of descent. Sequences from naive B cells will not generally be able to be used to produce such an evolutionary tree, as they have not been activated and therefore provide little to no information on the active, ongoing immune response.

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Activated B cells first become blasting cells, which are activated and are larger in size. These blasting cells then go on to become either memory B cells or plasma cells. In humans, although memory B cells and plasma cells result from an immune response, they join large pools of memory B cells and plasma cells that have resulted from responses to previous immunological insults, making it difficult to distinguish memory B cells and plasma cells against recent or previous immune responses. Therefore, in humans, blasting cells are a preferred candidate for sequencing to obtain evolutionary trees of an ongoing immune response. In research animals bred in controlled conditions (e.g., mice) however, blasting B cells, memory B cells, and plasma cells are all candidates for sequencing to obtain evolutionary trees as they are bred in a clean environment, making it possible for the majority of memory B cells and plasma cells after a rigorous immune response to be against the insult, especially after booster shots, as they should not have large memory or plasma cell populations that have seen any major immunological challenge before.

[00531] Similarly for T cells, in humans, the preferred cells to sequence to obtain an evolutionary tree of an ongoing immune response will be blasting T cells. For mice, activated, blasting, and memory T cells are all preferred candidates to sequence to obtain an evolutionary tree.

[00532] Blasting B cells are known to be larger than typical B cells. The size of a small lymphocyte, of which a resting B cell is one, is typically between 6-8 μm in size. Blasting lymphocytes (T and B cells) are typically between 8-14 μm in size. (See Figure 41, also Tzur et al, PLoS ONE, 6(1): e16053. doi:10.1371/journal.pone.0016053, 2011;Teague et al, Cytometry, 14:7 2005). Plasmablasts can have the following expression pattern: $CD19^{low/+}$, $CD20^{low/-}$, $CD27^{+}$ and $CD38^{high}$. Although use of all of these markers results in the purest population for single cell sorting, not all of the above markers need to be used to isolate plasmablasts.

[00533] As exemplified in Figure 39, plasmablasts can be gated on by using an FSC^{hi} for larger cells, resulting in a 37% pure plasmablast population. Gating on $FSC^{hi}CD19^{hi}$ cells gives 72% plasmablast purity. Gating on FSC^{hi} and $CD27^{+}$, $CD38^{hi}$, or $CD20^{-}$ gives 44, 80, and 71 percent plasmablast purity, respectively. Combination of any of these markers or other markers found to be able to distinguish plasmablasts from other B cells can be used to increase the purity of sorted plasmablasts, however any one of these markers alone can distinguish plasmablasts from other B cells, albeit with a lower purity.

Example 14: Alternative platform for sequencing and analysis.

[00534] Heavy chain reads from a single plate, plate 44, was prepared for PacBio sequencing run using the methods described in "Touchdown PCR" in the materials and methods to amplify

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gamma heavy chain cDNA. 48 2nd PCRs were done to obtain sufficient DNA for PacBio run. Pooling and cleanup of DNA was done as described in "Preparing for PacBio sequencing run". DNA was sent to PacBio for prep and sequencing. CCS reads were obtained from PacBio and assigned to wells and assembled according to "Assignment of sequences to wells" and "Assembly of sequences" in the materials and methods. Results of the assignment is in Figure 38. This shows that our methods and compositions are not platform-specific for high-throughput sequencing.

Example 15: Sequencing and analysis on 454 XL+ runs

[00535] Sequencing can be adapted to 454 XL+ runs by following the method described in "Preparing for 454 XL+ sequencing run". This needs to be done as 454 XL+ runs currently only support Lib-L chemistry, while our 454 XLR70 runs utilize the Lib-A chemistry. This can also generally be adapted to situations where Lib-L chemistry is preferred to the typical Lib-A chemistry for amplicon sequencing on XLR70 runs. Reads from 454 XL+ runs can still be assigned to wells and assembled following the methods described in "Assignment of sequences to wells" and "Assembly of sequences". Reads from XLR70 and XL+ runs after 454 filtering can be used in identical fashion, i.e. downstream selection of antibodies for cloning and expression and assaying of antibody functional properties can still proceed as per Figures 6 and 9.

Example 16: Cloning of paired immunoglobulin genes.

[00536] Assuming that each clonal family recognizes the same epitope, and that sequence variance within each family is due to somatic hypermutation, we can first clone and express the most frequent clone of each clonal family for screening of antibodies that bind the antigen of interest (Figure 6). We use the most frequent clone because, during affinity maturation and selection in the germinal center, centrocytes that bind antigen with the highest affinity out compete other centrocytes for survival factors. Therefore, we expect the highest frequency clone to also have the highest binding affinity. Once a clone has been identified as an antibody capable of binding antigen, representative paired immunoglobulin sequences from the entire clonal family are then cloned, expressed, and screened for being neutralizing antibodies (Figure 6). This process may involve cloning and expression of sequences representing multiple sub-clones within the clonal family, or encoding antibodies of different isotypes within the clonal family, to enable direct testing and comparison of the binding and functional properties of specific clones representing the spectrum of antibodies contained within the clonal family. The specific clone exhibiting the desired binding and functional properties are then selected for further characterization and consideration for development as a therapeutic human antibody.

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[00537] An alternative approach to selecting candidates for cloning from a family (or any other set of antibodies of interest) is to build a phylogenetic tree for the antibodies (rooted at the germline sequence in the case of a clonal family). Leaf nodes in such a tree correspond to the antibody family members. Candidates for cloning are then selected by descending from the top of the tree, always choosing the branch with the largest number of leaf nodes underneath (choosing randomly in case of a tie), then at the last node above the leaves choosing the leaf with the largest number of mutations, or choosing randomly in the event of a tie. Additional candidates could then be selected, if desired, by repeatedly selecting candidates until the desired number is achieved, as follows. For every node in the tree, if none of the leaves that are descendants of the node have been selected, count the number of leaves that are descendants. For the node with the largest such count (choosing randomly in case of a tie), descend, always choosing the branch with the largest number of descendant leaf nodes (choosing randomly in case of a tie between branches). Then, at the last node above the leaves, choose the leaf with the largest number of mutations or choose randomly in case of a tie.

[00538] Yet another approach to selecting candidates from a family of antibodies (or any other set of antibodies of interest) is to list the antibodies by descending number of non-silent mutations relative to germline and select from the list in order, thereby choosing the antibodies that are most evolved.

Example 17: Permanent transfection and expression of candidate human antibodies

[00539] Desired clones are selectively amplified from a pooled plate of sequences by using cloning primers specific to a given sample-ID; these primers also incorporate different 5' and 3' restriction sites into the clone. The restriction sites are then used for inserting the clone into vectors. Because the amplified clones may contain only a partial constant region sequence, vectors already contain either the kappa, lambda or gamma constant regions with the appropriate restriction sites needed for inserting the amplified clones in the open reading frame. Multiple restriction sites are engineered into the vector, because clones have variable sequences, to avoid the potential problem of the restriction site existing also in the clone itself, which would then also be cut by the restriction enzyme. This allows as many clones to be inserted as possible. Vectors used are either two separate vectors with different mammalian selectable markers (modified Invitrogen pcDNA3.3 or pOptivec vectors that contain constant-region gene with engineered restriction sites) or a dual-expression vector containing both the genes (Lonza GS system; pEE6.4 and pEE12.4). See Tables 16 and 17 respectively for sequences of the constant region inserts. Selection markers are amplifiable, such as dihydrofolate reductase (DHFR) in pOptivec or glutamine synthetase (GS) in Lonza GS system, to allow for gene amplification and

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efficient production of antibodies for further screening purposes requiring large amounts of antibody (e.g. an *in vivo* screen). Mammalian cells are either transfected using a double transfection, with a light chain in one and the heavy chain in the other vector (modified pOptivec and pcDNA3.3), or a dual-expression vector (Lonza GS system) containing both genes.

[00540] *Modified Invitrogen Vectors.* Vectors are two separate vectors with different mammalian selectable markers and engineered restriction sites). pcDNA3.3 has a Neomycin resistance gene as a selectable marker, and pOptivec has a DHFR-selectable marker. CHO DHFR - cells are co-transfected with modified pcDNA3.3 and pOptivec under selection from Geneticin. Only DHFR - cells transfected with pOptivec, which contains a copy of DHFR, will survive, and the Neomycin resistance gene in pcDNA3.3 confers resistance to Geneticin, This allows for selection of cells that are successfully transfected with both vectors (containing the light chain in one vector and heavy chain in the other vector), and therefore will produce functional immunoglobulins.

[00541] *Lonza GS System.* Lonza GS system utilizes the vectors pEE12.4 and pEE6.4. Vector pEE12.4 contains the GS gene as the amplifiable selection marker, and pEE6.4 is a supplementary vector. The light chain will be cloned into one of the vectors and the heavy chain into the other vector. Thereafter, both vectors are cut with restriction enzymes and ligated together to form a single vector that can express both heavy and light chain genes on separate promoters. Therefore, is a dual-expression vector system, , allowing for expression of both genes from a single vector.CHO cells are transfected with the dual-expression vector under the selection of methionine sulfoximine. Transfected cells are thus selected for.

[00542] *Gene amplification*

[00543] Both dihydrofolate reductase (DHFR) and GS are amplifiable selection markers. Under selection pressure from increasing amounts of methotrexate and methionine sulfoximine respective, transfected cell lines that have duplicated genomic regions containing the DHFR and GS genes will survive because they are more resistant to the selection reagents. Genes near the selection markers, such as the inserted heavy- and light-chain immunoglobulin genes are also amplified, resulting in higher gene copies and greater production rates of immunoglobulins. Clones producing antibodies that have been found to have neutralizing properties in the *in vitro screens* (see below) are amplified so that more antibodies can be obtained for subsequent *in vivo* studies.

Example 18: Identifying the specificity of the expressed human antibodies

[00544] Antibody screening occurs in two stages. We are utilizing a novel 'selective screening' process, in which we first select appropriate clonal families to be used in the screen

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for neutralizing antibodies. We screen the most frequent 1-3 clones of each clonal family for its ability to bind to the antigen. Our screen typically takes the format of an indirect ELISA, although flow cytometry may be used to identify cell-binding antibodies. This comprises first binding the appropriate antigen to an ELISA plate, then incubating it with supernatants containing the expressed antibodies. Antibodies that bind to the antigen are detected by a specific secondary antibody.

[00545] Once binding antibodies have been identified, the entire clonal family of that clone is cloned and expressed in the screening stage of the 'selection screen'. Although all antibodies in a clonal family are expected to bind to the same epitope, they may differ slightly in avidity of antigen binding and in their positioning over the antigen, differences that may affect the binding properties and/or neutralization ability of the antibodies; thus, in most cases, several different antibodies (possessing minor differences in their CDR3 regions) are expressed and screened for binding and neutralizing properties.

[00546] For neutralizing antibodies that target a specific ligand/receptor pair, 293T cells are first stably transfected with a signaling pathway reporter construct, such as a plasmid containing the luciferase gene linked to NF-kB transcription response elements. Activation of NF-kB in the transfected cell induces the expression of luciferase, whose levels can be determined in a luciferase assay. This measures NF-kB signaling activated by ligand-receptor binding. NF-kB is the signaling element of choice because most signaling events activate NF-kB. For assaying other signaling pathways, the luciferase gene promoter region contains the appropriate transcriptional binding site, such as that for AP-1, for example. Next, the 293T cells are transfected with the target receptor. 293T cells are then incubated with the ligand and binding antibodies of interest in 96-well plates. After 24 or 48 hours, a luciferase assay is done to determine expression of luciferase gene. Wells with neutralizing antibodies have minimal to no luciferase expression. Results are verified by Western blotting for phosphorylated signaling proteins in the NF-kB signaling pathway. A neutralizing antibody prevents ligand-receptor signaling; and consequently abrogates phosphorylation of signaling proteins.

[00547] For antigens present on live cells, such as cancer antigens and bacterial antigens, the *in vitro* neutralization assay takes the form of an assay that detects live/dead cells, and can be done in a high-throughput format. Cancer cells or bacteria are incubated in 96-well plates with a candidate antibody. A stain that distinguishes live from dead cells and is compatible with flow cytometry can then be applied to each well. Live and dead cells are stained with different fluorophores and screened using flow to give percentages of live and dead cells. Antibodies that pass the *in vitro* screen will then be screened *in vivo* for their neutralizing activity.

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[00548] Virus *in vitro* neutralization assay may be conducted using a standard plaque neutralization assay. By doing plaque neutralization assays in 96-well plates, each well can be imaged using a microscope and plaque counting can be automated with image-analysis software. Neutralizing antibodies reduce plaque formation. These antibodies are then further screened *in vivo* for neutralizing activity.

[00549] See example 9 section "Fluzone ELISA", example 11 section "Flow cytometry ..." and example 12 section "Staph Flow Cytometry" for successful assays of binding activity using ELISA (example 7) and flow cytometry (examples 9 and 10). See example 9 section "Influenza microneutralization assay" for a successful assay of antibodies with neutralizing activity.

Example 19: Sequencing of B cells with more than one cell per well

[00550] Individual samples having multiple B cells are separately reverse transcribed in containers. Reverse transcription adds a sample-ID and a 5' universal primer region to all 1st strand cDNA. cDNA from all containers of a set of containers are pooled and undergo 2 rounds of PCR. Steps are as described in "Touchdown PCR and non-touchdown PCR" , "Preparing for 454 XLR70 sequencing run" in the materials and methods. Sequences for primers are also shown in Fig. 9. Note that regardless of which gene is amplified, the forward primers remain constant (b). After RT and 2 PCRs, amplicons from all container sets are pooled and 454-sequenced. Assignment to wells and assembly of sequences follow the protocol as described in "Assignment of sequences to wells" and "assembly of sequences" in the materials and methods. The combination of plate-IDs and sample-IDs allows for identification of sequences that originate from the same sample.

[00551] Even though there are multiple cells in a well, we can pair individual heavy chains with light chains. The heavy chains from B cells derived from a common progenitor will be clonally related, as will the light chains. Therefore, we can associate a heavy chain clonal family to a light chain clonal family by observing the correlation across wells. Once an association is established between the heavy chains of a clonal family and the light chains of a clonal family, pairs are assigned in each well by selecting the heavy chain that is a member of the heavy chain clonal family and a light chain that is a member of the light chain clonal family. The selection of the pair is unambiguous when only a single instance of the heavy chain family and a single instance of the light chain family is present in a well. After determining which heavy and light chains are associated with one another, evolutionary trees may be drawn and antibodies selected for downstream characterization of their functional properties.

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Example 20: Sequencing of B cells with one or more cells per well

[00552] Samples could be sorted with one B cell per well in some plates, and multiple B cells per well in other plates, yet heavy and light chains could still be paired for those wells having more than one B cell. We examined the sequences generated from the flu vaccination patient of example 9 above, where some wells had more than one distinct heavy chain sequence assembly or more than one distinct light chain sequence assembly observed. RT, PCR, sequencing and assignment to wells and assembly of sequences followed the protocol in example 9 above. For determining which heavy and light chains were associated with each other, heavy chains were assigned to clonal families by grouping all heavy chains with the same V and J gene usage, and the same number of nucleotides between the end of the V gene segment and the beginning of the J gene segment. Light chains were assigned to clonal families by grouping all light chains with the same V and J gene usage, and the same number of nucleotides between the end of the V gene segment and the beginning of the J gene segment. Pairing relationships between heavy and light chains were first assigned for wells with exactly one heavy chain and one light chain, based on them sharing a well (i.e. having the same compound barcodes). Then, a score was computed for each possible pairing of a heavy chain clonal family with a light chain clonal family. The score was determined by counting the number of times a member of the heavy chain family and the light chain family share a well. Then, each heavy chain family was associated with the light chain family with which the highest score was achieved, or the heavy chain family was not associated to a light chain family if the highest score was achieved with more than one light chain family. Individual heavy and light chains were then paired by starting with the overall highest-scoring heavy chain family, and proceeding well by well through the family assigning pairs, then continuing on with the next heavy chain family. For a given heavy chain family, for each well, if there was a single heavy chain within the well that was a member of the heavy chain family, then the light chain from that well which belonged to the heavy chain family's associated light chain family was assigned to be the heavy chain's pair. If more than one such light chain existed, no pairing was assigned. This process of associating heavy chains with light chains was continued until all families and all chains within those families had been considered. If, for a given heavy or light chain, the process resulted in more than one candidate for pairing, both heavy and light chain were discarded. Evolutionary trees were generated from the paired chains, and antibodies selected for downstream characterization of their functional properties. A portion of the evolutionary tree is shown in Figure 25A.

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Example 21: Use of sorted plasmablasts to generate human monoclonal antibodies

[00553] From a subject with a recent or current condition resulting in acute, subacute, or ongoing generation of circulating plasmablast, flow cytometry is performed on peripheral blood (either whole blood or peripheral blood mononuclear cells (PBMCs)) to identify the plasmablast population. This population of B cells is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. During RT, well-specific sample-ID adaptor oligonucleotides are added to the reaction. These adaptors have well-specific barcode sequences (sample-IDs) that can identify sequences as originating from different wells. Utilizing the 3' tailing and template-switching activity of MMLV H- reverse transcriptases, sample-IDs are added to the 3' end of the 1st strand cDNA. cDNA from each plate are pooled together. During the first round of PCR, a plate-specific FW long primer1 adds the plate-ID to the 5' end of the amplicons. Thus, FW long primer1 with different plate-IDs are added to different plates giving each PCR product an identifying barcode sequence. Gene specific reverse primers are used to amplify the kappa, lambda and gamma chains, they are kappa GSP1, lambda GSP1 and gamma GSP1 respectively. These primers bind to the constant region of the immunoglobulin genes. Products from the first round of PCR are diluted and used for a second nested PCR. FW primer2 is used as the forward primer and reverse primers kappa, lambda, and gamma GSP long primers are used to amplify their respective amplicons. Notably, the GPS long primer2 for each plate adds a common plate ID to the 3' end of each amplicon for each plate, thus each will end up having two plate-IDs and a sample-ID barcode. Further details for RT, 1st and 2nd PCR are found in "non-touchdown PCR" in the materials and methods. Multiple plates are then pooled according to the method detailed in "preparing for 454 XLR70 sequencing run" and subjected to high-throughput 454 DNA sequencing and individual sequences identified with their barcodes serving as identifiers of which heavy and/or light chain is obtained from each well thus providing a guide for matching individual variable heavy and light chains derived from the same initial cell, according to the methods detailed in "Assignment of sequences to wells" and "Assembly of sequences" in the materials and methods. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity (see Figures 6-8).

[00554] Candidate heavy and light chain genes from particular cells of origin are then cloned and expressed for screening of desired properties as in example 8. Once stably or transiently transfected, the expression of the paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants

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containing secreted antibodies are then screened for desired properties including but not limited to antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 demonstrate how this was done using the compositions and methods herein to obtain human monoclonal antibodies against hemagglutinin from single cell sorted plasmablasts from an influenza-vaccinated human.

Example 22: Use of sorted unbiased or antigen-specific memory B cells to generate human monoclonal antibodies

[00555] From a subject with a documented or suspected exposure to an antigen of interest, FACS is performed on peripheral blood (either whole blood or isolate peripheral blood mononuclear cells, PBMCs) to identify the memory B cell population (defined as $CD19^+CD20^+CD27^+$). Additionally, memory B cells specific against antigens of interest may also be sorted by staining peripheral blood or PBMCs with memory B cell surface markers and with fluorophore-conjugated antigen(s) ($CD19^+CD20^+CD27^+antigen^+$). This population of cells is then sorted by FACS either as single cells or multiple cells into wells. The process described in detail in Example 21 is repeated to barcode and obtain sequences from 454 sequencing and assign sequences to wells and assemble sequences. HighV-QUEST is used to identify VDJ gene usage, and a few members of each clonal family on an evolutionary tree selected for cloning and expression as in example 8. Cloning and expression is done as detailed in example 8. Once transfected, the expression of the entire paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing antibodies will be screened for antigen specificity against the target antigen(s) of interest as well as functionality by appropriate functional assays. Figures 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining human monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 23: Use of sorted unbiased or antigen-specific total B cells to generate human monoclonal antibodies

[00556] From a subject with or without documented or suspected prior exposure an antigen of interest, FACS is performed on peripheral blood (either whole blood or isolate peripheral blood mononuclear cells, PBMCs) to identify the $CD19^+$ B cell population. This population of cells is then sorted by FACS either as single cells or multiple cells into wells. The process described in detail in Example 21 is repeated. The process described in detail in Example 21 is repeated to barcode and obtain sequences from 454 sequencing and assign sequences to wells and assemble

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sequences. HighV-QUEST is used to identify VDJ gene usage, and a few members of each clonal family on an evolutionary tree selected for cloning and expression as in example 8. Once transfected, the expression of paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing expressed antibodies will be screened for antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figures 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining human monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 24: Use of plasma cells to generate human monoclonal antibodies

[00557] From a subject with or without documented or suspected prior exposure an antigen of interest, FACS is performed on peripheral blood (either whole blood or isolate peripheral blood mononuclear cells, PBMCs) or bone marrow cells to identify the $CD138^{+}$ plasma cell population. This population of cells is then sorted by FACS either as single cells or multiple cells into wells. The process described in detail in Example 21 is repeated to barcode and obtain sequences from 454 sequencing and assign sequences to wells and assemble sequences. HighV-QUEST is used to identify VDJ gene usage, and a few members of each clonal family on an evolutionary tree selected for cloning and expression. Once transfected, the expression of paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing expressed antibodies will be screened for antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining human monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 25: Use of blasting B cells to generate human monoclonal antibodies

[00558] From a subject with or without documented or suspected prior exposure an antigen of interest, FACS is performed on peripheral blood (either whole blood or isolate peripheral blood mononuclear cells, PBMCs) to identify the FSC^{hi} blasting B cell population. Blasting cells are activated B cells, and therefore are cells that have responded against the antigen and are actively proliferating. These B cells consist of clonal families and their paired heavy and light chains can be used to obtain evolutionary trees. Other markers of B cell activation, such as $CD69^{hi}$ and $CD44^{hi}$ may also be used in conjunction. Additionally DNA content, which may be stained using cell permeable DNA stains such as SYTO Blue (Invitrogen), to determine cells that are

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activated, proliferating and in the cell cycle may also be used in conjunction to delineate blasting B cells. This population of cells is then sorted by FACS either as single cells or multiple cells into wells. The process described in detail in Example 21 is repeated to barcode and obtain sequences from 454 sequencing and assign sequences to wells and assemble sequences. HighV-QUEST is used to identify VDJ gene usage, and a few members of each clonal family on an evolutionary tree selected for cloning and expression. Once transfected, the expression of paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing expressed antibodies will be screened for antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figures 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining human monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 26: Use of murine B cells to generate monoclonal antibodies

[00559] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine B cells. Murine B cells may be obtained from blood, from splenocytes or from the bone marrow. Flow cytometry is performed to obtain $CD19^+$ or $B220^+$ B cells. This population of B cells is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR is performed as detailed in "non-touchdown PCR" in the materials and methods. Mouse gene-specific primers are found in Table 11 and other primers used for RT and PCR are found in Table 1. Multiple plates are then pooled according to the method detailed in "preparing for 454 XLR70 sequencing run" and subjected to high-throughput 454 DNA sequencing and individual sequences identified with their barcodes serving as identifiers of which heavy and/or light chain is obtained from each well thus providing a guide for matching individual variable heavy and light chains derived from the same initial cell, according to the methods detailed in "Assignment of sequences to wells" and "assembly of sequences" in the materials and methods. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity.

[00560] Sequences for cloning can either be obtained through synthetic gene synthesis or amplified from the 1st PCR products using cloning primers. The forward cloning primer is sample-ID specific and can amplify specific sequences from a pool of amplicons. The sequence for each heavy and light chain is then cloned into an expression vector containing

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complementary restriction sites for those introduced by the cloning primers. Vectors also contain the either the heavy or light chain constant region, which the heavy or light chain sequences are cloned into (reading frame aligned) to produce the entire antibody. Vectors contain either heavy or light chain clones are then dual transfected into a mammalian expression system or alternately, both amplicons can be cloned into a dual expression vector to allow for a single transfection into mammalian cells.

[00561] Candidate heavy and light chain genes from particular cells of origin are then expressed using the for screening of desired properties as above. Once stably or transiently transfected, the expression of the paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing secreted antibodies are then screened for desired properties including but not limited to antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 27: Use of murine plasma cells to generate monoclonal antibodies

[00562] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine B cells. Murine plasma cells may be obtained from blood, from splenocytes or from the bone marrow, although the splenocytes and bone marrow are typically used. Flow cytometry is performed to obtain $CD19^{low/-}B220^{low/-}CD138^{+}$ plasma cells. This population of plasma cells is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR is performed as detailed in "non-touchdown PCR" in the materials and methods. Mouse gene-specific primers are found in Table 11, and other primers used for RT and PCR are found in Table 1. Multiple plates are then pooled according to the method detailed in "preparing for 454 XLR70 sequencing run" and subjected to high-throughput 454 DNA sequencing and individual sequences identified with their barcodes serving as identifiers of which heavy and/or light chain is obtained from each well thus providing a guide for matching individual variable heavy and light chains derived from the same initial cell, according to the methods detailed in "Assignment of sequences to wells" and "assembly of sequences" in the materials and methods. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity.

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[00563] Sequences for cloning can either be obtained through synthetic gene synthesis or amplified from the 1st PCR products using cloning primers as described in example 26. Candidate heavy and light chain genes from particular cells of origin are then expressed using the for screening of desired properties as above. Once stably or transiently transfected, the expression of the paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing secreted antibodies are then screened for desired properties including but not limited to antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 28: Use of unbiased or antigen-specific murine memory B cells to generate monoclonal antibodies

[00564] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine B cells. Murine memory B cells may typically be obtained from splenocytes or lymph nodes. Flow cytometry is performed to obtain $CD19^+$ or $B220^+$ and $CD38^+IgG^+$ memory B cells. Other markers, such as CD45RO, may also be used. Antigen-specific memory B cells may also be visualized by staining with fluorophore-conjugated antigen and sorted for. This population of memory B cells is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR , followed by sequencing, assignment of sequences to wells and sequence assembly is performed as in example 26. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity.

[00565] Sequences for cloning can either be obtained through synthetic gene synthesis or amplified from the 1st PCR products using cloning primers as described in example 26. Candidate heavy and light chain genes from particular cells of origin are then expressed using the for screening of desired properties as above. Once stably or transiently transfected, the expression of the paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing secreted antibodies are then screened for desired properties including but not limited to antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 3 and 9 each provide one example of a general methodology for carrying out this

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method. Figures 26-27 provide another example of obtaining monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 29: Use of murine short-lived plasmablasts to generate monoclonal antibodies

[00566] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine B cells. Murine short-lived plasmablasts may typically be obtained from splenocytes. These plasmablasts have been variously described as $CD19^{low/-}B220^{low/-}$ and $CD22^{low}$ or $CD11c^{+}$, and also as $CD138^{+}$. Flow cytometry is performed to obtain plasmablasts. This population of plasmablasts is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR, followed by sequencing, assignment of sequences to wells and sequence assembly is performed as in example 26. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity.

[00567] Sequences for cloning can either be obtained through synthetic gene synthesis or amplified from the 1st PCR products using cloning primers as described in example 26. Candidate heavy and light chain genes from particular cells of origin are then expressed using them for screening of desired properties as above. Once stably or transiently transfected, the expression of the paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing secreted antibodies are then screened for desired properties including but not limited to antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 30: Use of murine blasting B cells to generate monoclonal antibodies

[00568] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine B cells. Murine blasting B cells may typically be obtained from splenocytes. Blasting cells are activated B cells, and therefore are cells that have responded against the antigen and are actively proliferating. These B cells consist of clonal families and their paired heavy and light chains can be used to obtain evolutionary trees. Blasting B cells may be gated as FSC^{hi}, and may also be further identified via cell surface markers such as $CD44^{hi}$ $CD69^{hi}$ and as blasting B cells are proliferating, they may also be identified as having entered the cell cycle by having increased DNA content as stained by cell

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permeably DNA stains such as SYTO Blue. Flow cytometry is performed to obtain blasting B cells. This population of plasmablasts is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR , followed by sequencing, assignment of sequences to wells and sequence assembly is performed as in example 26. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity.

[00569] Sequences for cloning can either be obtained through synthetic gene synthesis or amplified from the 1st PCR products using cloning primers as described in example 26. Candidate heavy and light chain genes from particular cells of origin are then expressed using the for screening of desired properties as above. Once stably or transiently transfected, the expression of the paired heavy and light chains will result in generation of monoclonal antibodies recapitulating the specificity of the initially sorted cell. Supernatants containing secreted antibodies are then screened for desired properties including but not limited to antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees

Example 31: Obtaining monoclonal antibodies from unbiased or antigen-specific human IgA+ B cells

[00570] From a subject with or without documented or suspected prior exposure an antigen of interest, FACS is performed on peripheral blood (either whole blood or isolate peripheral blood mononuclear cells, PBMCs) or on bone marrow to isolate IgA+ B cells. These B cells may be memory B cells, plasma cells, or plasmablasts. These IgA^+ B cells may also be antigen-specific, by sorting for antigen-positive B cells using a fluorophore-conjugated antigen to stain for the IgA+ B cells. This population of cells is then sorted by FACS either as single cells or multiple cells into wells. The process described in detail in Example 21 is repeated to barcode and obtain sequences from 454 sequencing and assign sequences to wells and assemble sequences, and IgA constant region specific primers used for PCR are in Table 10. HighV-QUEST is used to identify VDJ gene usage, and a few members of each clonal family on an evolutionary tree selected for cloning and expression as in example 8. Supernatants containing expressed antibodies will be screened for antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of

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obtaining human monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 32: Obtaining monoclonal antibodies from unbiased or antigen-specific human IgM+ B cells

[00571] From a subject with or without documented or suspected prior exposure an antigen of interest, FACS is performed on peripheral blood (either whole blood or isolate peripheral blood mononuclear cells, PBMCs) to isolate IgM+ B cells. These B cells may be memory B cells, plasma cells, or blasting B cells. These IgM^+ B cells may also be antigen-specific, by sorting for antigen-positive B cells using a fluorophore-conjugated antigen to stain for the IgM+ B cells. This population of cells is then sorted by FACS either as single cells or multiple cells into wells. The process described in detail in example 21 is repeated to barcode and obtain sequences from 454 sequencing and assign sequences to wells and assemble sequences, and IgM constant region specific primers used for PCR are in Table 10. HighV-QUEST is used to identify VDJ gene usage, and a few members of each clonal family on an evolutionary tree selected for cloning and expression as in example 8. Supernatants containing expressed antibodies will be screened for antigen specificity against the target antigen of interest as well as functionality by appropriate functional assays. Figs. 9 and 6 each provide one example of a general methodology for carrying out this method. Figures 26-27 provide another example of obtaining human monoclonal antibodies from functional characterization of selected cloned and expressed antibodies from evolutionary trees.

Example 33: Obtaining monoclonal antibodies from unbiased or antigen-specific murine IgA+ B cells

[00572] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine IgA^+ B cells. These B cells may be memory B cells, plasma cells, plasmablasts or blasting B cells, and can typically be obtained from splenocytes. These IgA^+ B cells may also be antigen-specific, by sorting for antigen-positive B cells using a fluorophore-conjugated antigen to stain for the IgA^+ B cells. This population of IgA^+ B cells is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR , followed by sequencing, assignment of sequences to wells and sequence assembly is performed as in example 26, and IgA constant region specific primers used for PCR are in Table 11. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity. Figs. 9 and 6 provides one example of a general methodology for carrying out this method.

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Example 34: Obtaining monoclonal antibodies from unbiased or antigen-specific murine IgM+ B cells

[00573] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine IgM^+ B cells. These B cells may be memory B cells, plasma cells, plasmablasts or blasting B cells, and can typically be obtained from splenocytes. These IgM^+ B cells may also be antigen-specific, by sorting for antigen-positive B cells using a fluorophore-conjugated antigen to stain for the IgM+ B cells. This population of IgM^+ B cells is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR , followed by sequencing, assignment of sequences to wells and sequence assembly is performed as in example 26, and IgA constant region specific primers used for PCR are in Table 11. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity. Figs. 9 and 6 provides one example of a general methodology for carrying out this method.

Example 35: Sequencing of more than one sequence from human T cells

[00574] From a subject with a recent or current condition resulting in acute, subacute, or ongoing generation of circulating T cells, flow cytometry is performed on peripheral blood (either whole blood or peripheral blood mononuclear cells (PBMCs)) to identify the T cell population of interest. This population of T cells may be activated T cells or blasting T cells. Activated T cells may be identified using $CD44^{hi}$, $CD69^{hi}$, $CD154^+$, $CD137^+$, or blasting T cells, which are also activated T cells may be delineated by their size or FSC^{hi}, and may also be identified as being in the cell cycle using a cell permeant DNA dye such as SYTO Blue. Activated T cells should consist of clonal families which then can be clustered into an evolutionary tree, with identical family members in clonal families, which can be used to select clones for downstream functional analysis. T cells are then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT and PCR to barcode the TCR genes are detailed in "Non-touchdown PCR" in the materials and methods, sequencing prep is detailed in "Preparing for 454 XLR70 sequencing run" in the materials and methods, and assignment of sequences to wells and assembly of reads are detailed in "Assignment of sequences to wells" and "assembly of sequences" in the materials and methods, TCR gene-specific primers are found in Table 10. Evolutionary trees are then constructed and candidate genes from particular cells of origin are then chosen to be cloned and expressed for screening of desired properties. Sequences for cloning can either be gene-synthesized or amplified from the

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1st PCR products with cloning primers. Specific clones can be isolated from a pool of clones by having the forward cloning primer specific for the sample-ID barcode sequence. Reverse cloning primers are complementary for the appropriate gene. Both forward and reverse primers contain flanking restriction sites to integrate the clone (coding frame aligned) into a vector. Cells are either doubly transfected with two expression vectors, each containing either a gene of interest, or singly transfected with a dual expression vector that expresses both genes of interest, e.g., T cell alpha and beta chains.

[00575] Once stably or transiently transfected, the genes of interest can be expressed and screened for function properties using the desired screens.

Example 36: Sequencing of more than one sequence from murine T cells

[00576] A mouse is challenged with an antigen of interest, and may be given booster shots several times before sacrificing the mouse to obtain murine T cells. T cells are $CD3^+$, and helper T cells are $CD4^+$ and cytotoxic T cells are $CD8^+$. This population of T cells may be memory or activated T cells or blasting T cells. Memory T cells may be identified as $CD45RO^+$. Activated T cells may be identified using $CD44^{hi}$, $CD69^{hi}$, or blasting T cells, which are also activated T cells may be delineated by their size or FSC^{hi}, and may also be identified as being in the cell cycle using a cell permeant DNA dye such as SYTO Blue. All these T cells in a mouse kept in a clean environment after repeated antigen exposure should have a large fraction of clonal families which can then be displayed as an evolutionary tree, which can then be used to select TCRs for cloning and expression and downstream functional analysis.

[00577] T cells are sorted by flow cytometry using the suggested markers above. as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT and PCR to barcode the TCR genes are detailed in "Non-touchdown PCR" in the materials and methods, sequencing prep is detailed in "Preparing for 454 XLR70 sequencing run" in the materials and methods, and assignment of sequences to wells and assembly of reads are detailed in "Assignment of sequences to wells" and "assembly of sequences" in the materials and methods, TCR gene-specific primers are found in Table 11. Evolutionary trees are then constructed and candidate genes from particular cells of origin are then chosen to be cloned and expressed for screening of desired properties. Sequences for cloning can either be gene-synthesized or amplified from the 1st PCR products with cloning primers. Specific clones can be isolated from a pool of clones by having the forward cloning primer specific for the sample-ID barcode sequence. Reverse cloning primers are complementary for the appropriate gene. Both forward and reverse primers contain flanking restriction sites to integrate the clone (coding frame aligned) into a vector.

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Cells are either doubly transfected with two expression vectors, each containing either a gene of interest, or singly transfected with a dual expression vector that expresses both genes of interest, e.g., T cell alpha and beta chains.

[00578] Once stably or transiently transfected, the genes of interest can be expressed and screened for function properties using the desired screens.

Example 37: Sequencing of more than one sequence from a sample

[00579] A single sample comprising nucleic acids of interest is identified. The single sample can have a single cell or a population of cells. The sample can be sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. This population of B cells is then sorted by flow cytometry as single cells into wells containing a hypotonic buffer with an RNAse inhibitor. Sorted cells can be frozen at this time or used immediately for RT-PCR to create cDNA. RT, 1st and 2nd PCR is performed as detailed in "non-touchdown PCR" in the materials and methods. Multiple plates are then pooled according to the method detailed in "preparing for 454 XLR70 sequencing run" and subjected to high-throughput 454 DNA sequencing and individual sequences identified with their barcodes serving as identifiers of which heavy and/or light chain is obtained from each well thus providing a guide for matching individual variable heavy and light chains derived from the same initial cell, according to the methods detailed in "Assignment of sequences to wells" and "assembly of sequences" in the materials and methods. Evolutionary trees are then drawn and antibodies selected for cloning, expression and determination of functional activity.

[00580] Candidate genes from particular cells of origin are then chosen to be cloned and expressed for screening of desired properties or other needs. Sequences for cloning can either be gene-synthesized or amplified from the 1st PCR products with cloning primers. Specific clones can be isolated from a pool of clones by having the forward cloning primer specific for the sample-ID barcode sequence. Reverse cloning primers are complementary for the appropriate gene. Both forward and reverse primers contain flanking restriction sites to integrate the clone (coding frame aligned) into a vector. Cells are either transfected with two or more expression vectors, each containing either a gene of interest, or singly transfected with an expression vector that expresses the genes of interest.

[00581] Once stably or transiently transfected, the genes of interest can be expressed and screened where desired. Figs. 9 and 6 provides one example of a general methodology for carrying out this method.

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Example 38: Cloning of immunoglobulin V(D)J regions by DNA synthesis.

[00582] The desired immunoglobulin light chain and heavy chain V(D)J regions can be synthetically generated by DNA synthesis for cloning into expression vectors. The sequence used for the synthesis can be derived directly from the high-throughput 454 sequences, or alternatively cDNA encoding the heavy and light chain immunoglobulins from the sample(s) of interest can be re-sequenced from the individual sample or pooled samples for further verification of the sequence, and this sequence is used to synthesize the selected light chain and heavy chain V(D)J regions. Variable regions of Ig genes may be cloned by DNA synthesis, and incorporating the synthesized DNA into the vector containing the appropriate constant region using restriction enzymes and standard molecular biology. During synthesis, the exact nucleotide sequence need not be followed as long as long as the amino acid sequence is unchanged, unless mutagenesis is desired. This allows for codon optimization that may result in higher expression levels. This also allows for adding in restriction sites for the purpose of cloning. Non-translated sequences such as 5' UTR and barcode sequences need not be synthesized, leader sequences can also be swapped for other signal peptide sequences known for higher expression levels. These result in an Ig nucleotide sequence that can be very different from the high-throughput reads but give identical amino acid sequenced when expressed.

[00583] In one embodiment, the amplified V(D)J regions are inserted into vectors that already contain either the kappa, lambda, gamma or other heavy chain isotype constant regions with the appropriate restriction sites needed for inserting the amplified clones in the open reading frame. In another embodiment, the entire variable region may be gene synthesized with constant region and cloned into an expression vector for expression and downstream functional testing of antibody properties.

Example 39: Cloning of immunoglobulin V(D)J regions by using restriction site already present in sample identifcation adaptor.

[00584] In another aspect, the desired immunoglobulin light chain and heavy chain V(D)J regions can be cloned using restriction site already incorporated in the sample-ID adaptor added on during reverse transcription. This results in an adaptor with a restriction site 3' of the well-ID barcode in the PCR amplicon pool. During cloning with cloning primers, desired amplicons are amplified from a plate-specific amplicon pool using 5' primers that are complementary to the well-ID barcode sequences, and chain specific 3' primers (for the kappa, lambda and gamma chains). 3' primers will add on 3' restriction sites. 5' primers do not need to add restriction sites as the 5' primer already contains a restriction site 3' of the well-ID barcode. Following this amplification, restriction enzymes are used to cut the amplicon for ligation into the vector

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containing the constant region insert. During the restriction enzyme digest, sequences added on to the 5' end of the Ig gene sequences, such as barcodes and universal sequences are cut as they are 5' of the 5' restriction site.

Example 40: Identification of clonal families by sequencing of just one immunoglobulin chain (heavy chain or light chain), followed by cloning of paired immunoglobulin heavy and light chain V(D)J regions

[00585] Antibody heavy and light chains are reversed transcribed from mRNA, incorporating distinct sample-IDs on the cDNAs generated from each sample, and sample cDNAs pooled for amplifying PCR. The immunoglobulin cDNAs are amplified and either the immunoglobulin heavy chain or the light chain is sequenced using 454 high-throughput sequencing, and the sequences grouped according to their use of immunoglobulin heavy chain V(D)J or light chain V(D)J sequences that exhibit use of the same genome-encoded V(D)J segments. Bioinformatics is used to identify clonal families of interest, and the desired immunoglobulin light and heavy chain V(D)J regions from the same sample are then selectively amplified for sequencing and/or cloning. For PCR amplification, the forward primer includes the sample-ID and the reverse primer is specific for the light chain or heavy chain constant region. The primers can incorporate restriction sites into the amplicons. Amplicons can then be inserted into the appropriate expression vectors that already contain a heavy or light chain constant region. Antibodies can then be expressed and screened for desired properties.

Example 41: Identification of clonal families from immunoglobulin heavy and light chain V(D)J sequencing for cloning and expression of antibodies using only sample-IDs (and no plate-IDs)

[00586] Antibody heavy and light chains are reversed transcribed from mRNA in each sample, incorporating distinct sample-IDs into the cDNA generated from each sample. Each sample-ID is at least 6 nucleotides long and 1 base-pair different, resulting in 4096 distinct potential sample-IDs. A distinct sample-ID is used for each sample, and the unique sample-IDs identify cDNA derived from different samples and enables paired sequencing and cloning of 2 or more cDNAs expressed in an individual sample. Heavy and light chain amplicons are amplifed using PCR, which adds on the Titanium adaptors A and B required for 454 high-throughput sequencing and all samples are then sent for sequencing. Sequences are assigned to wells and assembled following "Assignment of sequences to wells" and "Assembly of sequences" sections in the materials and methods. V(D)J assignments are made using HighV-QUEST and grouped into clonal families based on their V(D)J usage. Selected clones are then specifically amplified with cloning primers, which also adds in restriction sites into the amplicon. Amplicons are then inserted in-frame into expression vectors which already contain

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the appropriate heavy or light constant regions for expression of the antibodies for screening for desired properties.

Example 42: Cloning of paired sequences by ligating on the universal primer sequence.

[00587] Antibody heavy and light chain genes are reversed transcribed from mRNA, which adds a 3' sequence to the newly synthesized cDNA consisting of an adaptor region and a sample-ID barcode. Samples are then pooled together and a universal primer sequence added to the 3' end of the 1st strand cDNA using T4 DNA ligase and a 5' phophorylated anti-sense universal primer oligonucleotide. Alternatively, 2nd strand cDNA synthesis may be done to obtain double stranded cDNA instead of an mRNA/cDNA hybrid before ligating on the universal primer sequence. Two rounds of PCR are then performed to amplify the cDNA and to add on plate-IDs and Titanium primers A and B for 454 sequencing. Alternatively, plate-IDs and Titanium Primers may also be added by DNA ligation instead of incorporated during PCR by using T4 DNA ligase. After 454 sequencing, sequences are assembled and clonal families identified. Selected clones from clonal families may be specifically cloned using cloning primers that add restriction sites to the amplicons. Sequences are then inserted in-frame into expression vectors that already have the appropriate heavy or light chain constant regions. Antibodies are then expressed and screened for desired properties.

Example 43: Testing of gene-specific primers for reverse transcription (RT-GSPs) of immunoglobulin heavy and light chains.

[00588] RT-GSPs were used instead of oligo(dT)s as primers in reverse transcription of heavy and light chain genes. cDNA were then amplified by PCR and visualized on an agarose gel. RT-GSP primers were IgKC_v3(a), IgLC_v5, IgLC_v6, IgLC_v7 and IgLC_v8 in lanes 1-4 respectively (b), IgHGC_v10, IgHGC_v11, IgHGC_v13 and IgGC_v15 in lanes 1-4 respectively (c) and IgHGC_v16 (d). KC, LC and GC in the primer names indicate that the primer is specific for the kappa chain, lambda chain and gamma heavy chain respectively. White bands in gel photos indicate where non-relevant lanes had been cropped out. *See* Figure 10 and Table 6.

Example 44: Testing of adaptor region sequences.

[00589] RNA was reversed transcribed with oligonucleotides comprising a universal primer region and an adaptor region at the 3' terminal end. cDNA was then amplified using the universal primer region sequence as a forward primer and gene-specific sequences as reverse primers. Amplified products were visualized on an agarose gel. Adaptor region consists of G (a), GGGGG and rGrGrG in lanes 1 and 2 respectively (b). rG indicates RNA nucleotides instead of DNA nucleotides. *See* Figure 11 and Table 6.

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Example 45: Testing of universal primer sequences.

[00590] RNA was reverse transcribed with oligonucleotides comprising a universal primer sequence and an adaptor region at the 3' terminal end. cDNA were then amplified by PCR using a forward primer complementary to the universal primer region and a reverse primer complementary to the gene specific sequence. Univ_seq_4 (a), univ_seq_5 (b) and univ_seq_f (c). Vertical white bands in gel photos indicate where non-relevant lanes have been cropped out. Otherwise lanes belong to the same gel photo. *See* Figure 12 and Table 6.

Example 46: Testing of gene-specific primer sequences for 1st PCR reaction.

[00591] Gene-specific reverse primers were used in amplification of sequences in the first PCR reaction. Either the 1st PCR reaction or the subsequent 2nd nested PCR products were run and visualized on an agarose gel. Reverse primers used were IgKC_v4, IgLC_v5, IgHGC_v13 on lanes 1-3 respectively (a), K_GSP1, L_GSP1, G_GSP1 on lanes 1-3 respectively (b), K_GSP1c, L_GSP1c on lanes 1-2 respectively (c), G_GSP1 (d), L_GSP1d, G_GSP1g on lanes 1-2 respectively (e), G_GSP1h, G_GSP1k, L_GSP1f, L_GSP1g on lanes 1-4 respectively (f), G_GSP1d (g) L_GSP1h-o on lanes 1-8 respectively (h), G_GSP1m-q and G_GSP1t on lanes 1-6 respectively (K, L and G in the primer names indicate that the primers are specific for the kappa, lambda and gamma immunoglobulin constant regions respectively). Each gel starts with a lane marker on the left followed by sample lanes. White bars between lanes on the same gel photo indicate where non-relevant lanes in-between have been cropped out. *See* Figure. 13. Also, more primers were tested in Figure 43. These primers were used for the 1st PCR, and then the 2nd PCR was done using the primers from Table 1 and PCR products ran on a 2% agarose gel and image was taken. Primers used for 1st PCR are Kappa GSP1, kappa GSP1e, kappa GSP1f, lambda GSP1, lambda GSP1x and lambda GSP1y respectively. Also see Table 6 for sequences used.

Example 47: Testing of gene-specific sequences for the 2nd PCR reaction.

[00592] Gene-specific reverse primers were used in amplification of sequences in the 2nd PCR reaction. PCR products were run and visualized on an agarose gel. Reverse primers used were K_GSP2, L_GSP2, G_GSP2 in lanes 1-3 respectively (a), K_GSP2v2a, K_GSP2v2b, L_GSP2v2 in lanes 1-3 respectively (b), K_GSP2v2c, K_GSP2v2c, G_GSP2v2c1, G_GSP2v2c2 in lanes 1-4 respectively (c), K_GSP2v2d-f in lanes 1-3 respectively (d),K_GSP2v2g, L_GSP2v2d and G_GSP2b in lanes 1-3 respectively (e). K, L, G in the primer names indicates that they are specific for the kappa, lambda and gamma immunoglobulin constant regions respectively. Each gel starts with a lane marker on the left followed by sample

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lanes. White bars between lanes on the same gel photo indicate that non-relevant lanes in-between have been cropped out. *See* Figure 14 and Table 6.

Example 48: Testing of gene-specific primers for other human variable region genes

[00593] 1st and 2nd PCR were done using gene-specific reverse primers and products ran on a 2% agarose gel and imaged. Lanes are from left: marker, mu, alpha constant regions, TCR alpha (a) and marker, TCR beta (b). sequences of 3' primers used are in table 10. White bars between lanes on the same gel photo indicate where non-relevant lanes in-between have been cropped out. See Figure 44.

Example 49: Testing of gene-specific primers for mouse variable region genes

[00594] 1st and 2nd PCR were done and products ran on a 2% agarose gel and imaged. Lanes are from left: marker, kappa, lambda, lambda, lambda, lambda light chains and mu heavy chain (a). The 4 lambda lanes had this combination of primers used: mouse_lambda_GSP1a with mouse_lambda GSP2a, mouse_lambda_GSP1a with mouse_lambda GSP2b, mouse_lambda_GSP1b with mouse_lambda GSP2a and mouse_lambda_GSP1b with mouse_lambda GSP2a. Marker and alpha heavy chain (b). Gamma1, 2a, 2c heavy chains with 2nd PCR using mo_g12_GSP2d and mo_g12_GSP2e respectively, marker (c). Gamma 3 heavy chain with 2nd PCR using mo_g3_GSP2d, mo_g3_GSP2e respectively followed by gamma 2b heavy chain with 2nd PCR using mo_g2b_GSP2d, mo_g2b_GSP2e respectively, followed by marker (d). Marker, TCR alpha (e). Marker, TCR beta(f). White bars between lanes on the same gel photo indicate where non-relevant lanes in-between have been cropped out. See Figure 45 and Table 11.

Example 50: Generation of linked pairs of antibody heavy and light chains with a barcode at one end.

[00595] As shown in Figure 1, individual B cells can be sorted by flow cytometry from blood, bulk peripheral blood mononuclear cells (PBMCs), bulk B cells, plasmablasts, plasma cells, memory B cells, or other B cell populations. B cells are single-cell-sorted into 96-well PCR plates, leaving one column of wells empty, as a negative control. Figure 17 describes the general methodology for a method that can be used to link two polynucleotide sequences of interest and add a barcode at one end.

[00596] Single step multiplex overlap-extension RT-PCR can be performed using a commercially available one-step RT-PCR kit (e.g., Qiagen one-step RT PCR kit) according to the manufacturer's recommendations. In this particular example, a polynucleotide synthesis reaction, such as a reverse transcription reaction, is used to generate cDNA templates from an mRNA sample. Referring to Figure 17, the forward gene specific primer for the RT-PCR

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reaction contains a restriction enzyme site (RE1), a sequencing primer site, and a barcode, to add these elements to a first cDNA of interest. Two additional primers (shown as containing RE3) have complementary overlap-extension tails. Use of these primers in a PCR reaction results in the two cDNAs of interest carrying overlap extension tails, which allow the two cDNAs of interest to anneal and link during amplification. In the example shown, a product of the indicated structure would be generated in which the LC and HC chains are physically linked with a barcode at one end.

[00597] The RE1 and RE2 restriction sites can be used clone the PCR product into suitable vectors for sequencing.

Example 51: Generation of linked pairs of antibody heavy and light chains with an internal barcode.

[00598] As shown in Figure 1, individual B cells can be sorted by flow cytometry from blood, bulk peripheral blood mononuclear cells (PBMCs), bulk B cells, plasmablasts, plasma cells, memory B cells, or other B cell populations. B cells are single-cell-sorted into 96-well PCR plates, leaving one column of wells empty, as a negative control. Figure 18 describes the general methodology for a method that can be used to link two polynucleotides of interest with a barcode located in between. Primers and oligonucleotides which can be used for antibody heavy and light chains are shown in Table 30. Restriction sites AsiSI and PacI are included in the RT oligonucleotides. Sample-ID sequences are shown in Table 2.

[00599] The method shown in Figure 18 relies on the 3' tailing and template switching activities of reverse transcriptase during a cDNA synthesis reaction. The 3' C tail added to the synthesized cDNA can be used for the annealing of an adaptor molecule carrying an overlap extension sequence and a barcode. Two types of adaptor molecules are used to link two cDNAs. A first adaptor carrying an overlap extension and barcode sequence is added to a first cDNA. A second adaptor carrying the reverse complement of the overlap extension without a barcode sequence is added to a second cDNA. The template switching property of reverse transcriptase adds these sequences to the 3' ends of their respective cDNAs.

[00600] In a PCR reaction, as shown in Figure 18, the complementary overlap extension sequences anneal and corresponding strands of DNA are synthesized from the site of annealing. Subsequent rounds of PCR using external primers results in amplification of the linked cDNA molecules.

[00601] Through the addition of appropriate restriction sites and the addition of sequencing primer sites incorporated into primers for the amplification reaction or later by ligation, the PCR products can be cloned into suitable vectors for sequencing.

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Example 52: Generation of linked pairs of antibody heavy and light chains with two internal barcodes using universal sequence overlap-extension primers.

[00602] As shown in Figure 1, individual B cells can be sorted by flow cytometry from blood, bulk peripheral blood mononuclear cells (PBMCs), bulk B cells, plasmablasts, plasma cells, memory B cells, or other B cell populations. B cells are single-cell-sorted into 96-well PCR plates, leaving one column of wells empty, as a negative control. Figure 19 describes the general methodology for a method that can be used to introduce two internal barcodes in between two linked polynucleotides of interest. Primers and oligonucleotides which can be used for antibody heavy and light chains are shown in Table 31. Restriction sites AsiSI and PacI are included in the RT oligonucleotide. Sample-ID sequences are shown in Table 2.

[00603] The method shown in Figure 19 relies on the 3' tailing and template switching activities of reverse transcriptase during a cDNA synthesis reaction. In this example, the 3' C tail added to oligo (dT) primed cDNA can be used for the annealing of an adaptor molecule carrying a universal sequence and a barcode to each of the cDNAs to be joined. The template switching property of reverse transcriptase adds these sequences to the 3' ends of their respective cDNAs. Subsequent overlap-extension PCR using primers to the universal sequence which carry complementary overlap-extension sequences in combination with external LC and HC specific primers results in a structure in which LC is linked to HC with two internal barcodes between them as shown in Figure 19.

[00604] Through the addition of appropriate restriction sites and the addition of sequencing primer sites incorporated into primers for the amplification reaction or later by ligation, the PCR products can be cloned into suitable vectors for sequencing.

Example 53: Generation of linked pairs of antibody heavy and light chains with two internal barcodes using overlap-extension adaptors.

[00605] As shown in Figure 1, individual B cells can be sorted by flow cytometry from blood, bulk peripheral blood mononuclear cells (PBMCs), bulk B cells, plasmablasts, plasma cells, memory B cells, or other B cell populations. B cells are single-cell-sorted into 96-well PCR plates, leaving one column of wells empty, as a negative control. Figure 20 describes the general methodology for another method that can be used to introduce two internal barcodes in between two linked polynucleotides of interest. Primers and oligonucleotides which can be used for antibody heavy and light chains are shown in Table 32. Restriction sites AsiSI and PacI are included in the RT oligonucleotides. Sample-ID sequences are shown in Table 2.

[00606] The method shown in Figure 20 also relies on the 3' tailing and template switching activities of reverse transcriptase during a cDNA synthesis reaction. In this example, the 3' C

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tail added to cDNA synthesized using gene specific primers can be used for the annealing of adaptor molecules carrying self complementary or palindromic overlap-extension sequences and a barcode to each of the cDNAs to be joined. The template switching property of reverse transcriptase adds these sequences to the 3' ends of their respective cDNAs. Subsequent annealing of the overlap-extension sequences added to the LC and HC cDNAs links them together at the site of overlap. Overlap-extension PCR using external primers to LC and HC results in a structure in which LC is linked to HC with two internal barcodes between them as shown in Figure 20.

[00607] Through the addition of appropriate restriction sites and the addition of sequencing primer sites incorporated into primers for the amplification reaction or later by ligation, the PCR products can be cloned into suitable vectors for sequencing.

Example 54: Studies on different methods of adding barcodes.

[00608] We investigated a variety of methods through which barcode sequences could be added during the course of a reverse transcription or amplification reaction using an oligonucleotide comprising the barcode sequence. We tested the addition of barcodes by incorporating them into gene-specific primers (GSPs) and into oligonucleotides containing one or more Gs that can be added to the 3' end of cDNAs by template switching. Based on the literature and our scientific knowledge, our expectation was that we would be able to effectively barcode cDNA using either 5' barcoded oligonucleotides or 3' barcoded GSPs.

[00609] As demonstrated in Figure 21, RT was performed with 1 μg of total PBMC RNA and 0.5 μM of univ_seq_2 template-switching oligo and 0.1 μM of IgKC_v3 GSP (lanes 1-2) and IgLC_v5 GSP (lanes 3-4) with an additional 5' flanking sequence, of which the first part is the Fixed_PCR3 sequence, and the last 8 bp AACAATAC is the barcode. The RT reaction was cleaned up using NucleoTraPCR (Macherey-Nagel) and dissolved in a final volume of 50 μl. 2 μl of this reaction was used in each subsequent PCR reaction, with either an internal 5' V_K (lane 1) or V_L (lane 3) primer or the Univ_seq_2 (lanes 2 and 4) as the 5' primer, and Fixed_PCR3 as the 3' primer. Note that the V_K primer is specific for kappa V genes 1 and 2, and the V_L primer is specific for lambda V gene 2. Sequences are in Table 33. As can be seen, the PCR products in lanes 2 and 4 ran as a smear. In contrast, the internal 5' primers produced distinct bands (lanes 1 and 3), showing that the primer pairs do work, and the smearing shown in lanes 1 and 3 cannot be attributed to poorly designed primers. As the oligonucleotide is added to all full-length reverse transcribed cDNA sequences, when a smear is obtained during PCR amplification with the univ_seq_2 and 3' barcoded GSPs, this suggests that reverse transcription with barcoded GSPs results in non-specifically primed nucleic acid sequences in the RT reaction.

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Our results suggest that use of 5' universal sequence adaptors and 3' barcoded primers is not a good strategy for the barcoding and specific amplification of immunoglobulin or other genes expressed by a B cell or other cell.

[00610] In hindsight, several biologic properties of DNA and the molecular reactions used likely contribute to our observations. Reverse transcription is usually performed at low temperatures such as 42°C or 56°C. Unlike PCR, where the annealing step is usually performed at a temperature just slightly below the Tm of the primers to promote priming specificity, this cannot be done for reverse transcription, as reverse transcriptases are inactivated at high temperatures. Therefore, gene specific primers used during RT are typically not very specific for the gene of interest because the reaction proceeds at a temperature much lower than the Tm of the primer. In such a situation, the primer can also bind to off-target mRNA sequences with some mismatches, and mispriming occurs. If the barcode is added on the GSP, the primer also has to have a fixed sequence 5' of the barcode for use in subsequent PCR. This makes the primer very long (~60 nt), resulting in a primer with even a much higher degree of mispriming. However, specific amplification during PCR usually can still be achieved by using a highly gene-specific forward primer; as long as one member of the primer pair is specific, there usually can be specific amplification, as shown in lanes 1 and 3.

[00611] If a template switching technique is used to add an adaptor, this adaptor is added to all mRNAs that are synthesized to form first-strand cDNA. As mentioned above, the barcoded GSPs will have significant amounts of mispriming, especially as the RT enzyme Superscript III loses its template-switching activity at 56°C, and reverse transcription proceeds at 42°C. Specific nested 5' or 3' primers cannot be used as one would either lose the ability to PCR amplify all immunoglobulin genes (thus having to resort to multiplex PCR with multiple degenerate 5' primers due to variable V genes) or else lose the 3' barcode.

Therefore, barcoded GSPs are not suitable for use with template-switch added adaptors, or any other 5' adaptors, as other methods to add 5' adaptors such as TdT tailing or blunt end cloning also add adaptors non-discriminately. Therefore, internal 3' primers or a nested or semi-nested PCR amplification strategy is also required, and barcoded 3' GSPs do not allow for the use of these strategies for specific amplification of genes from a B cell or other cell. Based on our results, one would also anticipate that barcoded oligo(dT)s would also perform poorly for many of the same reasons we believe barcoded GSPs perform poorly. These reasons include but are not limited to an inability to use internal 3' primers or a nested or semi-nested PCR strategy for specific amplification of genes from a B cell or other cell.

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[00612] In contrast, our results (see other examples) demonstrate the superiority of barcode sequence addition during the course of a reverse transcription reaction using a primer comprising the barcode sequence and an adaptor that anneals to the 3' tail of a cDNA generated during a reverse transcription reaction. In such an embodiment, the adaptor sequence can comprise a barcode sequence and be used to label genes encoding antibody heavy and light chains. Thus, as disclosed herein, template switching, or any other methods of tailing a cDNA adds a sequence that can be used for PCR amplification without prior knowledge of the 5' sequences themselves, enabling efficient and unbiased representation of the antibody repertoire. Furthermore, this approach allows one to obtain the repertoire of other co-expressed genes encoding proteins in addition to antibodies. Further, the approach of using template-switch adaptors has clear advantages over methods disclosed in the art that use sets of degenerate forward primers to amplify multiple V genes. These methods also fail to capture the entire antibody repertoire, because the known 5' primer sets: a) cover most but not the entire repertoire set; b) are not able to cover as yet known V genes variants (polymorphisms) in the human population; and c) may not be able to effectively amplify antibody sequences that have undergone extensive somatic hypermutation (SHM). See, e.g., Scheid et al., Sciencexpress, 14 July 2011 for an example of the effect of SHM.

[00613] Accordingly, the use of template-switch adaptors for the preparation of libraries of expressed genes, e.g., antibody heavy and light chains, provides clear advantages over other methods known in the art by allowing for unbiased representation of particular gene families and other co-expressed genes. The use of template-switch adaptors or 5' adaptors added using any other methods such as but not limited to TdT tailing and blunt end ligation are also more compatible with the use of barcoded 5' adaptors rather than barcoded 3' GSPs or barcoded oligo(dT)s for the reasons discussed above.

Example 55: Sorting of plasmablasts by forward-scatter and/or side-scatter on flow cytometer and/or in conjunction with other cell surface markers

[00614] Plasmablasts are blasting B cells that are activated, have proliferated/are proliferating and have undergone affinity maturation. Plasmablasts represent the active immune response and by practicing the methods and compositions herein allow for the bioinformatic construction of evolutionary trees with clonal families of antibodies that bind to target antigens of interest, whether it is an infection, a vaccine, autoimmune or cancer antigens.

[00615] Plasmablasts are blasting B cells and are larger than resting B cells (Figure 40A-B). Therefore, they can be sorted on a flow cytometer using their forward- and side-scatter properties. As shown in Figure 40c, plasmablasts have a median FSC-A that is ~1.29-1.66x

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larger than the median FSC-A of other $CD20^+$ B cells, with a median FSC-W that is 1.04-1.16x larger than resting $CD20^+$ B cells. Plasmablasts also have a median FSC-A that is 0.85-0.98x that of monocytes, and a median FSC-W that is 0.92-1.02x that of monocytes as determined by the 95% confidence interval. Here FSC-A and FSC-H could be interchangeable and equivalent as FSC-A and FSC-H are scaled on calibrated flow cytometers to give the same values. Similarly for SSC-A (and SSC-H, due to scaling) and SSC-W, plasmablasts have a median SSC-A that is 0.74-2.56x that of $CD20^+$ B cells and 0.21-0.84x that of monocytes, and a median SSC-W that is 1.01-1.20x that of $CD20^+$ B cells and 0.82-1.03x that of monocytes. The ratio of plasmablasts to B cells is representative of that to lymphocytes, as resting lymphocytes are similar in size.

[00616] An alternative approach to identify plasmablasts is to use the 20th percentile FSC or SSC of plasmablasts to the median FSC or SSC of $CD20^+$ B cells or monocytes (Figure 44D), which is 1.04-1.50x (1.02-1.11x) that of median FSC-A (FSC-W) for $CD20^+$ B cells and 0.70-0.88x (0.88-1.00x) for monocytes. Plasmablasts have a 20th percentile SSC-A (SSC-W) which is 0.67-1.89x (0.99-1.11x) that of median SSC-A (SSC-W) for $CD20^+$ B cells and 0.20-0.62x (0.77-0.99x) for monocytes. These numbers allows a gating cutoff to include 80% of plasmablasts and exclude other lymphocytes. This allows for using FSC (and/or SSC) in conjunction with single or dual color stains to gate for plasmablasts in single cell sorting plasmablasts. Such combinations may include $FSC^{hi}CD19^{lo}$ (Figure 39b), $CD19^+FSC^{hi}$ (Figure 39c) $CD19^+FSC^{hi}CD20^-$ (Figure 39d), $CD19^+FSC^{hi}CD38^{hi}$ (Figure 39e) and $CD19^+FSC^{hi}CD27^+$ (Figure 39f). Sorted cells may then undergo RT, PCR for barcoding as carried out as in "non-touchdown" PCR in the materials and methods. Downstream preparation for sequencing, cloning and expression are as follows in examples 6 and 8. Note that ratios given are that of the 95% confidence interval, or where 95% of ratios should fall within this range.

Example 56: Sorting of plasmablasts by size on any sieving device, such as a microfluidics device

[00617] Plasmablasts are blasting B cells that are activated, have proliferated/are proliferating and have undergone affinity maturation. Plasmablasts represent the active immune response and by practicing the methods and compositions herein allow for the bioinformatic construction of evolutionary trees with clonal families of antibodies that bind to target antigens of interest, whether it is an infection, a vaccine, autoimmune or cancer antigens.

[00618] Plasmablasts are blasting B cells and are larger than resting B cells. Plasmablasts and $CD20^+$ B cells were FACS sorted and stained with trypan blue to exclude dead cells and imaged at 200x magnification. 52 plasmablasts and 51 $CD20^+$ B cells were imaged and cell area

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measured with ImageJ. Plasmablasts imaged were between 7.8-13uM in diameter, and between 48-121uM^2 in area, and between in 251-996uM^3 in volume. CD20$^+$ B cells are not blasting and are smaller, the majority is between 6-8uM in diameter, or smaller than 50uM^2, or smaller than 268uM^3, with only 4 cells of 51 larger than that (Figure 41). Any sieving device that is capable of separating cells larger or smaller than 8uM in diameter or 50uM^2 in area or 268uM^3 in volume is capable of separating plasmablasts from CD20$^+$ resting B cells, with 96% of the plasmablasts captured, and sieving out 92% of the resting B cells. Such a device may be a fine sieve with 8uM diameter holes, or a microfluidics device with channels that only allows or prevents cells greater than 8uM in diameter or 50uM^2 in area or 268uM^3 in volume in passing through. These cells can then be sorted by actuators/pumps in the microfluidics device into wells such that there is only 1 or a few cell(s)/well and RT, PCR for barcoding may then be carried out as in "non-touchdown" PCR in the materials and methods using the same concentrations of reagents. Downstream preparation for sequencing, cloning and expression are as follows in examples 6 and 8.

Example 57: Anti-*Staphylococcus aureus* antibodies enhance phagocytosis of *S. aureus* by a neutrophil cell line

[00619] Humans with *S. aureus* infections who mount effective immune responses against their *S. aureus* infection, for example humans who clear *S. aureus* without the need for antibiotic therapy, are used as sources for peripheral blood from which peripheral blood plasmablasts are stained and sorted. Plasmablasts are single-cell sorted and barcoded as detailed in "Non touchdown PCR" in the materials and methods, and prepared for sequencing as detailed in "Preparing for 454 XLR70 sequencing" in the materials and methods. Evolutionary trees are bioinformatically constructed and a few select representatives of each clonal family are selected and cloned for expression as recombinant antibodies as in example 8. *S. aureus* Wood strain, which is ~5% protein A positive, is plated on 5% trypticase soy agar (TCA) blood agar and a colony grown and kept at 4°C as stock. This stock is refreshed weekly by picking another colony. 1mL of this stock is used to inoculate *S. aureus* growth till OD550 = 0.5, which is approximately mid-log growth phase. *S. aureus* is lightly fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature and washed once with Hanks balanced salt solution (HBSS), before staining with 1uM CFSE for 15minutes at room temperature. Fixed bacteria are then washed and incubated with 10ug/ml of the expressed recombinant anti-*S. aureus* antibodies, or 10ug/ml of expressed anti-influenza virus antibodies as a negative control. Bacteria are then washed twice. HL-60, a neutrophil cell line, is activated for 96hr with 25uM retinoic acid, and incubated with labeled, fixed bacteria at 1:1 to 1:100 for 45 minutes at 37°C gently shaking at

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300rpm in 96-well plates. HL-60 is then washed twice and analyzed on a flow cytometer. The amount of CFSE labeling in HL-60s is indicative of the amount of *S. aureus* phagocytosed. Some expressed anti-*S. aureus* antibodies will binding to staph cell surface proteins and opsonize the bacteria, leading to increased phagocytosis.

Example 58: Anti-*Staphylococcus aureus* antibodies enhance neutrophil-mediated killing of *S. aureus*

[00620] Humans who were able to effectively control and/or clear their *S. aureus* infections were selected as in the relevant example above, and plasmablasts were isolated and single cell sorted for sequencing and cloning and expression as in the relevant example above. An *S. aureus* clinical isolate, was plated on 5% TCA blood agar and a colony grown and kept at 4°C as stock. This stock was refreshed weekly by picking another colony. 1mL of this stock was used to inoculate *S. aureus* growth till OD550 = 0.5, which is approximately mid-log growth phase. *S. aureus* was then incubated with 2ug/ml of expressed anti- *S. aureus* antibodies for 30 minutes at 4°C before washing twice. The HL-60 neutrophil cell was activated for 96hr with 25uM of retinoic acid, and incubated with baby rabbit complement and *S. aureus* in a 1:1 to 1:100 ratio for 45 minutes at 37°C shaking at 300rpm in 96-well plates. HL-60 cells were then rapidly put on ice and washed 3x to remove loosely attached *S. aureus*. Extracellular *S. aureus* was then serially diluted and plated on 5%TSA blood agar and cultured overnight at 37°C. Colonies were counted the next day to determine the number of colony forming units (CFU). A decrease in CFUs by specific anti-*S. aureus* recombinant antibodies (after incubation with *S. aureus*) demonstrate that those antibodies were effective in mediating enhanced phagocytosis and killing, or reducing growth, of *S. aureus* by HL-60 cells (Figure 46).

Example 59: Treatment of *Staphylococcus aureus*-infected mice using expressed anti-*S. aureus* antibodies in mouse model

[00621] Anti-*S. aureus* antibodies that demonstrate *in vitro* killing, reduced growth or binding activity as in example 58 may also have *in vivo* activity. Anti-*S. aureus* antibodies with killing activity are isolated from *S. aureus*-infected humans who are able to control their staph infection as in examples 55-58. Mice are given a lethal dose of *S. aureus* and are then treated with a control antibody or a recombinant anti-*S. aureus* antibody(ies) with demonstrated *in vitro* killing, reduced growth or binding activity. Mice are deemed to be protected if they have a longer survival or reduced severity of infection as determined by the Kaplan-Meier survival test. Anti-*S. aureus* antibodies derived from humans who control or reduce the severity of their *S. aureus* infections are thereby evaluated for their ability to confer passive protection against *S. aureus*.

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Example 60: Use of the antigen targets of effective anti-*Staphylococcus aureus* immune responses to develop vaccines.

[00622] *S. aureus* antigens that are targeted by anti-*S. aureus* antibodies that exhibit killing, reduced growth or binding activity are good candidates for a *S. aureus* vaccine. Vaccinees who develop a strong response against those specific antigens may be protected against or exhibit reduced severity of infection with *S. aureus*. Anti-*S. aureus* antibodies with killing, reduced growth or binding activity are isolated from *S. aureus*-infected humans who are able to control their *S. aureus* infection, and their target antigens identified using mass spectrometry as in examples 55-58. Mice are either vaccinated with a mock vector or vaccinated with candidate *S. aureus* antigens and then boosted twice over a period of a two months. Mice may be immunized with candidate *S. aureus* antigens individually or in combination. Anti-*S. aureus* antigen antibody titer is confirmed by ELISA. Mice are then challenged with a lethal dose of *S. aureus*. Mice are deemed to be protected against *S. aureus* if they have a longer survival as determined by the Kaplan-Meier survival test. Immunization against these selected *S. aureus* antigens therefore confers protection or reduces the severity of infection, showing that the compositions and methods herein can aid in vaccine design.

Example 61: Treatment of *Staphylococcus aureus*-infected humans using recombinant anti-*S. aureus* antibodies with *in vivo* killing, reduced growth or binding activity in humans

[00623] Antibodies derived from humans who control their *S. aureus* infection and exhibit *in vitro* and *in vivo* killing, reduced growth or binding activity as in examples 55-59 may be used to treat *S. aureus*-infected patients. Antibodies are obtained and tested for *in vitro* and *in vivo* killing activity as in examples 55-59. Good Manufacturing Practice (GMP) manufactured anti-*S. aureus monoclonal antibodies* may be given intravenously or subcutaneously to *S. aureus*-infected humans, especially patients infected with methicillin resistant *S. aureus* (MRSA) or other strains of drug-resistant-*S. aureus*, and compared to antibiotics along for efficacy. The anti-*S. aureus* antibodies are deemed to have therapeutic utility if patients are protected against invasive *S. aureus* infections, have less severe *S. aureus* infections, and/or recover more rapidly than patients given antibiotics alone or not given antibiotics. Recombinant anti-*S. aureus* antibodies can be given therapeutically to patients with active *S. aureus* infections to reduce the severity of infection and/or to enhance clearance of the infection as well as prophylactically to high-risk patient populations, such as patients on hemodialysis for renal failure, patients admitted to the hospital, or patients with a positive-screen for *S. aureus* or MRSA.

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Example 62: *Staphylococcal aureus* vaccination using identified *S. aureus* antigens to confer protective immunity in humans

[00624] *S. aureus* antigens that are targeted by anti-*S. aureus* antibodies that exhibit killing or binding activity *in vivo*, and when vaccinated against in a mouse model confer protection against *S. Aureus* challenge, may be good candidates for a prophylactic vaccine in humans. Anti-*S. aureus* antibodies are derived from humans who control their *S. aureus* infections and are cloned, expressed and tested for *in vivo* killing, reduced growth or binding activity and as vaccine candidates in mice as in examples 55-58 and 59. Humans are then given the *S. aureus* vaccine containing *S. aureus* antigens that are the targets of anti-*S. aureus* antibodies with killing, reduced growth or binding activity, with a placebo being the control. The cohorts are tracked for their incidence or severity of *S. aureus* infections. The vaccine is deemed successful if it lowers the vaccinated cohort's incidence or severity of *S. aureus* infections compared to the placebo cohort.

Example 63: Monitoring of immune responses induced by candidate *Staphylococcus aureus* vaccines as a correlate of protection.

[00625] After immunization of humans with a candidate *S. aureus* vaccine as in example 62, the vaccine response may be monitored by determining if robust clonal families against the target *S. aureus* antigens of interest is elicited. Blood is drawn between 7-14 days post-vaccination and plasmablasts are single cell sorted, barcoded and 454 sequenced as detailed in "non-touchdown PCR" and "preparing for 454 XLR70 sequencing" in the materials and methods. Evolutionary trees are drawn and 2-3 members of each clonal family are then cloned and expressed as in example 8 and tested for their binding to the staph antigens of interest in an ELISA. We expect that humans who have a strong vaccine-induced anti-*S. aureus* immune response will exhibit large clonal families against the *S. aureus* antigens targeted in effective human immune responses. Such an approach has the potential to provide a correlate of protection for a *S. aureus* vaccine and in doing so enable clinical trials and development to be streamlined. This antibody and/or TCR immune repertoire monitoring would enable rapid assessment of the likelihood that a candidate vaccine would provide efficacy.

Example 64: Treatment of mice with lung-adenocarcinoma using recombinant anti-lung adenocarcinoma antibodies.

[00626] The anti-lung adenocarcinoma antibody that binds to a cell surface protein or other lung adenocarcinoma proteins may be useful as a carrier to target toxins to lung adenocarcinoma cells or to target other molecules expressed by lung adenocarcinoma cells. Anti-lung adenocarcinoma antibodies with cell surface binding activity or other lung adenocarcinoma

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antigens are isolated from a long-term non-progressor lung adenocarcinoma cancer patient(s) as in example 11. Nude mice are given a subcutaneous injection of H1650 lung adenocarcinoma cell line and the tumor allowed to grow for one week. Anti-lung adenocarcinoma antibody is then conjugated to a toxin, such as diphtheria toxin lacking the R-domain, which is the cell-binding domain and allows diphtheria toxin into the cell. Diphtheria toxin lacking R-domain therefore is lethal only to lung adenocarcinoma cells which the antibody binds to and delivers the diphtheria toxin payload. Control antibody conjugated to diphtheria toxin without R domain is used as the control. The lung-adenocarcinoma antibody is deemed to have successfully delivered its payload to kill adenocarcinoma cells if the tumor load decreases more than in the control. Alternatively, in certain cases the recombinant antibody itself may be able to mediate tumor cell killing or to prevent tumor cell growth (in the absence of a conjugated toxin).

Example 65: Treatment of lung adenocarcinoma patients using expressed anti-lung adenocarcinoma antibody.

[00627] The anti-lung adenocarcinoma antibody that binds to cell surface antigen may be useful as a carrier to target toxins to lung adenocarcinoma cells. Anti-lung adenocarcinoma antibodies with cell surface binding activity are isolated from a long-term non-progressor lung adenocarcinoma cancer patient as in example 11. GMP monoclonal antibody or other anti-lung adenocarcinoma monoclonal antibodies may be given intravenously or subcutaneously to lung adenocarcinoma patients, especially to patients whose biopsied adenocarcinoma cells expressed high levels of the cell surface antigen targeted by the monoclonal antibody. The recombinant monoclonal antibody(ies) lung adenocarcinoma antigen(s), or other members of the clonal families from which they are derived, can be used to immunohistochemically stain a biopsy specimen of an individual patient's lung adenocarcinoma to gain information on tumor antigen expression levels, and this information can be used to determine whether an individual patient is likely to respond to therapy with this monoclonal antibody. Anti-lung adenocarcinoma antibodies can be conjugated to a toxin, such as diphtheria toxin lacking the R-domain, which is the cell-binding domain and allows diphtheria toxin into the cell. Diphtheria toxin lacking R-domain therefore is lethal only to lung adenocarcinoma cells which the antibody binds to and delivers the diphtheria toxin payload. Standard of care chemotherapy is used for treatment of the comparator group. The anti-adenocarcinoma antibodies are deemed to have delivered their payload and have therapeutic utility if patients survive longer or exhibit longer times prior to relapse or progression. Alternatively, in certain cases the recombinant antibody itself, against lung adenocarcinoma antigens, may be able to mediate tumor cell killing or prevent tumor cell growth (in the absence of a conjugated toxin).

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Example 66: Lung adenocarcinoma therapeutic vaccination using identified antigens in humans.

[00628] The cell surface antigens bound by the anti-lung adenocarcinoma antibodies may be used in a therapeutic vaccine to treat established, or to protect against development of, lung adenocarcinoma. Anti-lung adenocarcinoma antibodies with cell surface binding activity are isolated from a long-term non-progressor lung adenocarcinoma cancer patient(s) as in example 11, and the target antigen identified using immunoprecipitation or immunoblotting and mass spectrometry. Vaccinees are given either a vaccine containing the lung adenocarcinoma antigen(s) of interest or a control vaccine. Cohorts are then followed and their incidence or progression of lung adenocarcinoma tracked. The vaccine is deemed to be successful if humans vaccinated with the target antigen have prolonged survival or extended time to relapse compared to the standard-of-care comparator group.

Example 67: Immune monitoring of lung adenocarcinoma vaccination for efficacy of response.

[00629] After immunization of humans with lung adenocarcinoma vaccine as in example 66, the vaccine response may be monitored by determining if robust clonal families against adenocarcinoma antigen(s) of interest in the vaccine are elicited. Blood is drawn between 7-14 days post-vaccination and plasmablasts are single cell sorted and barcoded, and 454 sequencing is performed as detailed in "non-touchdown PCR" and "preparing for 454 XLR70 sequencing" in the materials and methods. Evolutionary trees are drawn and 2-3 members of each clonal family are then cloned and expressed as in example 8 and tested for their binding to the staph antigens of interest in an ELISA. We expect that humans who have a strong immune response will have large clonal families against lung adenocarcinoma antigen(s) of interest and/or many clonal families against the adenocarcinoma antigen(s) of interest. This immune monitoring allows us to rapidly predict the efficacy of a candidate vaccine.

Example 68. Use of Superscript III for template switching during reverse transcription.

[00630] In our methods, the sample-identification region and adaptor regions are added on during reverse transcriptase. This utilizes the 3' tailing activity and template switching activity of RNase H$^-$ reverse transcriptases. Most frequently, a reverse transcriptase such as Superscript II (Invitrogen) is used at its working temperature of 42°C. MMLV H$^-$ reverse transcriptases that have also been engineered for thermal stability, such as Superscript III, with a recommended working temperature of 50°C, have been reported not to have this 3' tailing activity and therefore no template switching ability

(http://tools.invitrogen.com/content/sfs/ProductNotes/F_Superscript%20III%20Enzyme%20RD-

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MKT-TL-HL0506021.pdf?ProductNoteId=36). However, in Figure 42, we showed that Superscript III does have 3' tailing and template switching activity. This property is weak at 50°C, the recommended reverse transcription temperature for Superscript III, and may explain why the 3' tailing activity of Superscript III has not been reported before. However, 3' tailing activity and template switching increases significantly as the RT temperature was lowered from 50°C to 45.5°C to 42°C. We would expect all MMLV RNase H- reverse transcriptases that have been engineered for thermal stability to also have 3' tailing activity at lower working temperatures, i.e. between 42°C to 50°C.

Example 69. Analysis of co-expressed genes to identify antibodies associated with memory B cell and plasma cell responses as well as homing to specific tissues.

[00631] Barcoding of all the cDNA produced by B cells, T cells or other cells sorted into individual wells as described by the methods and compositions herein enables characterization of gene co-expression in plasmablasts, other B cells, T cells and other cells at the single cell level. This enables use of co-expressed genes to identify the specific antibodies and TCRs expressed by B and T cells that have been induced to differentiate into memory B cells, plasma cells, memory T cells, specific types of effector T cells (for example, Th1, Th2, Th17 or T-regulatory T cells) or induced to home to a specific tissue or site (for example, the gastrointestinal tract, skin, or brain). The barcoding of all cDNA produced by the individual cell or collection of cells in a specific sample enables use of additional 3' PCR primers for both 1st and 2nd PCR to characterize the co-expression of specific such genes. 5' primers remain the same as those used to amplify variable regions genes. Furthermore, analysis of co-expressed genes enables bioinformatic analysis of the relationships between the affinity maturation of clonal families and the co-expression of genes associated with the differentiation of B cells to memory B cells, short-lived plasmablasts, and long-lived plasma cells (Table 34), the differentiation of naïve or memory T cells to Tregs or Th1, Th2, Th17 cells (Table 35), or the homing of B or T cells to specific sites. Such analysis can further pinpoint the critical antibodies or TCRs mediating an effective immune response.

[00632] For example, PMBCs derived from individuals mounting immune response are used to single cell sort plasmablasts. The methods and compositions herein are used to analyze co-expression of genes associated with homing of plasmablasts into different tissues (see Table 36). Bioinformatic analysis of the datasets identifies antibodies associated with secretion at different bodily locations. These antibody genes are then recombinantly expressed for characterization in in vitro screening assays as in Example 8.

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[00633] It will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the various aspects of the invention.

[00634] It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

[00635] All references, issued patents and patent applications cited within the body of the instant specification are hereby incorporated by reference in their entirety, for all purposes.

TABLES

Table 1 - Primers and adapter molecules

seq id no	DESCRIPTION	sequence
	Sample-ID adaptor	CACGACCGGTGCTCGATTTAG(sample-ID)GGG
	FW long primer1	GAGAGACTGACAGCGTATCGCCTCCCTCGCGCCATCAG (plate-ID)CACGACCGGTGCTCGATTTAG
	FW short primer1	GAGAGACTGACAGCGTATCGCCTC
	kappa GSP1	CGATTGGAGGGCGTTATCCAC
	lambda GSP1	TYTGTGGGACTTCCACTGCTC
	gamma GSP1	TCTTGTCCACCTTGGTGTTGCTG
	FW primer2	CGTATCGCCTCCCTCGCG
	kappa GSP long primer2	CTATGCGCCTTGCCAGCCCGCTCAG(plate-ID)TCAGATGGC GGGAAGATGAAGAC
	lambda GSP long primer2	CTATGCGCCTTGCCAGCCCGCTCAG(plate-ID)GAGGAGGGY GGGAACAGAGTGAC
	gamma GSP long primer2	CTATGCGCCTTGCCAGCCCGCTCAG(plate-ID)GGAAGTAGT CCTTGACCAGGCAG
	kappa GSP 2s	CTATGCGCCTTGCCAGCCCGCTCAGTCAGATGGCGGGAAGATGAAGAC
	lambda GSP 2s	CTATGCGCCTTGCCAGCCCGCTCAGGAGGAGGGY GGGAACAGAGTGAC
	gamma GSP 2s	CTATGCGCCTTGCCAGCCCGCTCAGGGAAGTAGTCCTTGACCAGGCAG
	RV primer2	CTATGCGCCTTGCCAGCCC

*For sample-ID sequences , see Table 2. For plate-ID sequences, see Table 3. Kappa GSP 2s, lambda GSP 2s and gamma GSP 2s are identical to the kappa, lambda and gamma GSP long primer 2 except that they do not have a plate-ID sequence. The plate-ID sequence is not necessary when doing XL+ runs, or when only forwards reads are desired when doing XLR70 runs with the Titanium LibA chemistry.

**primers sequences were designed to be able to amplify all different constant gene variants as found in IMGT database (http://imgt.cines.fr/).

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Table 2 – Sample-ID			
Well	SampleID	Sequence of Sample-ID	SEQ ID NO
A1	1	ACGTCTCATCA	
A2	2	ACTCATCTACA	
A3	3	AGAGCGTCACA	
A4	4	AGTAGTGATCA	
A5	5	ATAGATAGACA	
A6	6	ATCTACTGACA	
A7	7	CACGTGTCGCA	
A8	8	CATACTCTACA	
A9	9	CGAGACGCGCA	
A10	10	CGTCGATCTCA	
A11	11	CTACGACTGCA	
B1	12	TAGTGTAGATA	
B2	13	TCTAGCGACTA	
B3	14	TGTGAGTAGTA	
B4	15	ACAGTATATAA	
B5	16	AGCTCACGTAA	
B6	17	TCGATAGTGAA	
B7	18	TCGCTGCGTAA	
B8	19	TGAGTCAGTAA	
B9	20	TGTAGTGTGAA	
B10	21	TGTCGTCGCAA	
B11	22	ACGACAGCTCA	
C1	23	TACACGTGATTAGGGATT	
C2	24	TACAGATCGTTAGGGAAA	
C3	25	TAGTGTAGATTTGGGTTT	
C4	26	TCTAGCGACTTTGGGTTT	
C5	27	ACGCGATCGAAGGGTTT	
C6	28	AGCTCACGTATTGGGTTT	
C7	29	AGTGCTACGAAGGGAAA	
C8	30	TCTGACGTCAAAGGGAAA	
C9	31	ACGTCTCATCAAGGAAGGAA	
C10	32	TATAGACATCAACACACAAA	
C11	33	AGTGAGCTCGTTGGGTTT	
D1	34	ATCGTCTGTGTTGGGTTT	
D2	35	CACACGATAGTTGGGTTT	
D3	36	CTGCGTCACGATCTCTCTT	
D4	37	TAGCATACTGTTGGGTTT	
D5	38	TATCTGATAGTTGGGTTT	
D6	39	TGACATCTCGCAGTTCTTT	
D7	40	TGATAGAGCGTAACAACAGA	

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D8	41	TCACGCGAGAAAGGGAAA	
D9	42	ACACATACGCAGGGAAA	
D10	43	ACTAGCAGTAA	
D11	44	CGCAGTACGAA	
E1	45	TGAGTCAGTAAGGGAAA	
E2	46	ACTCATCTACAGGGAAA	
E3	47	ACTCGCGCACAGAGAGAA	
E4	48	AGAGCGTCACAGAGAGAA	
E5	49	AGCGACTAGCAACACACAAA	
E6	50	ATCTACTGACAACACACAA	
E7	51	CATACTCTACAACACACAAA	
E8	52	TCGAGCTCTCAGAGAGAAA	
E9	53	AGAGAGTGTGTTGGGTTT	
E10	54	ATCGTAGCAGAACACACAAA	
E11	55	CACTCGCACGTTGGGTTT	
F1	56	CAGACGTCTGAACACACAAA	
F2	57	CTCGATATAGTTGGGTTT	
F3	58	TCTGATCGAGAAGGGAAA	
F4	59	TACACACACTTAGGGATT	
F5	60	TACGTCATCAGGGAAA	
F6	61	CTACGCTCTAAGGGAAA	
F7	62	TAGTCGCATAAAGGGAAA	
F8	63	CGATCGTATAA	
F9	64	CGCGTATACAA	
F10	65	CTACGCTCTAA	
F11	66	TCACGCGAGAA	
G1	67	AGTATACATATTGGGTTT	
G2	68	TCGATAGTGAAGGGAAA	
G3	69	TCGCTGCGTAAAGGAGAAA	
G4	70	TGTAGTGTGAAGGGAAA	
G5	71	TGTCGTCGCAAGAGAGAG	
G6	72	CTACGACTGCAAGGGAAA	
G7	73	CTCTACGCTCA	
G8	74	TAGCTCTATCA	
G9	75	TATAGACATCA	
G10	76	TCACTCATACA	
G11	77	CTAGTCACTCAAGGGAAA	
H1	78	TGTGAGTAGTTTGGGTTT	
H2	79	TGTCACACGAAGGGAAA	
H3	80	CTGTGCGTCGAAGGGAAA	
H4	81	TAGTGTAGATTCGC	
H5	82	TCGAGCTCTCTCGC	

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H6	83	ATCACGTGCGTCGC	
H7	84	CAGACGTCTGTCGC	
H8	85	TATCACTCAGTCGC	
H9	86	TGCTATAGACTTGGGTTT	
H10	87	CAGTACTGCGTTGGGTTT	
H11	88	CGACAGCGAGAACACACAAA	
A12-H12	89	TATGCTAGTAA (negative control)	

Table 3 – Plate-ID		
Plate	Sequence of Plate-ID	SEQ ID NO
1	ACGAGTGCGT	
2	ACGCTCGACA	
3	AGACGCACTC	
4	AGCACTGTAG	
5	ATCAGACACG	
6	ATATCGCGAG	
7	CGTGTCTCTA	
8	CTCGCGTGTC	
9	TGATACGTCT	
10	CATAGTAGTG	
11	CGAGAGATAC	
12	ATACGACGTA	
13	TCACGTACTA	
14	CGTCTAGTAC	
15	TCTACGTAGC	
16	TGTACTACTC	
17	CGTAGACTAG	
18	TACGAGTATG	
19	TACTCTCGTG	
20	TAGAGACGAG	
21	TCGTCGCTCG	
22	ACATACGCGT	
23	ACGCGAGTAT	
24	ACTACTATGT	
25	ACTGTACAGT	
26	AGACTATACT	
27	AGCGTCGTCT	
28	AGTACGCTAT	
29	ATAGAGTACT	
30	CACGCTACGT	
31	CAGTAGACGT	

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32	CGACGTGACT	
33	TACACACACT	
34	TACACGTGAT	
35	TACAGATCGT	
36	TACGCTGTCT	
37	TAGTGTAGAT	
38	TCGATCACGT	
39	TCGCACTAGT	
40	TCTAGCGACT	
41	TCTATACTAT	
42	TGACGTATGT	
43	TGTGAGTAGT	
44	ACAGTATATA	
45	ACGCGATCGA	
46	ACTAGCAGTA	
47	AGCTCACGTA	
48	AGTATACATA	
49	AGTCGAGAGA	
50	AGTGCTACGA	
51	CGATCGTATA	
52	CGCAGTACGA	
53	CGCGTATACA	
54	CGTACAGTCA	
55	CGTACTCAGA	
56	CTACGCTCTA	
57	CTATAGCGTA	
58	TACGTCATCA	
59	TAGTCGCATA	
60	TATATATACA	

Table 4 - Cloning primers

seq id no	DESCRIPTION	sequence
	Clon_PacI	ACTGTTAATTAA(sample-ID)
	Clon_AscI	ATTAGGCGCGCC(sample-ID)
	Clon_FseI	ATTAGGCCGGCC(sample-ID)
	Clon_AsiSI	ATTAGCGATCGC(sample-ID)
	K_NheIa_DHFR	ACGTGCTAGCAGTTCCAGATTTCAACTGCTCATCAGA
	K_XhoId_DHFR	ACGTCTCGAGGATAGAAGTTATTCAGCAGGCACACAACA

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	L_XhoI_PspXI_DHFR	ACTTGCTCGAGTCTGCYTTCCARGCMACTGT
	L_NheI_DHFR	AGTCGCTAGCCGCRTACTTGTTGTTGCTYTGTTTG
	G_EcoRI_DHFR	AGTCGAATTCCACGACACCGTCACCGGTT
	G_SacII_DHFR	ATTACCGCGGGGAAGGTGTGCACGCCG
	G_XhoI_PspXI_Lonza	ACGTCTCGAGGGTGCCAGGGGGAAGACCGATG
	G_AgeI_Lonza	ACTGACCGGTTCGGGGAAGTAGTCCTTGACCAGGCA
	G_EcoRI_Lonza	TGCAGAATTCCACGACACCGTCACCG
	G_ApaI_Lonza	TGTAGGGCCCTGAGTTCCACGACACCGTC
	L_XmaI_Lonza	TGATCCCGGGATAGAAGTCACTKATSAGRCACACYAGTGTGG
	L_BstEII_Lonza	TGCAGGTCACCGCTCCCGGGTAGAAGTCACTKATSAGR
	L_XhoI_PspXI_v2_Lonza	TGATGCTCGAGTCTGCYTTCCARGCMACTGTC
	K_XmaI_Lonza	TAGTCCCGGGGATAGAAGTTATTCAGCAGGCACAC

*Cloning forward primers start with a 5' flanking restriction site and end with sample-ID sequences on the 3' end. This enables cloning primers to discriminate between sequences with different well origins and selectively amplify amplicons with specific sample-ID sequences. Therefore, there are multiple cloning forward primers, each specific for particular sample-ID(s). The 3' sequences of the cloning forward primer are complementary to the well-ID and are provided in Table 5. Primers with names starting with "Clon" are the forward primers. Primers with names starting with "K", "L" or "G" are the reverse primers that are constant region specific for kappa, lambda and gamma chains respectively. The name of the reverse primers also denote the restriction site that the primer will incorporate. Finally, "DHFR" or "Lonza" denotes whether the constant region primers are for the vector set pcDNA3.3 and pOptivec or Lonza vectors pEE12.4 and pEE6.4 respectively, with constant region inserts added in.

Table 5 - Cloning Primers Well-Specific Sequence

Well	Sequence	SEQ ID NO
A1	GGTGCTCGATTTAGACGTCTCATCAG	
A2	CGGTGCTCGATTTAGACTCATCTACAG	
A3	GTGCTCGATTTAGAGAGCGTCACAG	
A4	CGGTGCTCGATTTAGAGTAGTGATCA	
A5	ACCGGTGCTCGATTTAGATAGATAGACA	
A6	CGGTGCTCGATTTAGATCTACTGACAG	
A7	CTCGATTTAGCACGTGTCGCA	
A8	CGGTGCTCGATTTAGCATACTCTACA	
A9	CGATTTAGCGAGACGCGCA	
A10	TGCTCGATTTAGCGTCGATCTCA	
A11	GTGCTCGATTTAGCTACGACTGCA	

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B1	GACCGGTGCTCGATTTAGTAGTGTAGATAG	
B2	CGGTGCTCGATTTAGTCTAGCGACTAG	
B3	ACCGGTGCTCGATTTAGTGTGAGTAGTAG	
B4	CGACCGGTGCTCGATTTAGACAGTATATAA	
B5	GGTGCTCGATTTAGAGCTCACGTAAG	
B6	CGGTGCTCGATTTAGTCGATAGTGAA	
B7	TGCTCGATTTAGTCGCTGCGTAAG	
B8	CGGTGCTCGATTTAGTGAGTCAGTAA	
B9	CGGTGCTCGATTTAGTGTAGTGTGAA	
B10	GCTCGATTTAGTGTCGTCGCAA	
B11	GTGCTCGATTTAGACGACAGCTCA	
C1	CGGTGCTCGATTTAGTACACGTGATT	
C2	CGGTGCTCGATTTAGTACAGATCGTT	
C3	GACCGGTGCTCGATTTAGTAGTGTAGATTT	
C4	CGGTGCTCGATTTAGTCTAGCGACTTT	
C5	GCTCGATTTAGACGCGATCGAA	
C6	GGTGCTCGATTTAGAGCTCACGTATT	
C7	GGTGCTCGATTTAGAGTGCTACGAA	
C8	GGTGCTCGATTTAGTCTGACGTCAA	
C9	GGTGCTCGATTTAGACGTCTCATCAA	
C10	ACCGGTGCTCGATTTAGTATAGACATCAA	
C11	GGTGCTCGATTTAGAGTGAGCTCGT	
D1	GGTGCTCGATTTAGATCGTCTGTGT	
D2	GGTGCTCGATTTAGCACACGATAGT	
D3	GCTCGATTTAGCTGCGTCACGA	
D4	CCGGTGCTCGATTTAGTAGCATACTGT	
D5	GACCGGTGCTCGATTTAGTATCTGATAGT	
D6	GTGCTCGATTTAGTGACATCTCGC	
D7	CGGTGCTCGATTTAGTGATAGAGCGT	
D8	GCTCGATTTAGTCACGCGAGAAA	
D9	GGTGCTCGATTTAGACACATACGCA	
D10	CCGGTGCTCGATTTAGACTAGCAGTAA	
D11	TGCTCGATTTAGCGCAGTACGAA	
E1	CGGTGCTCGATTTAGTGAGTCAGTAA	
E2	CGGTGCTCGATTTAGACTCATCTACAG	
E3	GCTCGATTTAGACTCGCGCACA	
E4	GTGCTCGATTTAGAGAGCGTCACAG	
E5	GGTGCTCGATTTAGAGCGACTAGCA	
E6	CGGTGCTCGATTTAGATCTACTGACAA	
E7	CGGTGCTCGATTTAGCATACTCTACA	
E8	GTGCTCGATTTAGTCGAGCTCTCAG	
E9	CGGTGCTCGATTTAGAGAGAGTGTGT	

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E10	GGTGCTCGATTTAGATCGTAGCAGA	
E11	GCTCGATTTAGCACTCGCACGT	
F1	TGCTCGATTTAGCAGACGTCTGAA	
F2	CGGTGCTCGATTTAGCTCGATATAGT	
F3	GGTGCTCGATTTAGTCTGATCGAGA	
F4	CGGTGCTCGATTTAGTACACACACTT	
F5	CGGTGCTCGATTTAGTACGTCATCA	
F6	CGGTGCTCGATTTAGCTACGCTCTAA	
F7	CGGTGCTCGATTTAGTAGTCGCATAA	
F8	GGTGCTCGATTTAGCGATCGTATAA	
F9	GGTGCTCGATTTAGCGCGTATACAA	
F10	CGGTGCTCGATTTAGCTACGCTCTAA	
F11	GCTCGATTTAGTCACGCGAGAAG	
G1	ACGACCGGTGCTCGATTTAGAGTATACATAT	
G2	CGGTGCTCGATTTAGTCGATAGTGAA	
G3	GCTCGATTTAGTCGCTGCGTAAA	
G4	CGGTGCTCGATTTAGTGTAGTGTGAA	
G5	GCTCGATTTAGTGTCGTCGCAA	
G6	GTGCTCGATTTAGCTACGACTGCA	
G7	GGTGCTCGATTTAGCTCTACGCTCA	
G8	CCGGTGCTCGATTTAGTAGCTCTATCA	
G9	ACCGGTGCTCGATTTAGTATAGACATCAG	
G10	CGGTGCTCGATTTAGTCACTCATACA	
G11	CGGTGCTCGATTTAGCTAGTCACTCA	
H1	CGGTGCTCGATTTAGTGTGAGTAGTTT	
H2	GGTGCTCGATTTAGTGTCACACGAA	
H3	GCTCGATTTAGCTGTGCGTCGA	
H4	GACCGGTGCTCGATTTAGTAGTGTAGATTC	
H5	GGTGCTCGATTTAGTCGAGCTCTCTC	
H6	TGCTCGATTTAGATCACGTGCGT	
H7	GTGCTCGATTTAGCAGACGTCTGTC	
H8	CCGGTGCTCGATTTAGTATCACTCAGT	
H9	ACCGGTGCTCGATTTAGTGCTATAGACT	
H10	GGTGCTCGATTTAGCAGTACTGCGT	
H11	GCTCGATTTAGCGACAGCGAGA	

TABLE 6

SEQ ID NO	DESCRIPTION	SEQUENCE
	For kappa	
	IgKC_v3	CAGATGGCGGGAAGATGAAGAC
	For Lambda	
	IgLC_v5	CTCCCGGGTAGAAGTCAC
	IgLC_v6	TCCCGGGTAGAAGTCAC

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	IgLC v7	GCTCCCGGGTAGAAGTC
	IgLC v8	AGTGTGGCCTTGTTGG
	For gamma	
	IgHGC v10	GCCAGGGGGAAGACCGATG
	IgHGC v11	CAGGGGGAAGACCGATG
	IgHGC v13	AAGTAGTCCTTGACCAGGC
	IgHGC v15	GAAGACCGATGGGCCCTTGG
	IgHGC v16	AAGACCGATGGGCCCTTG
	Adaptor_v1	G
	Adaptor_v2	GGGGG
	Adaptor_v3	rGrGrG
	Univ seq 2	AACGCGTGACGAGAGACTGACAG
	Univ seq 4	TTGTTGCGTTCCTAGCCGCTATAG
	Univ seq 5	CTCTACGACCGGTGCTCGATTTAG
	Univ seq e	CCGTCGGTCGGCAGTG
	Kappa light chain specific	
	IgKC v4	ATGGCGGGAAGATGAAGAC
	K GSP1	GTGCTGTCCTTGCTGTCCTGCT
	K GSP1c	AGCAGGCACACAACAGAG
	K GSP1e	TTGTGTTTCTCGTAGTCTGCTTTGC
	K GSP1f	TCTCCCCTGTTGAAGCTCTTTGTG
	IgLC v5	CTCCCGGGTAGAAGTCAC
	L GSP1	ATCTGCCTTCCAGGCCACTGTC
	L GSP1c	CTCCCGGGTAGAAGTCAC
	L GSP1d	ACRGCTCCCGGGTAGAAGTCAC
	L GSP1f	TCCACGGTGCTCCCTTCAT
	L GSP1g	GGCCGCRTACTTGTTGTTGC
	L GSP1h	GCCTTCCAGGCCACTGTCAC
	L GSP1i	CTGCCTTCCAGGCCACTGTC
	L GSP1j	CTCCACGGTGCTCCCTTCA
	L GSP1k	GCTCCCTTCATGCGTGACC
	L GSP1l	TCTGTGGGACTTCCACTGCTC
	L GSP1m	GGGGCCACTGTCTTCTCCA
	L GSP1n	CTTCTGTGGGACTTCCACTGCT
	L GSP1o	ATCTGCCTTCCAGGCCACTGT
	L GSP1x	CTTYTGTGGGACTTCCACTGCTC
	L GSP1y	GCTTYTGTGGGACTTCCACTGCTC
	IgHGC v13	AAGTAGTCCTTGACCAGGC
	G GSP1c	TTCCACGACACCGTCAC
	G GSP1d	CACGCCGCTGGTCAG
	G GSP1g	GCTGCTGAGGGAGTAGAGTCCTGA
	G GSP1h	TCTTGTCCACCTTGGTGTTGCT
	G GSP1k	GCTGGAGGGCACGGTCAC
	G GSP1l	TCTTGTCCACCTTGGTGTTGCTG
	G GSP1m	TCTTGTCCACCTTGGTGTTGCT
	G GSP1n	GACTGTAGGACAGCCGGGAAGG
	G GSP1o	ACCACGCTGCTGAGGGAGTAG
	G GSP1p	TTGTCCACCTTGGTGTTGCTG
	G GSP1q	TGAGTTCCACGACACCGTCAC
	G GSP1t	GAGTTCCACGACACCGTCACC

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	Kappa specific	
	K_GSP2	ATGGCGGGAAGATGAAGAC
	K_GSP2v2a	ATGGCGGGAAGATGAAGAC
	K_GSP2v2b	TGGCGGGAAGATGAAGAC
	K_GSP2v2d	CGGAAGATGAAGACAGATGGT
	K_GSP2v2e	GCAGTTCCAGATTTCAACTG
	K_GSP2v2f	ATGGTGCAGCCACAGTT
	K_GSP2v2c	CAGATTTCAACTGCTCATCAGAT
	K_GSP2v2g	TCAGATGGCGGGAAGATGAAGAC
	Lambda specific	
	L_GSP2	CTCCCGGGTAGAAGTCAC
	L_GSP2v2c	AGGGYGGGAACAGAGTGAC
	L_GSP2v2	CTCCCGGGTAGAAGTCAC
	L_GSP2v2d	GAGGAGGGYGGGAACAGAGTGAC
	Gamma specific	
	G_GSP2v2c1	GCCAGGGGGAAGACCG
	G_GSP2v2c2	GGAAGTAGTCCTTGACCAGG
	G_GSP2b	GGAAGTAGTCCTTGACCAGGCAG
	G_GSP2	AAGTAGTCCTTGACCAGGC

Table 7	
Plate-ID	SEQ ID NO
TATGCTAGTA	
TCACGCGAGA	
TCGATAGTGA	
TCGCTGCGTA	
TCTGACGTCA	
TGAGTCAGTA	
TGTAGTGTGA	
TGTCACACGA	
TGTCGTCGCA	
ACACATACGC	
ACAGTCGTGC	
ACATGACGAC	
ACGACAGCTC	
ACGTCTCATC	
ACTCATCTAC	
ACTCGCGCAC	
AGAGCGTCAC	
AGCGACTAGC	
AGTAGTGATC	
AGTGACACAC	
AGTGTATGTC	

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ATAGATAGAC	
ATATAGTCGC	
ATCTACTGAC	
CACGTAGATC	
CACGTGTCGC	
CATACTCTAC	
CGACACTATC	
CGAGACGCGC	
CGTATGCGAC	
CGTCGATCTC	
CTACGACTGC	
CTAGTCACTC	
CTCTACGCTC	
CTGTACATAC	
TAGACTGCAC	
TAGCGCGCGC	
TAGCTCTATC	
TATAGACATC	
TATGATACGC	
TCACTCATAC	
TCATCGAGTC	
TCGAGCTCTC	
TCGCAGACAC	
TCTGTCTCGC	
TGAGTGACGC	
TGATGTGTAC	
TGCTATAGAC	
TGCTCGCTAC	
ACGTGCAGCG	
ACTCACAGAG	
AGACTCAGCG	
AGAGAGTGTG	
AGCTATCGCG	
AGTCTGACTG	
AGTGAGCTCG	
ATAGCTCTCG	
ATCACGTGCG	
ATCGTAGCAG	
ATCGTCTGTG	
ATGTACGATG	
ATGTGTCTAG	
CACACGATAG	
CACTCGCACG	
CAGACGTCTG	

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CAGTACTGCG	
CGACAGCGAG	
CGATCTGTCG	
CGCGTGCTAG	
CGCTCGAGTG	
CGTGATGACG	
CTATGTACAG	
CTCGATATAG	
CTCGCACGCG	
CTGCGTCACG	
CTGTGCGTCG	
TAGCATACTG	
TATACATGTG	
TATCACTCAG	
TATCTGATAG	
TCGTGACATG	
TCTGATCGAG	
TGACATCTCG	
TGAGCTAGAG	
TGATAGAGCG	
TGCGTGTGCG	
TGCTAGTCAG	
TGTATCACAG	
TGTGCGCGTG	

Table 8	
Sample-ID	SEQ ID NO
ACGAGTGCGT	
TAGACTGCAC	
TAGCGCGCGC	
TCATCGAGTC	
TCGCAGACAC	
TCTGTCTCGC	
TGATACGTCT	
TGAGTGACGC	
TGCTCGCTAC	
ACGTGCAGCG	
ACTCACAGAG	
AGACTCAGCG	
AGCTATCGCG	
AGTCTGACTG	
ATAGCTCTCG	
CATAGTAGTG	
CGATCTGTCG	

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CGCGTGCTAG	
CGCTCGAGTG	
CGAGAGATAC	
TGAGCTAGAG	
ATACGACGTA	
TGCGTGTGCG	
TGCTAGTCAG	
TGTATCACAG	
TGTGCGCGTG	
TCACGTACTA	
CGTCTAGTAC	
TCTACGTAGC	
TGTACTACTC	
ACGCTCGACA	
ACGACTACAG	
CGTAGACTAG	
TACTCTCGTG	
TAGAGACGAG	
TCGTCGCTCG	
ACATACGCGT	
ACGCGAGTAT	
ACTGTACAGT	
AGACGCACTC	
AGACTATACT	
AGCGTCGTCT	
AGTACGCTAT	
ATAGAGTACT	
CACGCTACGT	
CAGTAGACGT	
CGACGTGACT	
AGCACTGTAG	
TACGCTGTCT	
TCGATCACGT	
TCGCACTAGT	
TCTATACTAT	
ATCAGACACG	
AGTCGAGAGA	
CGTACAGTCA	
CGTACTCAGA	
ATATCGCGAG	
CTATAGCGTA	
TATATATACA	
CGTGTCTCTA	
ACAGTCGTGC	

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CTCGCGTGTC	
AGTGACACAC	
ATATAGTCGC	
CACGTAGATC	
CGACACTATC	
CTGTACATAC	

Table 9 - Primers to ligate on adaptors for XL+ sequencing		
seq id no	DESCRIPTION	sequence
	5LIB-LA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTATCGCCTCCCTCGCGCCAT
	5LIB-LB	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCGTATCGCCTCCCTCGCGCCAT
	3LIB-LA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATGCGCCTTGCCAGCCCGCTCA
	3LIB-LB	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCTATGCGCCTTGCCAGCCCGCTCA

Table 10 - 3' primers for other human genes		
seq id no	DESCRIPTION	sequence
	mu constant region specific	
	mu GSP1	CTCTCAGGACTGATGGGAAGCC
	mu GSP2	CTATGCGCCTTGCCAGCCCGCTCAGGGGAATTCTCACAGGAGACGAGG
	alpha constant region specific	
	alpha GSP1	ATTCGTGTAGTGCTTCACGTGGC
	alpha GSP2	CTATGCGCCTTGCCAGCCCGCTCAGCTCAGCGGGAAGACCTTGGG
	TCR alpha constant region specific	
	TR alpha GSP1a	cgtttgcacatgcaaagtcagatt
	TR alpha GSP2b	CTATGCGCCTTGCCAGCCCGCTCAGtcggtgaataggcagacagacttg
	TRC beta constant region specific	
	TR beta GSP1	CCTATCCTGGGTCCACTCGTCA
	TR beta GSP2	CTATGCGCCTTGCCAGCCCGCTCAGCTGCTTCTGATGGCTCAAACACA

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Table 11 - 3' primers for mouse genes		
seq id no	DESCRIPTION	sequence
	mu constant region specific	
	mouse_mu_GSP1	CTGAACCTTCAAGGATGCTCTTGG
	mouse_mu_GSP2	CTATGCGCCTTGCCAGCCCGCTCAGGGAAGACATTTGGGAAGGACTGAC
	alpha constant region specific	
	mouse_alpha_GSP1	TCTCCTTCTGGGCACTCGACAG
	mouse_alpha_GSP2	CTATGCGCCTTGCCAGCCCGCTCAGGGGAGTGTCAGTGGGTAGATGGTG
	gamma constant region specific	
	mo_g12b_GSP1d	AGGGGACAGTCACTGAGCTGCT
	mo_g2ac_GSP1d	TCGAGGTTACAGTCACTGAGCTGCT
	mo_g3_GSP1d	TGGAGGGTACAGTCACCAAGCTGCT
	mo_g12_GSP2d	CTATGCGCCTTGCCAGCCCGCTCAGGGGCCAGTGGATAGACHGATGG
	mo_g3_GSP2d	CTATGCGCCTTGCCAGCCCGCTCAGGGGACCAAGGGATAGACAGATGG
	mo_g12_GSP2e	CTATGCGCCTTGCCAGCCCGCTCAGCTGGACAGGGATCCAGAGTTCC
	mo_g3_GSP2e	CTATGCGCCTTGCCAGCCCGCTCAGCTGGACAGGGCTCCATAGTTCC
	kappa constant region specific	
	mouse_kappa_GSP1	GAAGTTGATGTCTTGTGAGTGGCCT
	mouse_kappa_GSP2	CTATGCGCCTTGCCAGCCCGCTCAGTGCTCACTGGATGGTGGGAA
	lambda constant region specific	
	mouse_lambda_GSP1a	ACTCTTCTCCACAGTGTCCCCTTCATG
	mouse_lambda_GSP1b	ACTCTTCTCCACAGTGTGACCTTCATG
	mouse_lambda_GSP2a	CTATGCGCCTTGCCAGCCCGCTCAGAGAGGAAGGTGGAAACASGGTGA
	mouse_lambda_GSP2b	CTATGCGCCTTGCCAGCCCGCTCAGAGGGGAAGGTGGAAACATGGTGA
	TCR alpha constant region specific	
	mo_TRA_GSP1b	TTGAAGATATCTTGGCAGGTGAAGCTT
	mouse_TRA_GSP2	CTATGCGCCTTGCCAGCCCGCTCAGCACAGCAGGTTCTGGGTTCTGG
	TRC beta constant region specific	
	mouse_TRB_GSP1	GAAAGCCCATGGAACTGCACTTG
	mouse_TRB_GSP2	CTATGCGCCTTGCCAGCCCGCTCAGGGGTGGAGTCACATTTCTCAGATC

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*lambda GSP1a and GSP1b are to be mixed 50:50, lambda GSP2a and lambda GSP2b ar also to be mixed 50:50 to amplify all lambda constant region alleles that are found in IMGT. All the gamma GSP1ds are also to be mixed equally to amplify al gamma 1, 2a, 2b, 2c and 3 constant region alleles. gamma GSP2ds are also to be mixed 50:50, and gamma GSP2es are also to be mixed 50:50 to amplify all gamma 1 2a, 2b, 2c and 3 constant regions alleles that are found in IMGT database.

Table 12 - Plate to patient referencing

PlateID	Patient
2	B Staph
3	B Staph
4	B Staph
5	B Staph
7	Lung Adeno
8	Lung Adeno
9	Lung Adeno
10	Lung Adeno
11	360 CCP+RA
12	360 CCP+RA
13	360 CCP+RA
14	361 Staph
15	361 Staph
16	361 Staph
17	361 Staph
18	368 CCP+RF+RA
19	368 CCP+RF+RA
21	368 CCP+RF+RA
22	368 CCP+RF+RA
26	372 CCP+RF+RA
27	372 CCP+RF+RA
40	375 CCP+RF+
41	375 CCP+RF+
43	375 CCP+RF+
44	369 CCP+RF+RA
46	372 CCP+RF+RA
47	372 CCP+RF+RA
48	375 CCP+RF+
49	Flu
51	Flu
52	Flu
53	Flu

Table 13

Plate ID	Plate Identification Region Regular Expression
1	ACGAGTGCGT
2	ACGCTCGACA
3	AGACGCACTC

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4	AGCACTGTAG
5	ATCAGACACG
6	ATATCGCGAG
7	CGTGTCTCTA
8	CTCGCGTGTC
9	TGATACGTCT
10	CATAGTAGTG
11	CGAGAGATAC
12	ATACGACGTA
13	TCACGTACTA
14	CGTCTAGTAC
15	TCTACGTAGC
16	TGTACTACTC
17	CGTAGACTAG
18	TACGAGTATG
19	TACTCTCGTG
20	TAGAGACGAG
21	TCGTCGCTCG
22	ACATACGCGT
23	ACGCGAGTAT
24	ACTACTATGT
25	ACTGTACAGT
26	AGACTATACT
27	AGCGTCGTCT
28	AGTACGCTAT
29	ATAGAGTACT
30	CACGCTACGT
31	CAGTAGACGT
32	CGACGTGACT
33	TACACACACT
34	TACACGTGAT

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35	TACAGATCGT
36	TACGCTGTCT
37	TAGTGTAGAT
38	TCGATCACGT
39	TCGCACTAGT
40	TCTAGCGACT
41	TCTATACTAT
42	TGACGTATGT
43	TGTGAGTAGT
44	ACAGTATATA
45	ACGCGATCGA
46	ACTAGCAGTA
47	AGCTCACGTA
48	AGTATACATA
49	AGTCGAGAGA
50	AGTGCTACGA
51	CGATCGTATA
52	CGCAGTACGA
53	CGCGTATACA
54	CGTACAGTCA
55	CGTACTCAGA
56	CTACGCTCTA
57	CTATAGCGTA
58	TACGTCATCA
59	TAGTCGCATA
60	TATATATACA

Table 14	
Sample ID	Sample Identification Region Regular Expression
1,31	ACGTCTCATC

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2,46	ACTCATCTAC
3,48	AGAGCGTCAC
4	AGTAGTGATC
5	ATAGATAGAC
6,50	ATCTACTGAC
7	CACGTGTCGC
8,51	CATACTCTAC
9	CGAGACGCGC
10	CGTCGATCTC
11,72	CTACGACTGC
12,81,25	TAGTGTAGAT
13,26	TCTAGCGACT
14,78	TGTGAGTAGT
15	ACAGTATATA
16,28	AGCTCACGTA
17,68	TCGATAGTGA
18,69	TCGCTGCGTA
19,45	TGAGTCAGTA
20,70	TGTAGTGTGA
21,71	TGTCGTCGCA
22	ACGACAGCTC
23	TACACGTGAT
24	TACAGATCGT
27	ACGCGATCGA
29	AGTGCTACGA
30	TCTGACGTCA
75,32	TATAGACATC
33	AGTGAGCTCG
34	ATCGTCTGTG
35	CACACGATAG
36	CTGCGTCACG

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37	TAGCATACTG
38	TATCTGATAG
39	TGACATCTCG
40	TGATAGAGCG
66,41	TCACGCGAGA
42	ACACATACGC
43	ACTAGCAGTA
44	CGCAGTACGA
47	ACTCGCGCAC
49	AGCGACTAGC
52,82	TCGAGCTCTC
53	AGAGAGTGTG
54	ATCGTAGCAG
55	CACTCGCACG
56,84	CAGACGTCTG
57	CTCGATATAG
58	TCTGATCGAG
59	TACACACACT
60	TACGTCATCA
65,61	CTACGCTCTA
62	TAGTCGCATA
63	CGATCGTATA
64	CGCGTATACA
67	AGTATACATA
73	CTCTACGCTC
74	TAGCTCTATC
76	TCACTCATAC
77	CTAGTCACTC
79	TGTCACACGA
80	CTGTGCGTCG
83	ATCACGTGCG

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85	TATCACTCAG
86	TGCTATAGAC
87	CAGTACTGCG
88	CGACAGCGAG
89	TATGCTAGTA

Table 15	
Sample ID	Sample Identification Region Regular Expression
1,31	ACGTCTCATCAAGGAAGGAAGG+
2,46	ACTCATCTACAGG+AA+GG+
3,48	AGAGCGTCACAGAGAGAAGG+
6,50	ATCTACTGACAACACACAAGG+
8,51	CATACTCTACAACACACAA+GG+
11,72	CTACGACTGCAAGG+AA+GG+
12,81,25	TAGTGTAGATT+GG+TT+GG+
12,81	TAGTGTAGATTCGCGG+
13,26	TCTAGCGACTT+GG+TT+GG+
14,78	TGTGAGTAGTT+GG+TT+GG+
16,28	AGCTCACGTATTGG+TT+GG+
17,68	TCGATAGTGAAGG+AA+GG+
18,69	TCGCTGCGTAA+GGAGAA+GG+
19,45	TGAGTCAGTAAGG+AA+GG+
20,70	TGTAGTGTGAAGG+AA+GG+
21,71	TGTCGTCGCAAGAGAGAGG+
75,32	TATAGACATCAACACACAA+GG+
66,41	TCACGCGAGAAAGG+AA+GG+
52,82	TCGAGCTCTCTCGCGG+
56,84	CAGACGTCTGTCGCGG+
65,61	CTACGCTCTAAGG+AA+GG+

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Table 16. Constant region insert sequences for DHFR vectors pcDNA3.3 and pOptivec		
Seq ID no	Description	Sequence
	IGHG1-G1m3 constant region insert sequence (restriction sites BstBI, EcoRI, SacII, BbvCI for splicing in variable region introduced via silent mutations)	TAAGCTTACTGATAAGGCGCGCCGCGATCGCGCCTCCACCAAGGACCCCTC TGTCTTCCCCCTGGCACCCTCTTCGAAGAGCACCTCTGGGGACACAGCAGC CCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCAGTGACAGTGAGTT GGAATTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCcGcGGTCCTTC AGTCTTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCA GCTTGGACACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACA CCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGACAAAACTCACACA TGCCCACCGTGCCCAGCACCTGAACTCCTGGAGGGACCGTCAGTCTTCCTC TTCCTCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCTAGAGGCC ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCAAGTTCAA CTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCTCGG GAGGAGCAGTACAACAGCACTTACCGTGTGGTCAGCGTCCTCACCGTCCT GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACA AAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAG CCCCGAGAACCACAGGTCTACACCCTGCCCCCATCCCGGGAGGAGATGAC CAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGA CATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAG ACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGC TCACCGTGGACAACAGCAGGTGGCAGCAGGGGAACGTCTTTTCATGCTCC GTGATGCATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTG TCCCCAGGTAAATGATAATGTACAACTGACTGAGGATCCT
	IGKC-Km3 constant region insert sequence (restriction sites NheI, XhoI for splicing in variable region introduced via silent mutations)	TAAGCTTACTGACTAGGCCGGCCTTAATTAACGTACGGTGGCTGCACCATC TGTCTTCATCTTCCCTCCATCTGATGAGCAGTTGAAATCTGGAACTGCTAGC GTCGTGTGCCTGCTGAATAACTTTTATCCTCGAGAGGCCAAAGTGCAGTGG AAGGTGGATAACGCCCTCCAATCCGGTAACTCCCAGGAGTCCGTCACAGA GCAGGACAGCAAGGACAGCACCTACAGCCTGAGCAGCACCCTGACACTGA GCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCAT CAGGGCCTGAGCTCCCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTG ATAAATCGATACTGACTGAGGATCCT
	IGLC-Mcg Ke Oz constant region insert sequence (restriction sites Bsu36I, XhoI, PspXI, NheI for splicing in variable region introduced via silent mutations)	TAAGCTTACTGACTAGGCCGGCCTTAATTAAGGTCAGCCTAAGGCTGCCCC CAGCGTCACTCTGTTCCCTCCCTCCTCTGAGGAGCTTCAAGCCAACAAGGC CACACTGGTGTGTCTCATCAGTGACTTCTACCCCGGAGCCGTGACAGTGGC TTGGAAAGCAGACTCGAGCCCCGTCAAGGCTGGAGTGGAGACCACCACAC CTTCCAAACAAAGCAACAACAAGTACGCAGCTAGCAGCTACCGCAGCCTG ACCCCTGAGCAGTGGAAGTCCCACAGAAGCTACTCCTGCCAGGTCACACAT GAAGGGAGCACCGTGGAGAAGACAGTGGCCCCCACAGAATGTTCATGAT AAATCGATACTGACTGAGGATCCT

Table17 . Constant region insert sequences for Lonza vectors		
seq id no	DESCRIPTION	sequence
	IGHG1-G1m3 constant region insert sequence (restriction sites EcoRI, ApaI, AgeI,	AAGCTTGGCGCGCCTTAATTAAGCCAGCACAAAAGGCCCCAGTG TGTTTCCCTTGGCACCCTCGAGCAAGAGTACATCTGGAGGTACA GCTGCCTTGGGCTGTTTGGTGAAAGACTATTTCCCCGAACCGGT TACTGTCTCTTGGAATTCCGGGGCCCTCACCAGTGGTGTCCATA

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	KpnI, XhoI for splicing in variable region introduced via silent mutations)	CCTTTCCCGCGGTGCTTCAGAGTTCCGGTTTGTATTCCCTGTCA AGTGTCGTGACGGTACCAAGTTCAAGTCTAGGCACCCAGACATA TATCTGTAACGTCAACCACAAGCCAAGCAACACCAAGGTTGACA AGCGGGTTGAACCTAAGTCCTGTGACAAGACCCATACCTGCCCC CCATGCCCCGCACCCGAGCTCCTCGGAGGGCCTTCCGTCTTTCT TTTCCCTCCCAAACCCAAGGACACTTTGATGATCTCAAGAACAC CAGAAGTCACTTGCGTCGTGGTTGACGTGTCTCACGAAGATCCC GAAGTGAAGTTCAACTGGTACGTGGATGGGGTAGAGGTTCATAA CGCCAAGACCAAACCCCGAGAGGAACAGTATAACTCCACCTATA GGGTAGTGTCCGTGCTCACCGTGCTCCACCAAGACTGGCTGAAT GGCAAGGAATACAAGTGCAAGGTGAGTAATAAGGCACTGCCTGC ACCCATTGAGAAGACAATATCTAAAGCAAAGGGACAGCCCAGAG AGCCCCAGGTTTATACTCTGCCACCTAGCAGAGAGGAAATGACT AAAAACCAGGTCAGCCTTACTTGTCTCGTAAAAGGCTTTTATCC AAGCGACATCGCTGTGGAGTGGGAATCAAATGGCCAACCTGAGA ATAATTATAAGACTACACCTCCCGTCCTTGACTCAGACGGTTCC TTCTTCCTGTATAGCAAGCTCACCGTCGATAAAAGTCGGTGGCA ACAGGGAAACGTGTTCTCATGCAGCGTCATGCACGAGGCCTTGC ACAATCATTACACCCAGAAGTCTCTGTCCCTGAGCCCTGGAAAG TGATCA
	IGKC-Km3 constant region insert sequence (restriction sites XmaI, EcoRI, BstEII, DraIII for splicing in variable region introduced via silent mutations)	AAGCTTAATTAAGGCGCGCCGAACAGTGGCTGCTCCTTCCGTGT TCATATTCCCCCCATCCGACGAGCAGCTTAAATCTGGGACTGCT AGCGTCGTGTGCCTGTTGAATAATTTTTATCCCCGGGAGGCTAA GGTACAGTGGAAGGTGGACAACGCCCTCCAATCAGGGAATTCCC AGGAGTCGGTCACCGAACAGGACAGCAAGGACTCAACCTACTCT CTGTCATCCACTCTCACACTCAGCAAAGCCGACTATGAAAAACA CAAAGTGTATGCTTGCGAGGTGACTCATCAAGGGCTCTCCAGTC CTGTGACTAAATCCTTCAACCGAGGCGAATGCTGATCA
	IGLC-Mcg Ke Oz constant region insert sequence (restriction sites DraIII, XmaI, BstEII, PspXI for splicing in variable region introduced via silent mutations)	AAGCTTGGCGCGCCTTAATTAAGGCCAGCCTAAAGCCGCACCCA GTGTGACCCTGTTTCCTCCCTCCTCTGAAGAGCTCCAGGCAAAC AAAGCTACTCTGGTGTGTCTTATTAGCGATTTCTATCCCGGGGC GGTGACCGTGGCTTGGAAGGCCGACTCGAGCCCAGTGAAGGCCG GAGTGGAAACTACAACCCCTTCCAAACAGTCAAACAATAAATAC GCCGCTAGCAGCTATCTCTCTCTCACCCCAGAACAGTGGAAATC CCACAGGTCCTATTCTTGCCAGGTCACACACGAGGGGTCAACCG TTGAGAAGACTGTTGCCCCAACAGAGTGCAGCTGATCA

Table 18. All expressed antibodies

SEQ ID NO	Antibody	Chain Type	Clone	V-GENE and allele	J-GENE and allele	D-GENE and allele
	LC1	Light chain	K8B8	IGKV3-11*01 F	IGKJ3*01 F	
	LC1	Heavy chain	G8B8	IGHV1-46*01 F, or IGHV1-46*03 F	IGHJ3*01 F, or IGHJ3*02 F	IGHD5-24*01 ORF
	LC2	Light chain	K8C11	IGKV3-11*01 F	IGKJ3*01 F, or IGKJ4*01 F	
	LC2	Heavy chain	G8C11	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ4*02 F	IGHD6-25*01 F
	LC3	Light chain	K8D6	IGKV3-20*01 F	IGKJ1*01 F	
	LC3	Heavy chain	G8D6	IGHV1-46*01 F, or IGHV1-46*03 F	IGHJ3*01 F, or IGHJ3*02 F	IGHD5-24*01 ORF
	LC5	Light chain	K10G5	IGKV3-11*01 F	IGKJ4*01 F	
	LC5	Heavy	G10G5	IGHV3-33*05 F	IGHJ4*02 F	IGHD6-13*01 F

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		chain				
	LC6	Light chain	K8D6	IGKV3-20*01 F	IGKJ1*01 F	
	LC6	Heavy chain	G10H2	IGHV3-11*01 F	IGHJ4*02 F	IGHD3-9*01 F
	LC7	Light chain	L8D9	IGLV2-8*01 F	IGLJ2*01 F, or IGLJ3*01 F or IGLJ3*02 F	
	LC7	Heavy chain	G8D9	IGHV3-53*02 F	IGHJ4*02 F	IGHD4-17*01 F
	LC9	Light chain	L10A1	IGLV10-54*01 F	IGLJ3*02 F	
	LC9	Heavy chain	G10A1	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ3*02 F	IGHD2-2*01 F
	LC10	Light chain	K9C11	IGKV3-11*01 F	IGKJ4*01 F	
	LC10	Heavy chain	G9C11	IGHV3-33*01 F, or IGHV3-33*06 F	IGHJ6*02 F	IGHD5-18*01 F
	LC11	Light chain	L10A6	IGLV2-14*01 F	IGLJ3*02 F	
	LC11	Heavy chain	G10A6	IGHV3-21*01 F, or IGHV3-21*04 F	IGHJ4*02 F	IGHD1-20*01 F
	LC12	Light chain	L9C9	IGLV2-14*01 F	IGLJ3*02 F	
	LC12	Heavy chain	G9C9	IGHV3-15*01 F	IGHJ4*02 F	IGHD1-26*01 F
	LC13	Light chain	L9B1	IGLV2-8*01 F	IGLJ1*01 F	
	LC13	Heavy chain	G9B1	IGHV3-66*01 F, or IGHV3-66*04 F	IGHJ3*02 F	IGHD2-8*01 F
	LC14	Light chain	L9A1	IGLV2-14*01 F	IGLJ3*02 F	
	LC14	Heavy chain	G9A1	IGHV3-15*01 F	IGHJ4*02 F	IGHD1-26*01 F
	LC15	Light chain	K10A9	IGKV3-11*01 F	IGKJ3*01 F	
	LC15	Heavy chain	G10A9	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ4*02 F	IGHD6-25*01 F
	LC16	Light chain	K10D2	IGKV3-20*01 F	IGKJ2*02 F	
	LC16	Heavy chain	G10D2	IGHV3-15*01 F	IGHJ4*02 F	IGHD4-23*01 ORF
	LC17	Light chain	K8D5	IGKV3-11*01 F	IGKJ3*01 F	
	LC17	Heavy chain	G8D5	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ4*02 F	IGHD4-23*01 ORF
	LC18	Light chain	L10D5	IGLV2-8*01 F	IGLJ3*02 F	
	LC18	Heavy chain	G10D5	IGHV3-53*02 F	IGHJ4*02 F	IGHD4-17*01 F
	Flu14	Light	L51A6	IGLV3-25*03 F	IGLJ1*01 F	

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		chain				
	Flu14	Heavy chain	G51A6	IGHV3-30-3*01 F	IGHJ3*01 F	IGHD1-14*01 ORF
	Flu15	Light chain	L51C4	IGLV3-1*01 F	IGLJ2*01 F, or IGLJ3*01 F or IGLJ3*02 F	
	Flu15	Heavy chain	G51C4	IGHV3-30*04 F	IGHJ6*02 F	IGHD3-10*01 F
	Flu16	Light chain	K51G11	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	
	Flu16	Heavy chain	G51G11	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ6*02 F	IGHD4-17*01 F
	Flu17	Light chain	K49F7	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	
	Flu17	Heavy chain	G49F7	IGHV1-69*02 F, or IGHV1-69*04 F	IGHJ3*02 F	IGHD3-22*01 F
	Flu18	Light chain	K51D7	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	
	Flu18	Heavy chain	G51D7	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ6*02 F	IGHD4-11*01 ORF
	Flu19	Light chain	K51D8	IGKV1-39*01 F, or IGKV1D-39*01 F	IGKJ4*01 F	
	Flu19	Heavy chain	G51D8	IGHV3-49*04 F	IGHJ6*02 F	IGHD3-22*01 F
	Flu20	Light chain	K51G10	IGKV3-15*01 F	IGKJ2*01 F	
	Flu20	Heavy chain	G51G10	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ6*01 F	IGHD3-16*02 F
	Flu21	Light chain	L49A9	IGLV3-21*02 F	IGLJ3*02 F	
	Flu21	Heavy chain	G49A9	IGHV3-74*01 F	IGHJ2*01 F	IGHD2-21*01 F
	Flu22	Light chain	L52A6	IGLV2-14*01 F	IGLJ3*02 F	
	Flu22	Heavy chain	G52A6	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ6*02 F	IGHD4-23*01 ORF
	Flu23	Light chain	K49F11	IGKV3-11*01 F	IGKJ3*01 F	
	Flu23	Heavy chain	G49F11	IGHV3-30*04 F, or IGHV3-30*08 F or IGHV3-30-3*01 F	IGHJ4*02 F	IGHD3-16*01 F
	Flu24	Light chain	K51C8	IGKV3-15*01 F	IGKJ2*01 F	
	Flu24	Heavy chain	G51C8	IGHV3-30*14 F	IGHJ5*02 F	IGHD3-22*01 F
	Flu25	Light chain	K51H1	IGKV1-39*01 F, or IGKV1D-39*01 F	IGKJ4*01 F	
	Flu25	Heavy chain	G51H1	IGHV3-9*01 F	IGHJ4*02 F	IGHD6-13*01 F
	Flu26	Light chain	K52A2	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	

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	Flu26	Heavy chain	G52A2	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ6*02 F	IGHD4-23*01 ORF
	Flu27	Light chain	L49A11	IGLV2-14*01 F	IGLJ3*02 F	
	Flu27	Heavy chain	G49A11	IGHV3-7*03 F	IGHJ5*02 F	IGHD3-3*01 F
	Flu28	Light chain	L49C4	IGLV3-10*01 F	IGLJ2*01 F, or IGLJ3*01 F	
	Flu28	Heavy chain	G49C4	IGHV3-43*01 F	IGHJ4*02 F	IGHD4-17*01 F
	Flu29	Light chain	L51H5	IGLV3-27*01 F	IGLJ2*01 F, or IGLJ3*01 F	
	Flu29	Heavy chain	G51H5	IGHV1-2*04 F	IGHJ6*02 F	IGHD2-21*02 F
	Flu30	Light chain	L52B8	IGLV2-14*01 F	IGLJ3*02 F	
	Flu30	Heavy chain	G52B8	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ6*02 F	IGHD4-17*01 F
	Flu31	Light chain	K49A5	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	
	Flu31	Heavy chain	G49A5	IGHV3-30-3*01 F	IGHJ4*02 F	IGHD5-12*01 F
	Flu32	Light chain	K49C11	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	
	Flu32	Heavy chain	G49C11	IGHV3-66*01 F, or IGHV3-66*04 F	IGHJ4*02 F	IGHD6-19*01 F
	Flu33	Light chain	L51E9	IGLV2-14*01 F	IGLJ3*02 F	
	Flu33	Heavy chain	G51E9	IGHV3-23*01 F	IGHJ4*02 F	IGHD3-16*01 F
	Flu34	Light chain	L52G10	IGLV2-14*01 F	IGLJ3*02 F	
	Flu34	Heavy chain	G52G10	IGHV3-30-3*01 F	IGHJ3*01 F, or IGHJ3*02 F	IGHD6-19*01 F
	Flu35	Light chain	L53F10	IGLV3-21*02 F	IGLJ3*02 F	
	Flu35	Heavy chain	G53F10	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ6*02 F	IGHD3-3*01 F
	Flu36	Light chain	L52G7	IGLV2-8*01 F	IGLJ2*01 F, or IGLJ3*01 F or IGLJ3*02 F	
	Flu36	Heavy chain	G52G7	IGHV3-66*02 F	IGHJ4*02 F	IGHD2-15*01 F
	Flu37	Light chain	K51E8	IGKV3-15*01 F	IGKJ1*01 F	
	Flu37	Heavy chain	G51E8	IGHV1-2*04 F	IGHJ6*02 F	IGHD2-21*02 F
	Flu39	Light chain	K53G7	IGKV1-5*03 F	IGKJ1*01 F	
	Flu39	Heavy chain	G53G7	IGHV1-46*01 F, or IGHV1-46*03 F	IGHJ4*02 F	IGHD3-9*01 F

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	Flu40	Light chain	L51A5	IGLV3-10*01 F	IGLJ2*01 F, or IGLJ3*01 F or IGLJ3*02 F	
	Flu40	Heavy chain	G51A5	IGHV4-34*01 F	IGHJ6*02 F	IGHD5-24*01 ORF
	Flu41	Light chain	L51B1	IGLV3-25*03 F	IGLJ1*01 F	
	Flu41	Heavy chain	G51B1	IGHV1-2*02 F	IGHJ4*02 F	IGHD3-10*01 F
	Flu43	Light chain	L51D3	IGLV3-22*01 F	IGLJ3*02 F	
	Flu43	Heavy chain	G51D3	IGHV4-39*01 F	IGHJ3*02 F	IGHD1-26*01 F
	Flu44	Light chain	L51D4	IGLV2-14*01 F	IGLJ3*02 F	
	Flu44	Heavy chain	G51D4	IGHV3-11*01 F	IGHJ6*02 F	IGHD4-17*01 F
	Flu45	Light chain	L52D4	IGLV2-14*01 F	IGLJ3*02 F	
	Flu45	Heavy chain	G52D4	IGHV3-30-3*01 F	IGHJ3*01 F, or IGHJ3*02 F	IGHD6-19*01 F
	Flu46	Light chain	L52H4	IGLV1-51*01 F	IGLJ3*02 F	
	Flu46	Heavy chain	G52H4	IGHV3-23*01 F, or IGHV3-23*04 F	IGHJ4*02 F	IGHD6-13*01 F
	S1	Light chain	K3G4	IGKV3-11*01 F	IGKJ3*01 F	
	S1	Heavy chain	G3G4	IGHV3-53*02 F	IGHJ4*02 F	IGHD2-21*01 F
	S2	Light chain	K4C4	IGKV2-28*01 F, or IGKV2D-28*01 F	IGKJ4*01 F	
	S2	Heavy chain	G4C4	IGHV3-23*04 F	IGHJ5*02 F	IGHD6-19*01 F
	S3	Light chain	K15C6	IGKV1-5*01 F	IGKJ4*01 F	
	S3	Heavy chain	G15C6	IGHV3-7*01 F	IGHJ1*01 F	IGHD2-2*01 F
	S4	Light chain	K15G1	IGKV1-6*01 F	IGKJ1*01 F	
	S4	Heavy chain	G15G1	IGHV3-7*03 F	IGHJ3*02 F	IGHD6-13*01 F
	S5	Light chain	K17C3	IGKV1-39*01 F, or IGKV1D-39*01 F	IGKJ2*02 F	
	S5	Heavy chain	G17C3	IGHV1-8*02 F	IGHJ4*02 F	IGHD3-16*01 F
	S6	Light chain	K3E11	IGKV3-15*01 F	IGKJ1*01 F	
	S6	Heavy chain	G3E11	IGHV3-30*04 F, or IGHV3-30-3*01 F	IGHJ4*02 F	IGHD4-23*01 ORF
	S7	Light chain	L4B8	IGLV2-8*01 F	IGLJ2*01 F, or IGLJ3*01 F	

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	S7	Heavy chain	G4B8	IGHV3-30*04 F, or IGHV3-30*10 F	IGHJ4*01 F, or IGHJ4*02 F	IGHD5-18*01 F
	S8	Light chain	L4D2	IGLV2-23*01 F, or IGLV2-23*03 F	IGLJ3*02 F	
	S8	Heavy chain	G4D2	IGHV3-33*03 F	IGHJ6*02 F	IGHD3-10*01 F
	S9	Light chain	L4D6	IGLV2-8*01 F	IGLJ2*01 F, or IGLJ3*01 F	
	S9	Heavy chain	G4D6	IGHV3-20*01 F	IGHJ4*02 F	IGHD2-2*01 F
	S10	Light chain	L4F4	IGLV3-1*01 F	IGLJ3*02 F	
	S10	Heavy chain	G4F4	IGHV4-59*01 F, or IGHV4-59*08 F	IGHJ4*02 F	IGHD3-3*01 F
	S11	Light chain	L15D1	IGLV8-61*01 F	IGLJ3*02 F	
	S11	Heavy chain	G15D1	IGHV3-7*01 F	IGHJ4*02 F	IGHD3-10*01 F
	S12	Light chain	L17C6	IGLV1-47*01 F, or IGLV1-47*02 F	IGLJ3*02 F	
	S12	Heavy chain	G17C6	IGHV3-7*03 F	IGHJ4*02 F	IGHD5-18*01 F
	S13	Light chain	L17C9	IGLV7-46*01 F	IGLJ3*02 F	
	S13	Heavy chain	G17C9	IGHV5-a*03 F	IGHJ6*02 F	IGHD6-13*01 F
	RA1	Light chain	K11G5	IGKV3-11*01 F	IGKJ5*01 F	
	RA1	Heavy chain	G11G5	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ4*02 F	IGHD4-23*01 ORF
	RA2	Light chain	K22C7	IGKV3-15*01 F	IGKJ1*01 F	
	RA2	Heavy chain	G22C7	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ4*02 F	IGHD6-25*01 F
	RA3	Light chain	K26B1	IGKV3-15*01 F	IGKJ4*01 F	
	RA3	Heavy chain	G26B1	IGHV4-39*07 F	IGHJ4*02 F	IGHD4-23*01 ORF
	RA4	Light chain	K26F5	IGKV3-15*01 F	IGKJ5*01 F	
	RA4	Heavy chain	G26F5	IGHV3-23*01 F	IGHJ4*02 F	IGHD6-19*01 F
	RA5	Light chain	K26H1	IGKV3-11*01 F	IGKJ4*01 F	
	RA5	Heavy chain	G26H1	IGHV3-9*01 F	IGHJ4*02 F	IGHD6-13*01 F
	RA6	Light chain	K40C5	IGKV1-39*01 F, or IGKV1D-39*01 F	IGKJ3*01 F	
	RA6	Heavy chain	G40C5	IGHV3-30*04 F, or IGHV3-30*08 F or IGHV3-30-3*01 F	IGHJ4*02 F	IGHD3-16*01 F

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	RA7	Light chain	K40G1	IGKV3-11*01 F	IGKJ1*01 F	
	RA7	Heavy chain	G40G1	IGHV4-39*01 F	IGHJ6*02 F	IGHD2-15*01 F
	RA8	Light chain	K40H4	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	
	RA8	Heavy chain	G40H4	IGHV1-2*02 F	IGHJ5*02 F	IGHD2-21*02 F
	RA9	Light chain	K41A2	IGKV1-33*01 F, or IGKV1D-33*01 F	IGKJ4*01 F	
	RA9	Heavy chain	G41A2	IGHV1-2*02 F	IGHJ6*02 F	IGHD1-1*01 F
	RA10	Light chain	K47A2	IGKV3-15*01 F	IGKJ5*01 F	
	RA10	Heavy chain	G47A2	IGHV3-23*01 F	IGHJ4*02 F	IGHD6-19*01 F
	RA11	Light chain	K47E2	IGKV3-15*01 F	IGKJ5*01 F	
	RA11	Heavy chain	G47E2	IGHV3-23*01 F	IGHJ4*02 F	IGHD6-19*01 F
	RA12	Light chain	K47F9	IGKV3-11*01 F	IGKJ4*01 F	
	RA12	Heavy chain	G47F9	IGHV4-39*02 F	IGHJ3*02 F	IGHD3-3*02 F
	RA13	Light chain	L13B10	IGLV3-1*01 F	IGLJ1*01 F	
	RA13	Heavy chain	G13B10	IGHV5-51*01 F	IGHJ6*02 F	IGHD6-25*01 F
	RA14	Light chain	L13G5	IGLV3-1*01 F	IGLJ1*01 F	
	RA14	Heavy chain	G13G5	IGHV5-51*01 F	IGHJ6*02 F	IGHD6-25*01 F
	RA15	Light chain	K40D6	IGKV3-20*01 F	IGKJ5*01 F	
	RA15	Heavy chain	G40D6	IGHV4-39*07 F	IGHJ4*02 F	IGHD4-23*01 ORF
	RA16	Light chain	K25D6	IGKV3-11*01 F	IGKJ3*01 F	
	RA16	Heavy chain	G26D6	IGHV3-72*01 F	IGHJ6*03 F	IGHD4-17*01 F
	RA17	Light chain	K25E9	IGKV3-11*01 F	IGKJ3*01 F	
	RA17	Heavy chain	G25E9	IGHV3-30*03 F, or IGHV3-30*18 F or IGHV3-33*05 F	IGHJ5*02 F	IGHD3-22*01 F
	RA18	Light chain	K25G4	IGKV1-27*01 F	IGKJ2*03 F	
	RA18	Heavy chain	G25G4	IGHV3-30*09 F	IGHJ4*02 F	IGHD1-1*01 F
	RA19	Light chain	K45D9	IGKV3-15*01 F	IGKJ1*01 F	

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	RA19	Heavy chain	G45D9	IGHV3-30*03 F, or IGHV3-30*18 F	IGHJ4*02 F	IGHD3-22*01 F
	RA21	Light chain	L13E11	IGLV2-23*02 F	IGLJ1*01 F	
	RA21	Heavy chain	G13E11	IGHV3-15*01 F	IGHJ4*02 F	IGHD6-13*01 F
	RA22	Light chain	L13G5	IGLV3-1*01 F	IGLJ1*01 F	
	RA22	Heavy chain	G13G5	IGHV3-7*01 F	IGHJ5*02 F	IGHD5-12*01 F
	RA23	Light chain	L44C5	IGLV2-23*01 F, or IGLV2-23*02 F or IGLV2-23*03 F	IGLJ1*01 F	
	RA23	Heavy chain	G44C5	IGHV3-30*14 F	IGHJ5*02 F	IGHD7-27*01 F
	RA24	Light chain	L44D6	IGLV2-23*01 F, or IGLV2-23*02 F or IGLV2-23*03 F	IGLJ1*01 F	
	RA24	Heavy chain	G44D6	IGHV3-30*04 F	IGHJ6*03 F	IGHD3-10*01 F
*VDJ identity as given by V-QUEST.						

Table19 - antibodies used in Fluzone ELISA

Antibody	Chain Type	Clone
Flu14	Light chain	L51A6
Flu14	Heavy chain	G51A6
Flu15	Light chain	L51C4
Flu15	Heavy chain	G51C4
Flu16	Light chain	K51G11
Flu16	Heavy chain	G51G11
Flu17	Light chain	K49F7
Flu17	Heavy chain	G49F7
Flu18	Light chain	K51D7
Flu18	Heavy chain	G51D7
Flu19	Light chain	K51D8
Flu19	Heavy chain	G51D8
Flu20	Light chain	K51G10
Flu20	Heavy chain	G51G10
Flu21	Light chain	L49A9
Flu21	Heavy chain	G49A9
Flu22	Light chain	L52A6
Flu22	Heavy chain	G52A6
Flu23	Light chain	K49F11
Flu23	Heavy chain	G49F11
Flu25	Light chain	K51H1
Flu25	Heavy chain	G51H1

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Flu26	Light chain	K52A2
Flu26	Heavy chain	G52A2
Flu27	Light chain	L49A11
Flu27	Heavy chain	G49A11
Flu29	Light chain	L51H5
Flu29	Heavy chain	G51H5
Flu30	Light chain	L52B8
Flu30	Heavy chain	G52B8
Flu33	Heavy chain	G51E9
Flu34	Light chain	L52G10
Flu34	Heavy chain	G52G10
Flu35	Light chain	L53F10
Flu35	Heavy chain	G53F10
Flu37	Light chain	K51E8
Flu37	Heavy chain	G51E8
Flu39	Light chain	K53G7
Flu39	Heavy chain	G53G7
Flu40	Light chain	L51A5
Flu40	Heavy chain	G51A5
Flu41	Light chain	L51B1
Flu41	Heavy chain	G51B1
Flu43	Light chain	L51D3
Flu43	Heavy chain	G51D3
Flu44	Light chain	L51D4
Flu44	Heavy chain	G51D4
Flu45	Light chain	L52D4
Flu45	Heavy chain	G52D4
Flu46	Light chain	L52H4
Flu46	Heavy chain	G52H4
S1	Light chain	K3G4
S1	Heavy chain	G3G4
S2	Light chain	K4C4
S2	Heavy chain	G4C4

Table 20- Antibodies used in surface plasmon resonance

Antibody	Chain Type	Clone
Flu14	Light chain	L51A6
Flu14	Heavy chain	G51A6
Flu15	Light chain	L51C4
Flu15	Heavy chain	G51C4
Flu16	Light chain	K51G11
Flu16	Heavy chain	G51G11
Flu17	Light chain	K49F7
Flu17	Heavy chain	G49F7

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Flu18	Light chain	K51D7
Flu18	Heavy chain	G51D7
Flu19	Light chain	K51D8
Flu19	Heavy chain	G51D8
Flu20	Light chain	K51G10
Flu20	Heavy chain	G51G10
Flu21	Light chain	L49A9
Flu21	Heavy chain	G49A9
Flu22	Light chain	L52A6
Flu22	Heavy chain	G52A6
Flu26	Light chain	K52A2
Flu26	Heavy chain	G52A2
Flu29	Light chain	L51H5
Flu29	Heavy chain	G51H5
Flu34	Light chain	L52G10
Flu34	Heavy chain	G52G10
Flu35	Light chain	L53F10
Flu35	Heavy chain	G53F10
Flu46	Light chain	L52H4
Flu46	Heavy chain	G52H4

Table 21 -Antibodies used in RA antigen array

Antibody	Chain Type	Clone
RA1	Light chain	K11G5
RA1	Heavy chain	G11G5
RA2	Light chain	K22C7
RA2	Heavy chain	G22C7
RA4	Light chain	K26F5
RA4	Heavy chain	G26F5
RA5	Light chain	K26H1
RA5	Heavy chain	G26H1
RA8	Light chain	K40H4
RA8	Heavy chain	G40H4
RA9	Light chain	K41A2
RA9	Heavy chain	G41A2
RA10	Light chain	K47A2
RA10	Heavy chain	G47A2
RA11	Light chain	K47E2
RA11	Heavy chain	G47E2
RA12	Light chain	K47F9
RA12	Heavy chain	G47F9
RA13	Light chain	L13B10
RA13	Heavy chain	G13B10
RA16	Light chain	K25D6

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RA16	Heavy chain	G26D6
RA19	Light chain	K45D9
RA19	Heavy chain	G45D9
RA22	Light chain	L13G5
RA22	Heavy chain	G13G5
RA23	Light chain	L44C5
RA23	Heavy chain	G44C5
Flu14	Light chain	L51A6
Flu14	Heavy chain	G51A6
Flu26	Light chain	K52A2
Flu26	Heavy chain	G52A2

Table 22 -Antibodies used in Histone 2A ELISA and CCP ELISA

Antibody	Chain Type	Clone
RA1	Light chain	K11G5
RA1	Heavy chain	G11G5
RA2	Light chain	K22C7
RA2	Heavy chain	G22C7
RA4	Light chain	K26F5
RA4	Heavy chain	G26F5
RA5	Light chain	K26H1
RA5	Heavy chain	G26H1
RA6	Light chain	K40C5
RA6	Heavy chain	G40C5
RA7	Light chain	K40G1
RA7	Heavy chain	G40G1
RA8	Light chain	K40H4
RA8	Heavy chain	G40H4
RA9	Light chain	K41A2
RA9	Heavy chain	G41A2
RA10	Light chain	K47A2
RA10	Heavy chain	G47A2
RA11	Light chain	K47E2
RA11	Heavy chain	G47E2
RA12	Light chain	K47F9
RA12	Heavy chain	G47F9
RA13	Light chain	L13B10
RA13	Heavy chain	G13B10
RA16	Light chain	K25D6
RA16	Heavy chain	G26D6
RA17	Light chain	K25E9
RA17	Heavy chain	G25E9
RA18	Light chain	K25G4
RA18	Heavy chain	G25G4

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RA19	Light chain	K45D9
RA19	Heavy chain	G45D9
RA22	Light chain	L13G5
RA22	Heavy chain	G13G5
RA23	Light chain	L44C5
RA23	Heavy chain	G44C5
RA24	Light chain	L44D6
RA24	Heavy chain	G44D6

Table 23 -Antibodies used in RF ELISA		
Antibody	Chain Type	Clone
RA1	Light chain	K11G5
RA1	Heavy chain	G11G5
RA2	Light chain	K22C7
RA2	Heavy chain	G22C7
RA4	Light chain	K26F5
RA4	Heavy chain	G26F5
RA5	Light chain	K26H1
RA5	Heavy chain	G26H1
RA6	Light chain	K40C5
RA6	Heavy chain	G40C5
RA8	Light chain	K40H4
RA8	Heavy chain	G40H4
RA9	Light chain	K41A2
RA9	Heavy chain	G41A2
RA10	Light chain	K47A2
RA10	Heavy chain	G47A2
RA11	Light chain	K47E2
RA11	Heavy chain	G47E2
RA12	Light chain	K47F9
RA12	Heavy chain	G47F9
RA14	Light chain	L13G5
RA14	Heavy chain	G13G5

Table 24 - Antibodies used in lung cancer Tissue IHC and flow cytometry of lung cancer cell lines		
Antibody	Chain Type	Clone
LC1	Light chain	K8B8
LC1	Heavy chain	G8B8
LC5	Light chain	K10G5
LC5	Heavy chain	G10G5
LC6	Light chain	K8D6
LC6	Heavy chain	G10H2
LC7	Light chain	L8D9

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LC7	Heavy chain	G8D9
LC9	Light chain	L10A1
LC9	Heavy chain	G10A1
LC10	Light chain	K9C11
LC10	Heavy chain	G9C11
LC11	Light chain	L10A6
LC11	Heavy chain	G10A6
LC12	Light chain	L9C9
LC12	Heavy chain	G9C9
LC13	Light chain	L9B1
LC13	Heavy chain	G9B1
LC14	Light chain	L9A1
LC14	Heavy chain	G9A1
LC15	Light chain	K10A9
LC15	Heavy chain	G10A9
LC16	Light chain	K10D2
LC16	Heavy chain	G10D2
LC17	Light chain	K8D5
LC17	Heavy chain	G8D5
LC18	Light chain	L10D5
LC18	Heavy chain	G10D5
Flu14	Light chain	L51A6
Flu14	Heavy chain	G51A6

Table 25 - Antibodies used in *S. aureus* surface staining

Antibody	Chain Type	Clone
S1	Light chain	K3G4
S1	Heavy chain	G3G4
S2	Light chain	K4C4
S2	Heavy chain	G4C4
S3	Light chain	K15C6
S3	Heavy chain	G15C6
S4	Light chain	K15G1
S4	Heavy chain	G15G1
S6	Light chain	K3E11
S6	Heavy chain	G3E11
S7	Light chain	L4B8
S7	Heavy chain	G4B8
S8	Light chain	L4D2
S8	Heavy chain	G4D2
S9	Light chain	L4D6
S9	Heavy chain	G4D6
S10	Light chain	L4F4
S10	Heavy chain	G4F4

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S11	Light chain	L15D1
S11	Heavy chain	G15D1
S12	Light chain	L17C6
S12	Heavy chain	G17C6
S13	Light chain	L17C9
S13	Heavy chain	G17C9
Flu14	Light chain	L51A6
Flu14	Heavy chain	G51A6
Flu26	Light chain	K52A2
Flu26	Heavy chain	G52A2

Table 26 - Antibodies used in microneutralization assay

Antibody	Chain Type	Clone
Flu15	Light chain	L51C4
Flu15	Heavy chain	G51C4
Flu16	Light chain	K51G11
Flu16	Heavy chain	G51G11
Flu18	Light chain	K51D7
Flu18	Heavy chain	G51D7
Flu19	Light chain	K51D8
Flu19	Heavy chain	G51D8
Flu20	Light chain	K51G10
Flu20	Heavy chain	G51G10
Flu21	Light chain	L49A9
Flu21	Heavy chain	G49A9

Table 27 - antibodies used in staph inhibition assay

Antibody	Chain Type	Clone
S6	Light chain	K3E11
S6	Heavy chain	G3E11
S9	Light chain	L4D6
S9	Heavy chain	G4D6
LC1	Light chain	K8B8
LC1	Heavy chain	G8B8

Table 28 - antibodies used in staph IP

Antibody	Chain Type	Clone
S1	Light chain	K3G4
S1	Heavy chain	G3G4
S2	Light chain	K4C4
S2	Heavy chain	G4C4

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S3	Light chain	K15C6
S3	Heavy chain	G15C6
S4	Light chain	K15G1
S4	Heavy chain	G15G1
S5	Light chain	K17C3
S5	Heavy chain	G17C3
S6	Light chain	K3E11
S6	Heavy chain	G3E11
S7	Light chain	L4B8
S7	Heavy chain	G4B8
S8	Light chain	L4D2
S8	Heavy chain	G4D2
S9	Light chain	L4D6
S9	Heavy chain	G4D6
S10	Light chain	L4F4
S10	Heavy chain	G4F4
S11	Light chain	L15D1
S11	Heavy chain	G15D1
S12	Light chain	L17C6
S12	Heavy chain	G17C6
S13	Light chain	L17C9
S13	Heavy chain	G17C9
Flu14	Light chain	L51A6
Flu14	Heavy chain	G51A6

Table 29 - Antibody used in staph mass spec		
Antibody	Chain Type	Clone
S4	Light chain	K15G1
S4	Heavy chain	G15G1

Table 30.	
Name	Sequence
RT oligo	CACGACCGGTGCTCGATTTAGTTAATTAA[sampleID]AGCGATCGCTGGG
RT oligo'	CTAAATCGAGCACCGGTCGTGTGGG
Fwd Primer (forkappa chain)	CGATTGGAGGGCGTTATCCAC
Fwd Primer (for lambda chain)	TYTGTGGGACTTCCACTGCTC

Table 31.	
Name	Sequence
RT oligo	CACGACCGGTGCTCGATTTAGTTAATTAA[sample-ID]AGCGATCGCTGGG
Overlap-extension	CGTATCGCTCCTAGGAGCGATACGCACGACCGGTGCTCGATTTAG

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primer	
LC Primer (for kappa chain)	CGATTGGAGGGCGTTATCCAC
LC Primer (for lambda chain)	TYTGTGGGACTTCCACTGCTC
HC Primer	TCTTGTCCACCTTGGTGTTGCTG

Table 32.	
Name	Sequence
RT oligo	CGTATCGCTCCTAGGAGCGATACGTTAATTAA[sample-ID]AGCGATCGCTGGG
LC Primer (for kappa chain)	CGATTGGAGGGCGTTATCCAC
LC Primer (for lambda chain)	TYTGTGGGACTTCCACTGCTC
HC Primer	TCTTGTCCACCTTGGTGTTGCTG

Table 33.	
Name	Sequence
Univ_seq_2	AACGCGTGACGAGAGACTGACAG
VK	ATGAGGSTCCCYGCTCAGCTGCTG G
VL	GGTCCTGGGCCCAGTCTGCCCTG
IgKC_v3_barcoded	AGGCCCTTACGACTGCGTCTTG AACAATAC CAGATGGCGGGAAGATGAAGAC
IgLC_v5_barcoded	AGGCCCTTACGACTGCGTCTTGAACAATAC CTCCCGGGTAGAAGTCAC
Fixed_PCR3	AGGCCCTTACGACTGCGTCTTG

Table 34. Co-expressed genes associated with B cell differentiation into memory B cells, short-live plasma cell, long-lived plasma cells and antibody secreting cells.

Generation of memory B cells	Generation of short-lived plasma cells	Generation of long-lived plasma cells	Antibody-secreting cells	
PAX-5	Blimp-1	Blimp-1	SLC7A7	CD36
Microphthalmia-assoc. transcription factor (MITF)	X-box binding protein 1 (XBP-1)	X-box binding protein 1 (XBP-1)	IL6R	BCL2L1
			RPN2	IL21R
	IRF-4	IRF-4	PDIA4	IKZF1
		BCMA	IG HD	BAC 2

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Table 35. Co-expressed genes associated with T cell differentiation into Treg, Th1, Th2, Th17 cells

Generation of Th1	Generation of Th2	Generation of Th17	Generation of Tregs	
T-bet	Gata-3	RORγt	FoxP3	GITR

Table 36. Co-expressed genes associated with plasmablast homing to specific tissues.

Homing of plasmablasts to the small intesting	Homing of plasmablasts to mucosal tissues	Homing of plasmablasts to ski
CCR9	CCR10	cutaneous lymphocyte-associated antigen (CLA)
α4β7		

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CLAIMS

1. A composition comprising a polynucleotide, wherein the polynucleotide comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises at least one identification region, and wherein the first region is coupled to the second region.

2. The composition of claim 1, wherein the variable immunoglobulin region comprises a VDJ region of an IgG immunoglobulin nucleotide sequence isolated from an activated human B cell greater than or equal to 8 μm in diameter, and wherein the 5' end of the immunoglobulin region is coupled to the 3' end of the identification region.

3. The composition of any one of claims 1-2, wherein the composition is comprised in a clonal family.

4. The composition of any one of claims 1-3, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is an activated B cell.

5. The composition of any one of claims 1-4, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasmablast.

6. The composition of any one of claims 1-5, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single B cell.

7. The composition of any one of claims 1-6, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell.

8. The composition of any one of claims 1-7, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell located in the blood of a subject.

9. The composition of any one of claims 1-8, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a human activated B cell.

10. The composition of any one of claims 1-9, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a memory B cell.

11. The composition of any one of claims 1-10, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasma cell.

12. The composition of any one of claims 1-11, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is an antigen--specific B cell.

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13. The composition of any one of claims 1-12, wherein the immunoglobulin region is isolated from a mammalian B cell.

14. The composition of any one of claims 1-13, wherein the immunoglobulin region is isolated from a human B cell.

15. The composition of any one of claims 1-14, wherein the immunoglobulin region is isolated from a mouse B cell.

16. The composition of any one of claims 1-15, wherein the immunoglobulin region is isolated from a B cell from a subject with a disease or condition of interest.

17. The composition of any one of claims 1-16, wherein the immunoglobulin region is isolated from a B cell from a subject recovering from or recovered from a disease or condition of interest.

18. The composition of any one of claims 1-17, wherein the immunoglobulin region is isolated from a B cell from a subject administered with at least one antigen of interest.

19. The composition of any one of claims 1-18, wherein the immunoglobulin region is isolated from a B cell from a subject administered with at least one antigen of interest and an adjuvant.

20. The composition of any one of claims 1-19, wherein the immunoglobulin region is isolated from a B cell located in the blood of a subject.

21. The composition of any one of claims 1-20, wherein the immunoglobulin region is isolated from a B cell located in the bone marrow of a subject.

22. The composition of any one of claims 1-21, wherein the immunoglobulin region is isolated from a B cell located in the spleen of a subject.

23. The composition of any one of claims 1-22, wherein the immunoglobulin region is isolated from a B cell located in at least one lymph node of a subject.

24. The composition of any one of claims 1-23, wherein the immunoglobulin region is isolated from a B cell located in lymphoid tissue of a subject.

25. The composition of any one of claims 1-24, wherein the immunoglobulin region is isolated from a B cell located in the gut of a subject.

26. The composition of any one of claims 1-25, wherein the immunoglobulin region is isolated from an activated B cell that is about 8-20 μm in diameter.

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27. The composition of any one of claims 1-26, wherein the immunoglobulin region is isolated from an activated B cell that is 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or greater than 20 μm in diameter.

28. The composition of any one of claims 1-27, wherein the immunoglobulin region is isolated from an activated B cell that is about 60, 70, 80, 90, 100, 120, 130, 140, 150, 200, 250, 300, 350, or greater than 350 μm^2 in area.

29. The composition of any one of claims 1-28, wherein the immunoglobulin region is isolated from an activated B cell that is about 250, 268, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, or greater than 4000 μm^3 in volume.

30. The composition of any one of claims 1-29, wherein the immunoglobulin region is isolated from an activated B cell that has a diameter of 10% or greater in size than the median diameter of a control resting B cell.

31. The composition of any one of claims 1-30, wherein the immunoglobulin region is isolated from an activated B cell that has a diameter of 15% or greater in size than the median diameter of a control resting B cell.

32. The composition of any one of claims 1-31, wherein the immunoglobulin region is isolated from an activated B cell that has a diameter of 20% or greater in size than the median diameter of a control resting B cell.

33. The composition of any one of claims 1-32, wherein the immunoglobulin region is isolated from an activated B cell capable of secreting immunoglobulin.

34. The composition of any one of claims 1-33, wherein the immunoglobulin region is isolated from a B cell in the gap 1 (G1), synthesis (S), gap 2 (G2), or mitosis (M) phase of the cell cycle.

35. The composition of any one of claims 1-34, wherein the immunoglobulin region is isolated from a B cell is not in the gap 0 (G0) phase of the cell cycle.

36. The composition of any one of claims 1-35, wherein the immunoglobulin region is isolated from a B cell characterized as having an FSC greater than 1.2x of the FSC mean of resting B lymphocytes by flow cytometry.

37. The composition of any one of claims 1-36, wherein the immunoglobulin region is isolated from a B cell characterized as having an FSC mean between 0.7 - 1.15x of the FSC mean of human monocytes by flow cytometry.

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38. The composition of any one of claims 1-37, wherein the immunoglobulin region is isolated from a single CD19 positive B cell.

39. The composition of any one of claims 1-38, wherein the immunoglobulin region is isolated from a single CD38 positive B cell.

40. The composition of any one of claims 1-39, wherein the immunoglobulin region is isolated from a single CD27 positive B cell.

41. The composition of any one of claims 1-40, wherein the immunoglobulin region is isolated from a single CD20 negative B cell.

42. The composition of any one of claims 1-41, wherein the immunoglobulin region is isolated from a single $CD19^{+}CD20^{-}CD27^{+}CD38^{hi}$ B cell.

43. The composition of any one of claims 1-42, wherein the 5' end of the immunoglobulin region is coupled to the 3' end of the identification region.

44. The composition of any one of claims 1-43, wherein the variable immunoglobulin region comprises a VDJ region of an immunoglobulin nucleotide sequence.

45. The composition of any one of claims 1-44, wherein the variable immunoglobulin region comprises a VJ region of an immunoglobulin nucleotide sequence.

46. The composition of any one of claims 1-45, wherein the variable immunoglobulin region comprises a V, D, and/or J region of an immunoglobulin nucleotide sequence.

47. The composition of any one of claims 1-46, wherein the variable immunoglobulin region comprises a heavy and/or light chain of an immunoglobulin nucleotide sequence.

48. The composition of any one of claims 1-47, wherein the variable immunoglobulin region comprises an IgG, IgM, IgD, IgE, or IgA immunoglobulin sequence.

49. The composition of any one of claims 1-48, wherein the variable immunoglobulin region comprises a human IgG1, IgG2, IgG3, or IgG4 immunoglobulin sequence.

50. The composition of any one of claims 1-49, wherein the variable immunoglobulin region comprises a mouse IgG1, IgG2a, IgG2b, or IgG3 immunoglobulin sequence.

51. The composition of any one of claims 1-50, wherein the immunoglobulin region is about 200-2000 nucleotides in length.

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52. The composition of any one of claims 1-51, wherein the immunoglobulin region is less than 200, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or greater than 2000 nucleotides in length.

53. The composition of any one of claims 1-52, wherein the identification region comprises a plurality of identification regions.

54. The composition of any one of claims 1-53, wherein the identification region comprises a plurality of identification regions, and wherein each identification region in the plurality has a distinct sequence.

55. The composition of any one of claims 1-54, wherein the identification region comprises at least one sample identification region and at least one plate identification region.

56. The composition of any one of claims 1-55, wherein the identification region comprises a sequence distinct from the sequence of the immunoglobulin region.

57. The composition of any one of claims 1-56, wherein the identification region is about 2 - 100 nucleotides in length.

58. The composition of any one of claims 1-57, wherein the identification region is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, or greater than 100 nucleotides in length.

59. The composition of any one of claims 1-58, wherein the identification region is 2 – 1,000 nucleotides in length.

60. The composition of any one of claims 1-59, wherein the identification region is equal to or greater than 100 nucleotides in length.

61. The composition of any one of claims 1-60, wherein the identification region comprises a contiguous non-coding nucleotide sequence.

62. The composition of any one of claims 1-61, wherein the identification region comprises a non-coding nucleotide sequence.

63. The composition of any one of claims 1-62, wherein the identification region comprises a non-contiguous, non-coding nucleotide sequence.

64. The composition of any one of claims 1-63, wherein the length of the sequence of the identification region is less than the length of the sequence of the immunoglobulin region.

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65. The composition of any one of claims 1-64, further comprising a third region, wherein the third region comprises an adapter region.

66. The composition of any one of claims 1-65, further comprising a third region, wherein the third region comprises an adapter region, and wherein the third region is located between the first region and the second region.

67. The composition of any one of claims 1-66, further comprising a third region, wherein the third region comprises an adapter region, and wherein the adapter region comprises at least one G nucleotide located at its 3' end.

68. The composition of any one of claims 1-67, wherein the identification region is 2-100 nucleotides long and has a sequence distinct from the immunoglobulin region sequence, and wherein the adaptor region comprises at least one G nucleotide at its 3' end and is located 3' of the sample identification region and 5' of the immunoglobulin region, and wherein the immunoglobulin variable region has undergone hypermutation and differs from the germline sequence of a naïve B cell.

69. The composition of any one of claims 1-68, wherein the composition is present in a container.

70. The composition of any one of claims 1-69, wherein a plurality of the compositions are present in a container.

71. The composition of any one of claims 1-70, wherein a plurality of the compositions are present in a single well of a single plate comprising a plurality of wells.

72. The composition of any one of claims 1-71, wherein the composition is in a library of compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide comprising a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region o is distinct from the nucleotide sequence of the other identification regions present in the library, and wherein the last nucleotide sequences of a plurality of variable immunoglobulin regions in the library share at least 80-99% sequence identity.

73. The composition of any one of claims 1-72, wherein the composition is comprised in a library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide,

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wherein the polynucleotide comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, and wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in each separate container in the library

74. A polynucleotide composition library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide, wherein the polynucleotide comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, and wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in each separate container in the library.

75. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide of the plurality is present in a separate container, wherein each polynucleotide of the plurality comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in the library, and wherein at least two variable immunoglobulin regions in the plurality share at least 80-99% sequence identity.

76. A polynucleotide library comprising a clonal family of polynucleotides, wherein each polynucleotide in the family comprises a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in the family, and wherein each of the variable immunoglobulin regions in the family exhibit at least 80-99% sequence identity.

77. The library of claim 76, wherein the library comprises a plurality of clonal families.

78. A clonal family of immunoglobulin sequences wherein each sequence in the family is coupled to an identification region.

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79. The clonal family of claim 78, wherein each identification region is distinct from the other identification regions.

80. The clonal family of any one of claims 78-79, wherein the immunoglobulin sequences comprise heavy chain immunoglobulin sequences.

81. The clonal family of any one of claims 78-80, wherein the immunoglobulin sequences comprise light chain immunoglobulin sequences.

82. The clonal family of any one of claims 78-81, wherein the immunoglobulin sequences comprise heavy chain and light chain immunoglobulin sequences.

83. The clonal family of any one of claims 78-82, wherein one or more of the identification regions comprise a light chain immunoglobulin sequence.

84. The clonal family of any one of claims 78-83, wherein one or more of the identification regions comprise a heavy chain immunoglobulin sequence.

85. A set of two or more of the clonal family of any one of claims 78-84.

86. A set of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more of the clonal family of any one of claims 78-85.

87. A clonal family of immunoglobulin sequences wherein each sequence in the family is operatively coupled to at least one contiguous nucleotide sequence.

88. The clonal family of claim 87, wherein the immunoglobulin sequences comprise heavy chain immunoglobulin sequences and the at least one contiguous nucleotide sequence comprises a light chain immunoglobulin sequence.

89. The clonal family of claim 87, wherein the immunoglobulin sequences comprise light chain immunoglobulin sequences and the at least one contiguous nucleotide sequence comprises a heavy chain immunoglobulin sequence.

90. A method of producing a clonal family of immunoglobulin sequences comprising obtaining a plurality of immunoglobulin sequences each having V, D, and/or J regions and each coupled to an identification region; and grouping two or more sequences from the plurality to produce the clonal family wherein each sequence in the clonal family is a mutated version of the same germline immunoglobulin sequence having a V, D, and/or J region or the germline immunoglobulin sequence having the V, D, and/or J region.

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91. The method of claim 90, wherein each identification region is distinct from the other identification regions

92. A method of producing a clonal family of immunoglobulin sequences comprising obtaining a plurality of immunoglobulin sequences each having V, D, and/or J regions and each coupled to an identification region, and wherein each identification region is distinct from the other identification regions; removing one or more identification regions; and grouping two or more sequences from the plurality to produce the clonal family wherein each sequence in the clonal family is a mutated version of the same germline immunoglobulin sequence having a V, D, and/or J region or the germline immunoglobulin sequence having the V, D, and/or J region.

93. A method of identifying a second cDNA coupled to a first identification region comprising selecting a first cDNA coupled to the first identification region and identifying the second cDNA based on the shared identity of the identification region coupled to each cDNA.

94. A method of producing a 3' tail on a second nucleotide sequence comprising obtaining a first nucleotide sequence and contacting the first nucleotide sequence with a thermal stable RNase H$^-$ reverse transcriptase having template switching activity at less than 50^{0}C, wherein the contacting produces the 3' tail and the second nucleotide sequence.

95. The method of claim 94, wherein the first nucleotide sequence is contacted at about less than 50, 49, 48, 47, 46, 45 ,44, 43, 42, or less than 42°C.

96. The method of any one of claims 94-95, wherein the first nucleotide sequence is contacted at 42°C.

97. The method of any one of claims 94-96, wherein the first nucleotide sequence is contacted at 45.5°C.

98. The method of any one of claims 94-97, wherein the transcriptase is a Moloney Murine Leukemia Virus (MMLV) RNase H$^-$ reverse transcriptase.

99. The method of any one of claims 94-98, wherein the transcriptase is SuperScript III.

100. A method for determining the naturally occurring sequence of a first sequence of interest comprising obtaining a plurality of sequences related to the first sequence and each coupled to a first identification region, wherein each first identification region is identical, and wherein one or more of the sequences in the plurality is distinct from the naturally

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occurring sequence; and comparing the sequences in the plurality to determine the naturally occurring sequence of the first sequence of interest.

101. The method of claim 100, wherein the plurality of sequences comprise immunoglobulin sequences.

102. The method of any one of claims 100-101, wherein plurality of sequences comprise immunoglobulin sequences.

103. The method of any one of claims 100-102, wherein plurality of sequences comprise immunoglobulin sequences.

104. The method of any one of claims 100-103, wherein plurality of sequences are each coupled to a second identification region and each second identification region is identical.

105. The method of any one of claims 100-104, wherein the first sequence of interest is an immunoglobulin sequence.

106. The method of any one of claims 100-105, wherein the plurality of sequences are immunoglobulin sequences.

107. A composition comprising a polynucleotide comprising a first region and a second region, wherein the first region comprises a B cell-derived variable immunoglobulin region and the second region comprises an identification region, and wherein the first region is coupled to the second region.

108. A polynucleotide composition library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide comprising a B cell-derived variable immunoglobulin region and an identification region, wherein the variable immunoglobulin region is coupled to the identification region, wherein the nucleotide sequence of each identification region is distinct from the nucleotide sequence of the other identification regions present in each separate container in the library.

109. A method for producing a polynucleotide composition, comprising:

obtaining a polynucleotide comprising a first region, wherein the first region comprises an expressed B cell variable immunoglobulin region associated with a subject; and

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generating the polynucleotide composition comprising the first region and a second region by coupling the first region to the second region, wherein the second region comprises an identification region.

110. The method of claim 109, wherein obtaining the polynucleotide comprises obtaining a B cell associated with the subject and processing the cell to prepare the polynucleotide.

111. The method of any one of claims 109-110, wherein obtaining the polynucleotide comprises receiving the polynucleotide directly or indirectly from a third party that has processed a B cell associated with the subject to prepare the polynucleotide.

112. The method of any one of claims 109-111, wherein obtaining the polynucleotide comprises receiving the polynucleotide directly or indirectly from a third party that has solubilized a B cell associated with the subject to prepare the polynucleotide.

113. The method of any one of claims 109-112, wherein obtaining the polynucleotide comprises obtaining a B cell using a flow cytometer.

114. The method of any one of claims 109-113, wherein obtaining the polynucleotide comprises obtaining a B cell using a microfluidic device.

115. The method of any one of claims 109-114, wherein the variable immunoglobulin region comprises a VDJ region of an IgG immunoglobulin nucleotide sequence is isolated from an activated human B cell greater than or equal to 8 μm in diameter, and wherein the 5' end of the immunoglobulin region is coupled to the 3' end of the identification region.

116. The method of any one of claims 109-115, wherein the composition is comprised in a clonal family.

117. The method of any one of claims 109-116, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is an activated B cell.

118. The method of any one of claims 109-117, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasmablast.

119. The method of any one of claims 109-118, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single B cell.

120. The method of any one of claims 109-119, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell.

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121. The method of any one of claims 109-120, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a single activated B cell located in the blood of a subject.

122. The method of any one of claims 109-121, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a human activated B cell.

123. The method of any one of claims 109-122, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a memory B cell.

124. The method of any one of claims 109-123, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is a plasma cell.

125. The method of any one of claims 109-124, wherein the immunoglobulin region is isolated from a B cell, and wherein the B cell is an antigen-specific B cell.

126. The method of any one of claims 109-125, wherein the immunoglobulin region is isolated from a mammalian B cell.

127. The method of any one of claims 109-126, wherein the immunoglobulin region is isolated from a human B cell.

128. The method of any one of claims 109-127, wherein the immunoglobulin region is isolated from a mouse B cell.

129. The method of any one of claims 109-128, wherein the immunoglobulin region is isolated from a B cell from a subject with a disease or condition of interest.

130. The method of any one of claims 109-129, wherein the immunoglobulin region is isolated from a B cell from a subject recovering from or recovered from a disease or condition of interest.

131. The method of any one of claims 109-130, wherein the immunoglobulin region is isolated from a B cell from a subject administered with an antigen of interest.

132. The method of any one of claims 109-131, wherein the immunoglobulin region is isolated from a B cell from a subject administered with an antigen of interest and an adjuvant.

133. The method of any one of claims 109-132, wherein the immunoglobulin region is isolated from a B cell located in the blood of a subject.

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134. The method of any one of claims 109-133, wherein the immunoglobulin region is isolated from a B cell located in the bone marrow of a subject.

135. The method of any one of claims 109-134, wherein the immunoglobulin region is isolated from a B cell located in the spleen of a subject.

136. The method of any one of claims 109-135, wherein the immunoglobulin region is isolated from a B cell located in at least one lymph node of a subject.

137. The method of any one of claims 109-136, wherein the immunoglobulin region is isolated from a B cell located in lymphoid tissue of a subject.

138. The method of any one of claims 109-137, wherein the immunoglobulin region is isolated from a B cell located in the gut of a subject.

139. The method of any one of claims 109-138, wherein the immunoglobulin region is isolated from an activated B cell that is about 8-20 μm in diameter.

140. The method of any one of claims 109-139, wherein the immunoglobulin region is isolated from an activated B cell that is 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or greater than 20 μm in diameter.

141. The method of any one of claims 109-140, wherein the immunoglobulin region is isolated from an activated B cell that is about 60, 70, 80, 90, 100, 120, 130, 140, 150, 200, 250, 300, 350, or greater than 350 μm^2 in area.

142. The method of any one of claims 109-141, wherein the immunoglobulin region is isolated from an activated B cell that is about 250, 268, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, or greater than 4000 μm^3 in volume.

143. The method of any one of claims 109-142, wherein the immunoglobulin region is isolated from an activated B cell that has a diameter of 10% or greater in size than the median diameter of a control resting B cell.

144. The method of any one of claims 109-143, wherein the immunoglobulin region is isolated from an activated B cell that has a diameter of 15% or greater in size than the median diameter of a control resting B cell.

145. The method of any one of claims 109-144, wherein the immunoglobulin region is isolated from an activated B cell that has a diameter of 20% or greater in size than the median diameter of a control resting B cell.

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146. The method of any one of claims 109-145, wherein the immunoglobulin region is isolated from an activated B cell capable of secreting immunoglobulin.

147. The method of any one of claims 109-146, wherein the immunoglobulin region is isolated from a B cell in the gap 1 (G1), synthesis (S), gap 2 (G2), or mitosis (M) phase of the cell cycle.

148. The method of any one of claims 109-147, wherein the immunoglobulin region is isolated from a B cell is not in the gap 0 (G0) phase of the cell cycle.

149. The method of any one of claims 109-148, wherein the immunoglobulin region is isolated from a B cell characterized as having an FSC mean greater than 1.2x of the FSC mean of resting B lymphocytes by flow cytometry.

150. The method of any one of claims 109-149, wherein the immunoglobulin region is isolated from a B cell characterized as having an FSC between 0.7 - 1.15x of the FSC mean of human monocytes by flow cytometry.

151. The method of any one of claims 109-150, wherein the immunoglobulin region is isolated from a single CD19 positive B cell.

152. The method of any one of claims 109-151, wherein the immunoglobulin region is isolated from a single CD38 positive B cell.

153. The method of any one of claims 109-152, wherein the immunoglobulin region is isolated from a single CD27 positive B cell.

154. The method of any one of claims 109-153, wherein the immunoglobulin region is isolated from a single CD20 negative B cell.

155. The method of any one of claims 109-154, wherein the immunoglobulin region is isolated from a single $CD19^{+}CD20^{-}CD27^{+}CD38^{hi}$ B cell.

156. The method of any one of claims 109-155, wherein the 5' end of the immunoglobulin region is coupled to the 3' end of the identification region.

157. The method of any one of claims 109-156, wherein the variable immunoglobulin region comprises a VDJ region of an immunoglobulin nucleotide sequence.

158. The method of any one of claims 109-157, wherein the variable immunoglobulin region comprises a VJ region of an immunoglobulin nucleotide sequence.

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159. The method of any one of claims 109-158, wherein the variable immunoglobulin region comprises a V, D, and/or J region of an immunoglobulin nucleotide sequence.

160. The method of any one of claims 109-159, wherein the variable immunoglobulin region comprises a heavy and/or light chain of an immunoglobulin nucleotide sequence.

161. The method of any one of claims 109-160, wherein the variable immunoglobulin region comprises an IgG, IgM, IgD, IgE, or IgA immunoglobulin sequence.

162. The method of any one of claims 109-161, wherein the variable immunoglobulin region comprises a human IgG1, IgG2, IgG3, or IgG4 immunoglobulin sequence.

163. The method of any one of claims 109-162, wherein the variable immunoglobulin region comprises a mouse IgG1, IgG2a, IgG2b, or IgG3 immunoglobulin sequence.

164. The method of any one of claims 109-163, wherein the immunoglobulin region is about 200-2000 nucleotides in length.

165. The method of any one of claims 109-164, wherein the immunoglobulin region is less than 200, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or greater than 2000 nucleotides in length.

166. The method of any one of claims 109-165, wherein the identification region comprises a plurality of identification regions.

167. The method of any one of claims 109-166, wherein the identification region comprises a plurality of identification regions, and wherein each identification region in the plurality has a distinct sequence.

168. The method of any one of claims 109-167, wherein the identification region comprises at least one sample identification region and at least one plate identification region.

169. The method of any one of claims 109-168, wherein the identification region comprises a sequence distinct from the sequence of the immunoglobulin region.

170. The method of any one of claims 109-169, wherein the identification region is about 2 - 100 nucleotides in length.

171. The method of any one of claims 109-170, wherein the identification region is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, or greater than 100 nucleotides in length.

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172. The method of any one of claims 109-171, wherein the identification region is 2 – 1,000 nucleotides in length.

173. The method of any one of claims 109-172, wherein the identification region is equal to or greater than 100 nucleotides in length.

174. The method of any one of claims 109-173, wherein the identification region comprises a contiguous non-coding nucleotide sequence.

175. The method of any one of claims 109-174, wherein the identification region comprises a non-coding nucleotide sequence.

176. The method of any one of claims 109-175, wherein the identification region comprises a non-contiguous, non-coding nucleotide sequence.

177. The method of any one of claims 109-176, wherein the length of the sequence of the identification region is less than the length of the sequence of the immunoglobulin region.

178. The method of any one of claims 109-177, further comprising a third region, wherein the third region comprises an adapter region.

179. The method of any one of claims 109-178, further comprising a third region, wherein the third region comprises an adapter region, and wherein the third region is located between the first region and the second region.

180. The method of any one of claims 109-179, further comprising a third region, wherein the third region comprises an adapter region, and wherein the adapter region comprises at least one G nucleotide located at its 3' end.

181. The method of any one of claims 109-180, wherein the identification region is 2-100 nucleotides long and has a sequence distinct from the immunoglobulin region sequence, and wherein the adaptor region comprises at least one G nucleotide at its 3' end and is located 3' of the sample identification region and 5' of the immunoglobulin region, and wherein the immunoglobulin variable region has undergone hypermutation and differs from the germline sequence of a naïve B cell.

182. The method of any one of claims 109-181, wherein the composition is present in a container.

183. The method of any one of claims 109-182, wherein a plurality of the compositions are present in a container.

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184. The method of any one of claims 109-183, wherein a plurality of the compositions are present in a single well of a single plate comprising a plurality of wells.

185. The method of any one of claims 109-184, wherein the composition is in a library of compositions, wherein each composition is present in a separate container, wherein each composition comprises a polynucleotide comprising a first region and a second region, wherein the first region comprises an expressed B cell variable immunoglobulin region and the second region comprises an identification region, wherein the first region is coupled to the second region, wherein the nucleotide sequence of each identification region o is distinct from the nucleotide sequence of the other identification regions present in the library, and wherein the nucleotide sequence of a plurality of variable immunoglobulin regions in the library share at least 80-99% sequence identity.

186. A method for producing a polynucleotide composition, comprising:

obtaining a B cell associated with a subject;

isolating polynucleotides from the cell comprising an expressed B cell variable immunoglobulin region; and

generating the polynucleotide composition comprising the variable immunoglobulin region and an identification region by coupling the variable immunoglobulin region to the identification region.

187. A method for producing a polynucleotide composition, comprising:

obtaining a polynucleotide comprising a B cell-derived variable immunoglobulin region associated with a subject; and

generating the polynucleotide composition comprising the variable immunoglobulin region and an identification region by coupling the variable immunoglobulin region to the identification region.

188. The method of claim 187, wherein obtaining the polynucleotide comprises obtaining a B cell and processing the cell to prepare the polynucleotide.

189. The method of claim 187, wherein obtaining the polynucleotide comprises receiving the polynucleotide directly or indirectly from a third party that has processed a B cell to prepare the polynucleotide.

190. A method for producing two or more polynucleotide compositions, comprising:

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obtaining a polynucleotide library comprising a plurality of polynucleotides associated with a plurality of samples obtained from one or more subjects, wherein one or more polynucleotides comprises an expressed B cell variable immunoglobulin region, wherein each sample is associated with a B cell, and wherein each polynucleotide associated with each sample is present in a separate container; and

generating two or more polynucleotide compositions each comprising a polynucleotide from the plurality of polynucleotides and an identification region by coupling the polynucleotide to the identification region, wherein the sequence of each identification region is distinct from the sequence of the identification regions coupled to the other polynucleotides in the library.

191. The method of claim 190, wherein obtaining the polynucleotide library comprises obtaining a plurality of B cells and processing the cells to prepare the polynucleotide library.

192. The method of claim 190, wherein obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has processed a plurality of B cells to prepare the polynucleotide library.

193. A method for producing two or more polynucleotide compositions, comprising:

obtaining a polynucleotide library comprising a plurality of polynucleotides associated with a plurality of samples obtained from one or more subjects, wherein one or more polynucleotides comprises a B cell-derived variable immunoglobulin region, and wherein each polynucleotide associated with each sample is present in a separate container; and

generating two or more polynucleotide compositions each comprising a polynucleotide from the plurality of polynucleotides and an identification region by coupling the polynucleotide to the identification region, wherein the sequence of each identification region is distinct from the sequence of the identification regions coupled to the other polynucleotides in the library.

194. The method of claim 193, wherein obtaining the polynucleotide library comprises obtaining a plurality of B cells and processing the cells to prepare the polynucleotide library.

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195. The method of claim 193, wherein obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has processed a plurality of B cells to prepare the polynucleotide library.

196. A polynucleotide composition library comprising a plurality of polynucleotide compositions, wherein each composition is present in a separate container, wherein each composition comprises a single sample-derived cDNA region comprising the nucleotide C at the 3' end of the cDNA region and a sample identification-adapter region comprising a sample identification region coupled to an adapter region, wherein the nucleotide sequence of the sample identification region of each sample identification-adapter region is distinct from the nucleotide sequence of the sample identification region of the other sample identification-adapter regions present in each separate container in the library, wherein the adapter region comprises the nucleotide G at the 3' end of the adapter region, and wherein the sample identification-adapter region is attached to the cDNA region by binding between the C and G.

197. The library of claim 196, wherein the cDNA region comprises an RNA polynucleotide hybridized to a DNA polynucleotide.

198. The library of claim 196 or claim 197, wherein the cDNA region comprises an mRNA polynucleotide hybridized to a cDNA polynucleotide.

199. The library of any one of claims 196-198, wherein the cDNA region comprises at least one C at the 3' end and wherein the adapter region comprises at least one G at the 3' end.

200. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a sample identification region, an adapter region, and a single sample-derived cDNA region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the cDNA region is coupled to the 3' end of the adapter region, wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample, and wherein the sample identification region is double-stranded.

201. The library of claim 200, wherein each polynucleotide comprises a plurality of sample identifications regions.

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202. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

203. The library of claim 202, wherein the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises the sequence CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

204. The library of any one of claims 202-203, wherein the sequence of the universal primer region is not fully complementary to any human gene exon, and wherein the universal primer region has minimal secondary structure that does not interfere with the adapter region.

205. The library of any one of claims 202-204, wherein the universal primer region is the sequence CACGACCGGTGCTCGATTTAG.

206. The library of any one of claims 202-205, wherein the amplicon region comprises a cDNA region comprising a cDNA nucleotide sequence.

207. The library of any one of claims 202-206, wherein the sequence of the sample identification region of each polynucleotide from a first single sample differs by at least 1 nucleotide from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

208. The library of any one of claims 202-207, wherein the sequence of the sample identification region of each polynucleotide from a first single sample differs by at least 2 nucleotides from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

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209. The library of any one of claims 202-208, wherein the sample identification region is at least 10 nucleotides in length.

210. The library of any one of claims 202-209, wherein the sample identification region is at least 1 nucleotide in length.

211. The library of any one of claims 202-210, wherein the sequence of each sample identification region is selected from Tables 2 and 7.

212. The library of any one of claims 202-211, wherein the sequence of the adapter region comprises at least one G nucleotide at its 3' end.

213. The library of any one of claims 202-212, wherein the amplicon region comprises an immunoglobulin heavy chain amplicon sequence, an immunoglobulin light chain amplicon sequence, a T cell receptor alpha amplicon sequence, or a T cell receptor beta amplicon sequence.

214. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-3', wherein A is a universal primer region, wherein B is a sample identification region, wherein C is an adapter region, wherein D is an amplicon region from a single sample, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

215. A polynucleotide comprising a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region.

216. The polynucleotide of claim 215, wherein the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

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217. A polynucleotide comprising the sequence 5'-A-B-C-D-3', wherein A is a universal primer region, wherein B is a sample identification region, wherein C is an adapter region, and wherein D is an amplicon region from a single sample.

218. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a first plate identification region, a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first plate identification region is operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

219. The library of claim 218, wherein the sequence of the first plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples.

220. The library of any one of claims 218-219, wherein the sequence of the first plate identification region of each polynucleotide from the first set of single samples differs by at least 1 nucleotide from the sequence of the first plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples.

221. The library of any one of claims 218-220, wherein the sequence of the first plate identification region of each polynucleotide from the first set of single samples differs by at least 2 nucleotides from the sequence of the first plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples.

222. The library of any one of claims 218-221, wherein the first plate identification region is at least 10 nucleotides in length.

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223. The library of any one of claims 218-222, wherein the sequence of the first plate identification region is selected from Tables 3 and 6.

224. The library of any one of claims 218-223, wherein the 3' end of the first plate identification region is coupled to the 5' end of the universal primer region, wherein the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, wherein the adapter region comprises at least one G, wherein each polynucleotide further comprises a second plate identification region, a first sequencing region, and a second sequencing region, wherein the 5' end of the second plate identification region is coupled to the 3' end of the amplicon region, wherein the 3' end of the first sequencing region is coupled to the 5' end of the first plate identification region, and wherein the 5' end of the second sequencing region is coupled to the 3' end of the second plate identification region.

225. The library of any one of claims 218-224, wherein the sequence of the second plate identification region is identical to the sequence of the first plate identification region on each polynucleotide.

226. The library of any one of claims 218-225, wherein the sequence of the second plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the second plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples.

227. The library of any one of claims 218-226, wherein the sequence of the second plate identification region of each polynucleotide from the first set of single samples differs by at least 1 nucleotide from the sequence of the second plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples.

228. The library of any one of claims 218-227, wherein the sequence of the second plate identification region of each polynucleotide from the first set of single samples differs by at least 2 nucleotides from the sequence of the second plate identification region of the other polynucleotides in the library from one or more single sample sets distinct from the first set of single samples.

229. The library of any one of claims 218-228, wherein the second plate identification region is at least 10 nucleotides in length.

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230. The library of any one of claims 218-229, wherein the sequence of the second plate identification region is selected from Tables 3 and 6.

231. The library of any one of claims 218-230, wherein the first sequencing region comprises GAGAGACTGACAGCGTATCGCCTCCCTCGCGCCATCAG.

232. The library of any one of claims 218-231, wherein the second sequencing region comprises CTATGCGCCTTGCCAGCCCGCTCAG.

233. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-E-3', wherein A is a plate identification region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, wherein E is an amplicon region from a single sample, and wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

234. A polynucleotide comprising a first plate identification region, a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first plate identification region is operatively coupled to the universal primer region, and wherein the amplicon region is operatively coupled to the adapter region.

235. The polynucleotide of claim 234, wherein the 3' end of the first plate identification region is coupled to the 5' end of the universal primer region, wherein the 5' end of the amplicon region is coupled to the 3' end of the adapter region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, wherein the adapter region comprises at least one G, wherein each polynucleotide further comprises a second plate identification region, a first sequencing region, and a second sequencing region, wherein the 5' end of the second plate identification region is coupled to the 3' end of the amplicon region, wherein the 3' end of the first sequencing region is coupled to the 5' end of the first plate identification region, and wherein the 5' end of the second sequecing region is coupled to the 3' end of the second plate identification region.

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236. A polynucleotide comprising the sequence 5'-A-B-C-D-E-3', wherein A is a plate identification region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, and wherein E is an amplicon region from a single sample.

237. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a first restriction site region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second restriction site region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first restriction site region is operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, wherein the second restriction site region is operatively coupled to the amplicon region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

238. The library of claim 237, wherein the first restriction site region comprises one or more restriction sites.

239. The library of any one of claims 237-238, wherein the first restriction site region comprises one or more restriction sites selected from the group consisting of: NheI, XhoI, BstBI, EcoRI, SacII, BbvCI, PspXI, AgeI, ApaI, KpnI, Acc65I, XmaI, BstEII, DraIII, PacI, FseI, AsiSI and AscI.

240. The library of any one of claims 237-239, wherein the second restriction site region comprises one or more restriction sites.

241. The library of any one of claims 237-240, wherein the second restriction site region comprises one or more restriction sites selected from the group consisting of: NheI, XhoI, BstBI, EcoRI, SacII, BbvCI, PspXI, AgeI, ApaI, KpnI, Acc65I, XmaI, BstEII, DraIII, PacI, FseI, AsiSI and AscI.

242. The library of any one of claims 237-241, wherein the 3' end of the first restriction site region is coupled to the 5' end of the universal primer region, wherein the 3'

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end of the adapter region is coupled to the 5' end of the amplicon region, wherein the 3' end of the amplicon region is coupled to the 5' end of the second restriction site region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

243. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-E-F-3', wherein A is a first restriction site region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, wherein E is an amplicon region from a single sample, wherein F is a second restriction site region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

244. A polynucleotide for insertion into a vector, comprising a first restriction site region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second restriction site region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first restriction site region is operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, and wherein the second restriction site region is operatively coupled to the amplicon region.

245. The polynucleotide of claim 244, wherein the 3' end of the first restriction site region is coupled to the 5' end of the universal primer region, wherein the 3' end of the adapter region is coupled to the 5' end of the amplicon region, wherein the 3' end of the amplicon region is coupled to the 5' end of the second restriction site region, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

246. A polynucleotide for insertion in a vector, comprising the sequence 5'-A-B-C-D-E-F-3', wherein A is a first restriction site region, wherein B is a universal primer region, wherein C is a sample identification region, wherein D is an adapter region, wherein E is an amplicon region from a single sample, and wherein F is a second restriction site region.

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247. A polynucleotide adapter molecule, comprising a universal primer region, a sample identification region, and an adapter region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, and wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region.

248. The molecule of claim 247, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, and wherein the adapter region comprises at least one G.

249. A polynucleotide primer, comprising a universal primer region and a plate identification region, and wherein the 3' end of the plate identification region is coupled to the 5' end of the universal primer region.

250. The primer of claim 247, wherein the universal primer region comprises CACGACCGGTGCTCGATTTAG, wherein the primer further comprises a sequencing region, and wherein the 3' end of the sequencing region is coupled to the 5' end of the plate identification region.

251. A vector comprising the polynucleotide of any one of claims 1-250.

252. The vector of claim 251, wherein the vector comprises a plurality of polynucleotides.

253. The vector of any one of claims 251-252, wherein the vector is selected from the group consisting of: pEE6.4 and pEE12.4.

254. An isolated host cell comprising the vector of any one of claims claim 251-253 or the polynucleotide of any one of claims 1-250.

255. The host cell of claim 254, wherein the host cell is selected from the group consisting of: CHO cells, CHO-K1 cells, CHO-S cells, NS0 cells, CHO cells that are dhfr-, CHO-dhfr-, DUKX-B11 CHO cells, and DG44 CHO cells.

256. A method for producing one or more polynucleotides of interest, comprising:

obtaining a cDNA library comprising a plurality of cDNAs associated with a plurality of samples obtained from one or more subjects, wherein each cDNA is associated with a single sample in the plurality of samples, and wherein each cDNA associated with each sample is present in a separate container; and

adding an adapter molecule to the cDNA associated with each sample to produce the one or more polynucleotides of interest, wherein the adapter molecule comprises a sample

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identification region and an adapter region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the sequence of the sample identification region of each adapter molecule is distinct from the sequence of the sample identification region of the other adapter molecules added to each cDNA in the library.

257. The method of claim 256, further comprising allowing the 3' end of the adapter region to attach to the 3' end of each cDNA in the library to produce the one or more polynucleotides of interest.

258. The method of any one of claims 256-257, wherein obtaining the cDNA library comprises obtaining the plurality of samples and processing the samples to prepare the cDNA library.

259. The method of any one of claims 256-258, wherein the adapter molecule further comprises a universal primer region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region.

260. The method of any one of claims 256-259, wherein each cDNA region comprises an mRNA polynucleotide hybridized to a cDNA polynucleotide.

261. The method of any one of claims 256-260, wherein each sample comprises a cell.

262. The method of any one of claims 256-261, wherein the cell is a B cell.

263. The method of any one of claims 256-262, wherein the B cell is a plasmablast, memory B cell, or a plasma cell.

264. The method of any one of claims 256-263, wherein each sample comprises a plurality of cells.

265. The method of of any one of claims 256-264, wherein obtaining the cDNA library comprises receiving the cDNA library directly or indirectly from a third party that has processed the plurality of samples to prepare the cDNA library.

266. The method of any one of claims 256-265, wherein the adaptor is added by annealing the adaptor to the '3 tail of a cDNA generated during a reverse transcription reaction.

267. The method of any one of claims 256-266, wherein each cDNA comprises at least one C nucleotide, wherein C is located at the 3' end of each cDNA, wherein the adapter

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region comprises at least one G nucleotide, wherein G is located at the 3' end of the adapter region, and wherein the adapter region is attached to each cDNA via binding between the G and C.

268. The method of any one of claims 256-267, wherein the adapter molecule is single-stranded, and further comprising incorporating the adapter molecule into each cDNA by allowing an enzyme to make the adapter molecule double-stranded.

269. The method of any one of claims 256-268, wherein the adapter molecule is incorporated into each cDNA to produce the polynucleotide of interest by an MMLV H- reverse transcriptase.

270. A method of producing one or more polynucleotides of interest for sequencing, comprising:

obtaining a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample; and

amplifying the polynucleotide library with a set of primers to produce the one or more polynucleotides of interest for sequencing, wherein the one or more polynucleotides of interest for sequencing comprises a first sequencing region, a first plate identification region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second sequencing region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the first plate identification region is operatively coupled to the universal primer region, wherein the amplicon region is operatively coupled to the adapter region, wherein the first sequencing region is located at the 5' end of the

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polynucleotide of interest, and wherein the second sequencing region is located at the 3' end of the polynucleotide of interest.

271. The method of claim 270, further comprising sequencing the one or more polynucleotides of interest.

272. The method of claim 270, further comprising sequencing the one or more polynucleotides of interest with 454 sequencing.

273. The method of claim 270, further comprising sequencing the one or more polynucleotides of interest with SMRT sequencing.

274. The method of claim 270, further comprising sequencing the one or more polynucleotides of interest with SOLiD sequencing.

275. The method of claim 270, further comprising sequencing the one or more polynucleotides of interest with SOLEXA sequencing.

276. The method of claim 270, further comprising sequencing the one or more polynucleotides of interest with tSMS sequencing.

277. The method of claim 270, wherein the set of primers is selected from the primers shown in Tables 1 and 5.

278. The method of claim 270, wherein obtaining the polynucleotide library comprises preparing the polynucleotide library in a laboratory.

279. The method of claim 270, wherein obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has prepared the polynucleotide library.

280. A method for analyzing sequencing data, comprising:

obtaining a dataset associated with a plurality of polynucleotides, wherein the dataset comprises sequencing data for the plurality of polynucleotides, wherein each polynucleotide in the plurality of polynucleotides comprises a sample identification region, and wherein each sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the plurality of polynucleotides from one or more samples distinct from the first single sample; and

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analyzing the dataset to match together polynucleotides with identical sample identification regions, wherein a match indicates that the polynucleotides originated from the same sample.

281. The method of claim 280, wherein each polynucleotide in the plurality of polynucleotides further comprises a first plate identification region, wherein each combination of each first plate identification region and sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the first plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the plurality of polynucleotides from one or more single sample sets distinct from the first set of single samples, and further comprising analyzing the dataset to match together polynucleotides with identical first plate identification regions and identical sample identification regions, wherein a match between both regions indicates that the polynucleotides originated from the same sample.

282. The method of claim 280, wherein obtaining the dataset comprises obtaining the plurality of polynucleotides and sequencing the plurality of polynucleotides to experimentally determine the dataset.

283. The method of claim 280, wherein obtaining the dataset comprises receiving the dataset directly or indirectly from a third party that has sequenced the plurality of polynucleotides to experimentally determine the dataset.

284. The method of claim 280, wherein the dataset is stored on an electronic storage medium.

285. The method of claim 280, wherein the single sample is a single cell.

286. The method of claim 280, wherein the single sample comprises a single cell.

287. The method of claim 280, wherein the single sample comprises a single B cell.

288. The method of claim 280, wherein the single sample comprises a plurality of B cells.

289. The method of claim 280, further comprising selecting one or more polynucleotides for cloning.

290. A method for identifying a second polynucleotide of interest based on selection of a first polynucleotide of interest, comprising:

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obtaining a dataset associated with a plurality of polynucleotides, wherein the dataset comprises sequencing data for the plurality of polynucleotides, wherein each polynucleotide in the plurality of polynucleotides comprises a sample identification region, and wherein each sample identification region on each polynucleotide is unique to a single sample thereby associating each polynucleotide in the plurality of polynucleotides with a distinct single sample, wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the plurality of polynucleotides from one or more samples distinct from the first single sample; and

selecting a first polynucleotide of interest associated with a first single sample from the dataset and identifying a second polynucleotide of interest in the first single sample based on the sample identification region of the first polynucleotide of interest.

291. The method of claim 290, wherein each polynucleotide in the plurality of polynucleotides further comprises a first plate identification region, wherein each combination of each first plate identification region and sample identification region on each polynucleotide is unique to a single sample, wherein the sequence of the first plate identification region of each polynucleotide from a first set of single samples is distinct from the sequence of the first plate identification region of the other polynucleotides in the plurality of polynucleotides from one or more single sample sets distinct from the first set of single samples, and further comprising identifying a second polynucleotide of interest in the first single sample based on the sample identification region and first plate identification region of the first polynucleotide of interest.

292. The method of claim 291, wherein the first single sample comprises a B cell.

293. The method of claim 291, wherein the first single sample comprises a plurality of B cells.

294. The method of claim 291, wherein the first single sample comprises a B cell, wherein the first polynucleotide of interest comprises an antibody heavy chain nucleotide sequence, and wherein the second polynucleotide of interest comprises an antibody light chain nucleotide sequence.

295. The method of claim 291, wherein the first single sample comprises a B cell, wherein the first polynucleotide of interest comprises an antibody light chain nucleotide

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sequence, and wherein the second polynucleotide of interest comprises an antibody heavy chain nucleotide sequence.

296. The method of claim 291, wherein obtaining the dataset comprises obtaining the plurality of polynucleotides and sequencing the plurality of polynucleotides to experimentally determine the dataset.

297. The method of claim 291, wherein obtaining the dataset comprises receiving the dataset directly or indirectly from a third party that has sequenced the plurality of polynucleotides to experimentally determine the dataset.

298. The method of claim 291, wherein the dataset is stored on an electronic storage medium.

299. A method of producing one or more polynucleotides of interest for cloning, comprising:

obtaining a polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises a universal primer region, a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region, wherein the sequence of the universal primer region is substantially identical on each polynucleotide in the plurality of polynucleotides, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample; and

amplifying the polynucleotide library with a set of primers to produce the one or more polynucleotides of interest for cloning, wherein the one or more polynucleotides of interest for cloning comprises a first restriction site region, a universal primer region, a sample identification region, an adapter region, an amplicon region from a single sample, and a second restriction site region, wherein the 3' end of the universal primer region is coupled to the 5' end of the sample identification region, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, wherein the amplicon region is operatively coupled to the adapter region, wherein the first restriction

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site region is located at the 5' end of the polynucleotide of interest, and wherein the second restriction site region is located at the 3' end of the polynucleotide of interest.

300. The method of claim 299, wherein obtaining the polynucleotide library comprises preparing the polynucleotide library in a laboratory.

301. The method of claim 299, wherein obtaining the polynucleotide library comprises receiving the polynucleotide library directly or indirectly from a third party that has prepared the polynucleotide library.

302. A method of producing a molecule of interest, comprising:

obtaining a host cell comprising a polynucleotide comprising a sample identification region, an adapter region, and an amplicon region from a single sample, wherein the 3' end of the sample identification region is coupled to the 5' end of the adapter region, and wherein the amplicon region is operatively coupled to the adapter region; and

culturing the host cell under conditions sufficient to produce the molecule of interest.

303. The method of claim 302, wherein obtaining the host cell comprises preparing the host cell comprising the polynucleotide in a laboratory.

304. The method of claim 302, wherein obtaining the host cell comprises receiving the host cell comprising the polynucleotide directly or indirectly from a third party that has prepared the host cell.

305. The method of claim 302, wherein the molecule of interest is a protein.

306. The method of claim 302, wherein the molecule of interest is an antibody.

307. The method of claim 302, wherein the molecule of interest is a human monoclonal antibody.

308. The method of claim 302, further comprising collecting the molecule of interest.

309. A kit, comprising a polynucleotide, a polynucleotide library, a vector, or a host cell of any one of claims 1-308 and instructions for use.

310. A method of linking and barcoding a plurality of non-contiguous polynucleotide sequences of interest, said method comprising:

(a) providing a plurality of cDNA molecules;

(b) physically linking cDNA molecules of interest; and

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(c) adding a barcode sequence to the cDNAs of interest prior to, during, or after physical linkage.

311. The method of claim 310, wherein the physical linking is by ligation.

312. The method of claim 310, wherein the physical linking is by recombination.

313. The method of claim 310, wherein the physical linking comprises using an overlap-extension sequence.

314. The method of claim 310, wherein the barcode sequence is located at one or both of the ends of the physically linked cDNAs.

315. The method of claim 310, wherein the barcode sequence is located in between the physically linked cDNAs.

316. The method of claim 311, wherein the ligation is performed by annealing and ligation of compatible ends.

317. The method of claim 310, wherein the compatible ends are a restriction site.

318. The method of claim 310, wherein the ligation is performed by blunt end ligation.

319. The method of claim 310, wherein the overlap-extension sequence is added during the course of amplification using a primer comprising the overlap-extension tail.

320. The method of claim 310, wherein the overlap-extension sequence is added during the course of reverse transcription using a primer comprising the overlap-extension tail.

321. The method of claim 310, wherein the overlap-extension sequence is added by annealing an adaptor to the 3' tail of a cDNA generated during a reverse transcription reaction.

322. The method of claim 310, wherein the barcode sequence is added by ligation.

323. The method of claim 310, wherein the ligation is performed by annealing and ligation of compatible ends.

324. The method of claim 310, wherein the compatible ends are a restriction site.

325. The method of claim 310, wherein the ligation is performed by blunt end ligation of an adaptor comprising the barcode sequence.

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326. The method of claim 310, wherein the barcode sequence is added during the course of an amplification reaction using a primer comprising the barcode sequence.

327. The method of claim 310, wherein the barcode sequence is added during the course of a reverse transcription reaction using a primer comprising the barcode sequence.

328. The method of claim 310, wherein the barcode sequence is added by annealing an adaptor to the 3' tail of a cDNA generated during a reverse transcription reaction.

329. The method of claim 310, wherein the 3' end of the cDNA comprises at least one C nucleotide, and wherein the 3' end of the adaptor comprises at least one G nucleotide, and wherein the adaptor is attached to each cDNA via binding between the C and G.

330. The method of claim 310, wherein the adaptor is single-stranded, and further comprising incorporating the adaptor into each cDNA by allowing an enzyme to make the adaptor double-stranded.

331. The method of claim 310, wherein the adaptor is incorporated into each cDNA by an MMLV H- reverse transcriptase.

332. The method of claim 310, wherein the overlap-extension sequence comprises a barcode sequence.

333. The method of claim 310, wherein the polynucleotide sequences of interest comprise antibody heavy and light chains.

334. The method of claim 310, further comprising (d) adding a sequencing region to the cDNAs of interest prior to, during, or after physical linkage.

335. The method of claim 310, wherein the sequencing region is added with an adaptor.

336. The method of claim 310, further comprising (e) sequencing of the physically linked cDNA molecules of interest using a NextGen sequencing platform.

337. The method of claim 310, wherein the NextGen sequencing platform is 454 sequencing.

338. The method of claim 310, wherein the NextGen sequencing platform is SMRT sequencing.

339. The method of claim 310, wherein the NextGen sequencing platform is SOLiD sequencing.

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340. The method of claim 310, wherein the NextGen sequencing platform is SOLEXA sequencing.

341. The method of claim 310, wherein the NextGen sequencing platform is tSMS sequencing.

342. The method of claim 310, wherein the plurality of cDNA molecules is from single samples contained in a plate with at least 6 wells, at least 12 wells, at least 24 wells, at least 48 wells, at least 96 wells, at least 384 wells, at least 1536 wells, or more wells.

343. The method of claim 310, wherein the plurality of cDNA molecules is from single samples contained in at least one, two, three, four, five, six, seven, eight, nine, ten, twenty, thirty, forty, fifty, seventy five, one hundred, or more plates with at least 96 wells each.

344. A method of linking and barcoding a plurality of samples containing polynucleotide sequences of interest, said method comprising: (a) distributing the samples into a plurality of containers; (b) synthesizing polynucleotide sequences of interest using templates from the sample, wherein said synthesis results in the addition of a barcode sequence; and (c) effecting linkage of the polynucleotide sequences of interest synthesized in step (b).

345. The method of claim 344, wherein each sample comprises a cell.

346. The method of claim 344, wherein the cell is a B cell.

347. The method of claim 344, wherein the B cell is a plasmablast, memory B cell, or a plasma cell.

348. The method of claim 344, wherein each sample comprises a plurality of cells.

349. The method of claim 344, wherein the polynucleotide sequences of interest comprise antibody heavy and light chains.

350. The method of claim 344, wherein said synthesis comprises an RT-PCR amplification.

351. The method according to claim 344, wherein said RT-PCR amplification is performed in a single step.

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352. The method of claim 344, wherein said linkage of the polynucleotide of interest is performed during the course of an RT-PCR amplification using an overlap-extension primer.

353. The method of claim 344, further comprising (d) adding a sequencing region to the polynucleotide sequences of interest prior to, during, or after barcode sequence addition or linkage.

354. The method of claim 344, wherein the sequencing region is added with an adaptor.

355. The method of claim 344, further comprising (e) sequencing of the linked polynucleotide sequences of interest using a NextGen sequencing platform.

356. The method of claim 344, wherein the NextGen sequencing platform is 454 sequencing.

357. The method of claim 344, wherein the NextGen sequencing platform is SMRT sequencing.

358. The method of claim 344, wherein the NextGen sequencing platform is SOLiD sequencing.

359. The method of claim 344, wherein the NextGen sequencing platform is SOLEXA sequencing.

360. The method of claim 344, wherein the NextGen sequencing platform is tSMS sequencing.

361. The method of claim 344, wherein the plurality of samples are single samples contained in a plate with at least 6 wells, at least 12 wells, at least 24 wells, at least 48 wells, at least 96 wells, at least 384 wells, at least 1536 wells, or more wells.

362. The method of claim 344, wherein the plurality of samples are single samples contained in at least one, two, three, four, five, six, seven, eight, nine, ten, twenty, thirty, forty, fifty, seventy five, one hundred, two hundred, five hundred or more plates with at least 96 wells each.

363. A method of linking and barcoding a plurality of non-contiguous polynucleotide sequences of interest, said method comprising: (a) distributing cells into a plurality of containers to obtain isolated one or more cells; (b) amplifying polynucleotide sequences of interest using templates from said isolated one or more cells, wherein said amplification

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results in the addition of a barcode sequence; and (c) effecting linkage of the polynucleotide sequences of interest amplified in step (b).

364. The method of claim 363, wherein the nucleotide sequences of interest comprise antibody heavy and light chains.

365. The method of claim 363, wherein said amplification comprises an RT-PCR amplification.

366. The method according to claim 363, wherein said RT-PCR amplification is performed in a single step.

367. The method of claim 363, wherein said linkage of the nucleotide of interest is performed during the course of an RT-PCR amplification using an overlap-extension primer.

368. The method of claim 363, further comprising (d) adding a sequencing region to the polynucleotide sequences of interest prior to, during, or after barcode sequence addition or linkage.

369. The method of claim 363, wherein the sequencing region is added with an adaptor.

370. The method of claim 363, further comprising (e) sequencing of the linked polynucleotide sequences of interest using a NextGen sequencing platform.

371. The method of claim 363, wherein the NextGen sequencing platform is 454 sequencing.

372. The method of claim 363, wherein the NextGen sequencing platform is SMRT sequencing.

373. The method of claim 363, wherein the NextGen sequencing platform is SOLiD sequencing.

374. The method of claim 363, wherein the NextGen sequencing platform is SOLEXA sequencing.

375. The method of claim 363, wherein the NextGen sequencing platform is tSMS sequencing.

376. The method of claim 363, wherein the one or more cells are contained in a plate with at least 6 wells, at least 12 wells, at least 24 wells, at least 48 wells, at least 96 wells, at least 384 wells, at least 1536 wells, or more wells.

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377. The method of claim 363, wherein the one or more cells are contained in at least one, two, three, four, five, six, seven, eight, nine, ten, twenty, thirty, forty, fifty, seventy five, one hundred, or more plates with at least 96 wells each.

378. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-3', wherein A is a sample identification region (barcode sequence), wherein B is a first cDNA region from a single sample, wherein C is a linker region, wherein D is a second cDNA region from the same single sample, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

379. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-D-3', wherein A is a first cDNA region from a single sample, wherein B is a linker region, wherein C is a second cDNA region from the same single sample, wherein D is a sample identification region (barcode sequence), and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

380. A polynucleotide library comprising a plurality of polynucleotides, wherein each polynucleotide comprises the sequence 5'-A-B-C-3', wherein A is a first cDNA region from a single sample, wherein B is a linker region comprising a sample identification region (barcode sequence), wherein C is a second cDNA region from the same single sample, and wherein the sequence of the sample identification region of each polynucleotide from a first single sample is distinct from the sequence of the sample identification region of the other polynucleotides in the library from one or more samples distinct from the first single sample.

381. The polynucleotide library of claim 378, wherein said first cDNA region comprises an antibody heavy chain and said second cDNA region comprises an antibody light chain.

382. The polynucleotide library of claim 378, wherein the library comprises at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, at least 100,000, at least 300, 000, at least 1,000,000, at least 3,000,000, at least 10,000,000, at least 30,000,000, or more members.

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383. The polynucleotide library of claim 378, wherein the library comprises at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 3000, at least 10,000, at least 30,000, or more genes of a cell sample's whole transcriptome.

384. The polynucleotide library of claim 378, wherein the library comprises at least 1, at least 2, at least 3, at least 10, at least 30, at least 100, at least 300, at least 1000, at least 10,000, at least 100,000, at least 1,000,000, at least 10,000,000, at least 100,000,000 or more of the different antibody species present in the blood of an individual.

385. The antibody species of claim 378, wherein the antibodies are expressed by plasmablasts, plasma cells, memory B cells, long-lived plasma cells, other B lineage cells or combinations thereof.

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Figure 1

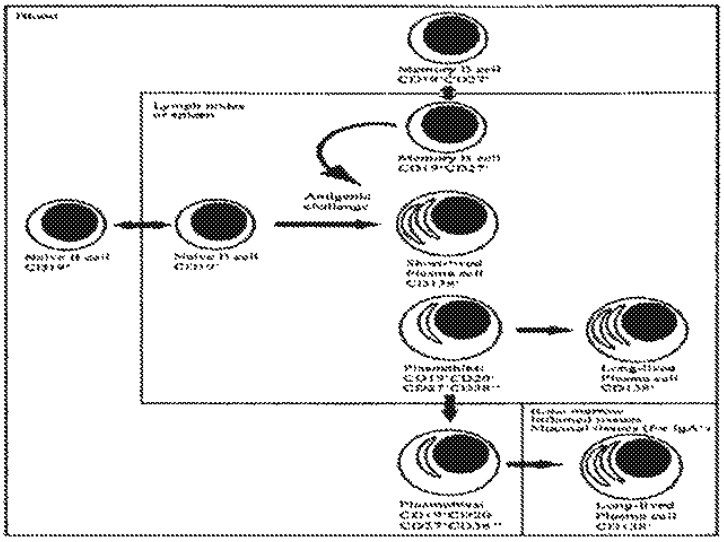
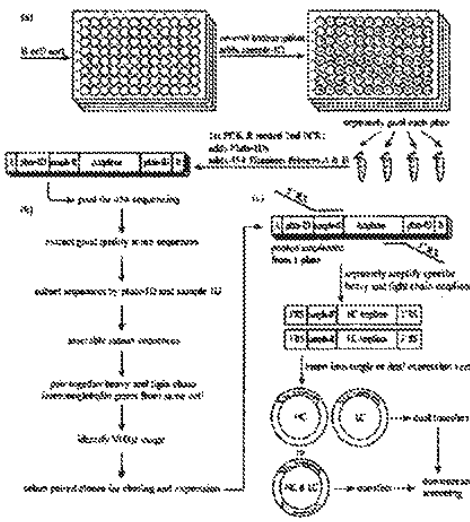
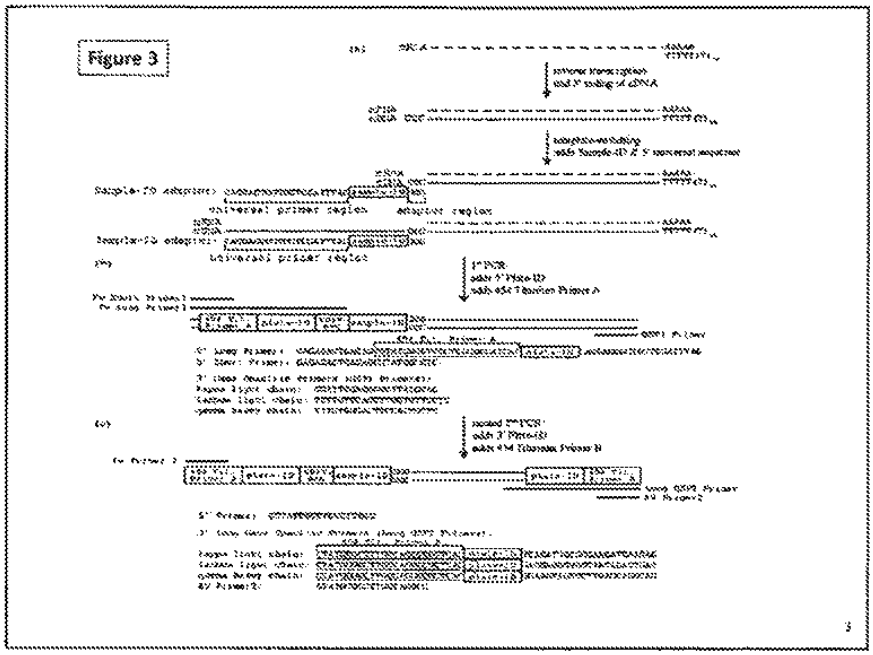

Memory B cell
CD19+CD27+
Lymph nodes or spleen
Memory B cell
CD19+CD27+
Antigenic challenge
Naive B cell
CD19+
Naive B cell
CD19+
Short-lived Plasma cell
CD138+
Plasmablast
CD19+CD20-
CD27+CD38++
Long-lived Plasma cell
CD138+
Plasmablast
CD19+CD20-
CD27+CD38++
Long-lived Plasma cell
CD138+

Figure 2



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Figure 3

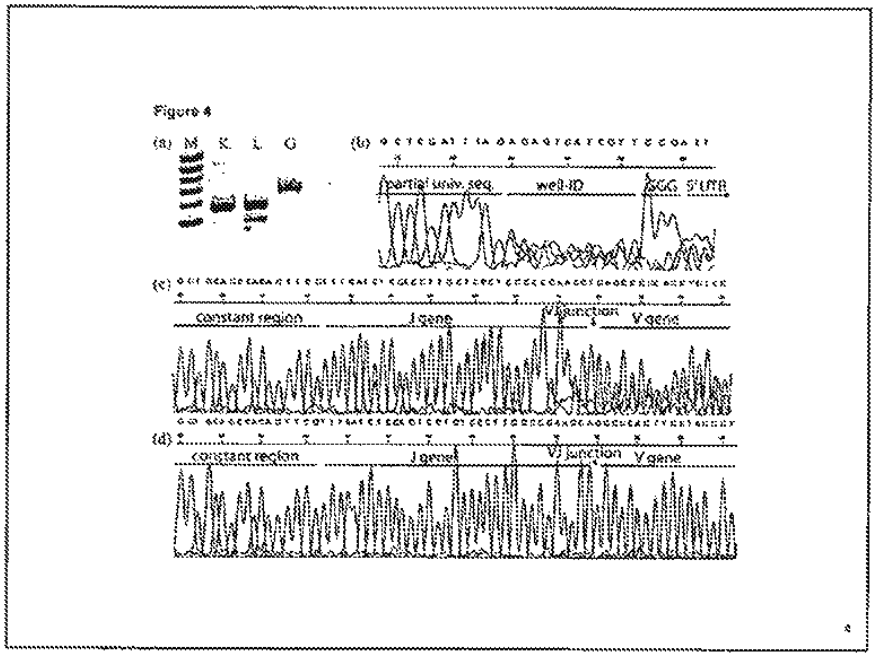
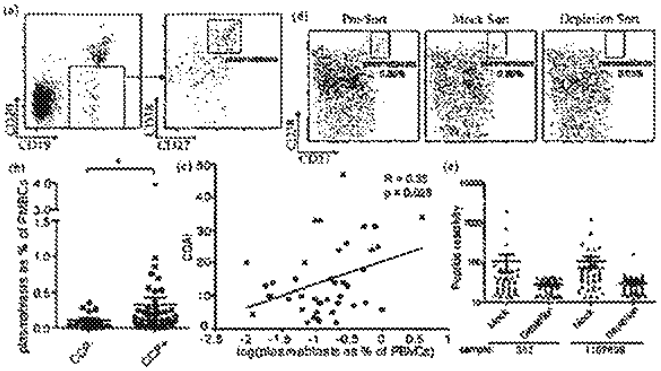
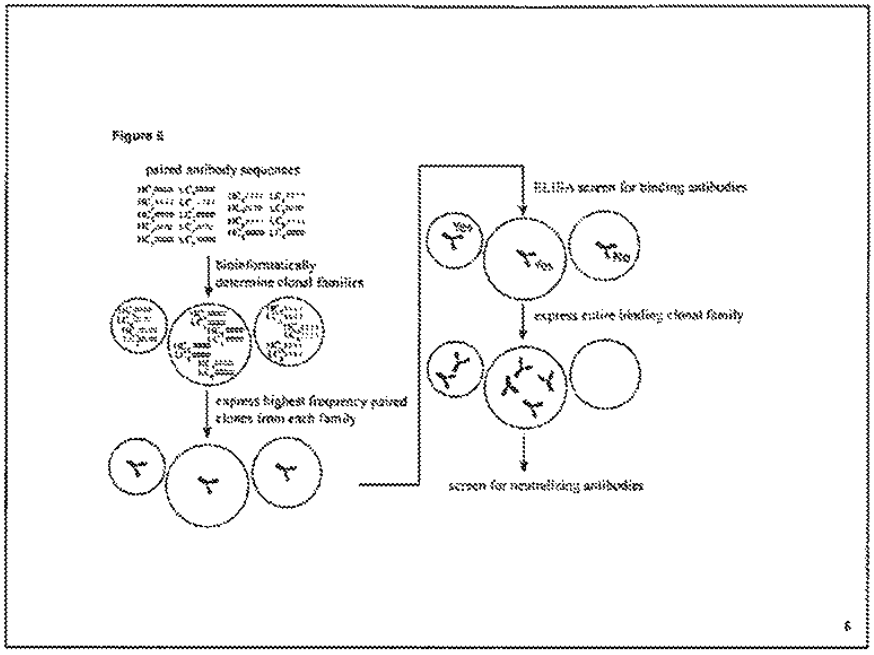
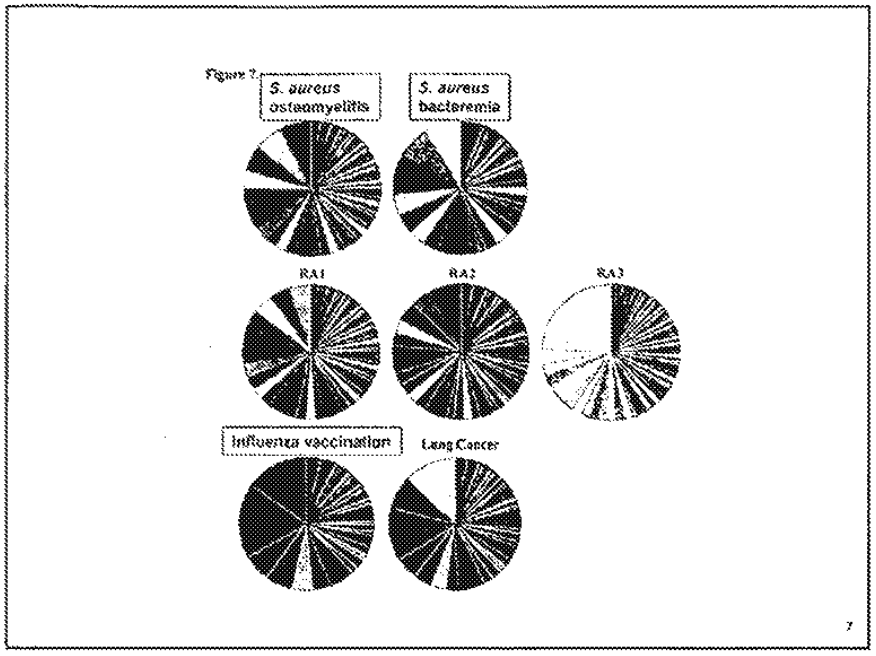

Figure 4
(a) M K L G
(b)
partial read seq
well-ID
constant region
J gene
VJ junction
V gene
(c)
(d)

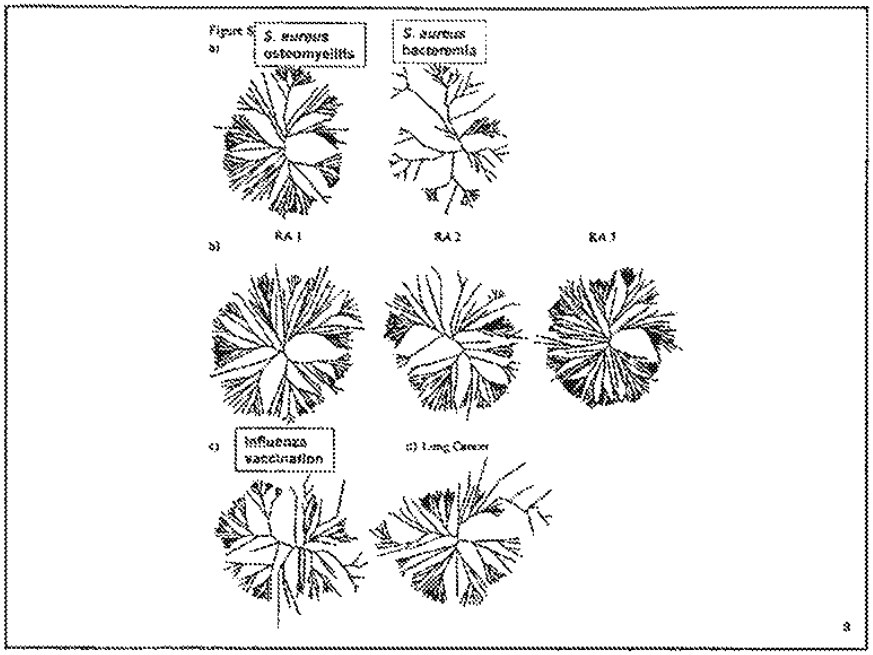
Figure 5

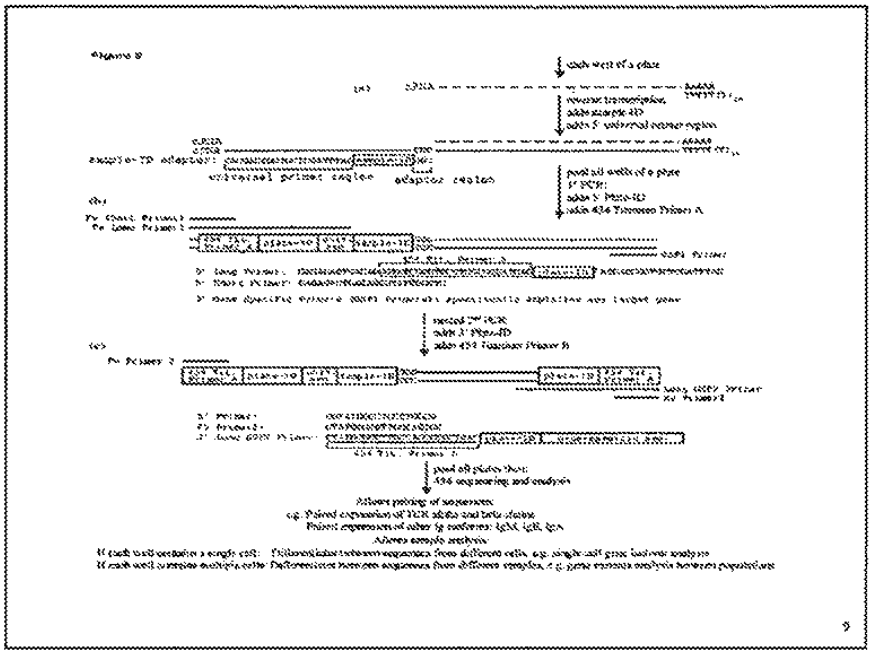


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Figure 6
paired antibody sequences
bioinformatically determine clonal families
express highest frequency paired clones from each family
ELISA screen for binding antibodies
express entire binding clonal family
screen for neutralizing antibodies
6

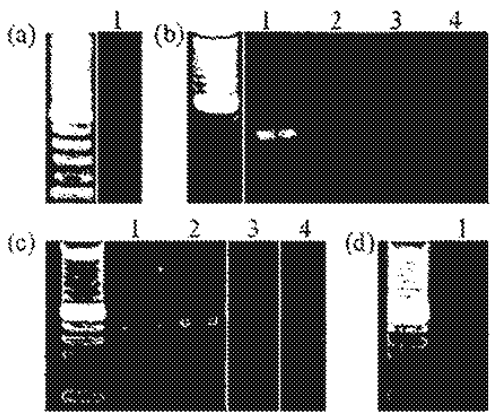

Figure 7
S. aureus osteomyelitis
S. aureus bacteremia
RA1
RA2
RA3
Influenza vaccination
Lung Cancer
7


Figure 8
a)
S. aureus osteomyelitis
S. aureus bacteremia
b)
RA 1
RA 2
RA 3
c)
Influenza vaccination
d) Lung Cancer
8



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Figure 10



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Figure 11

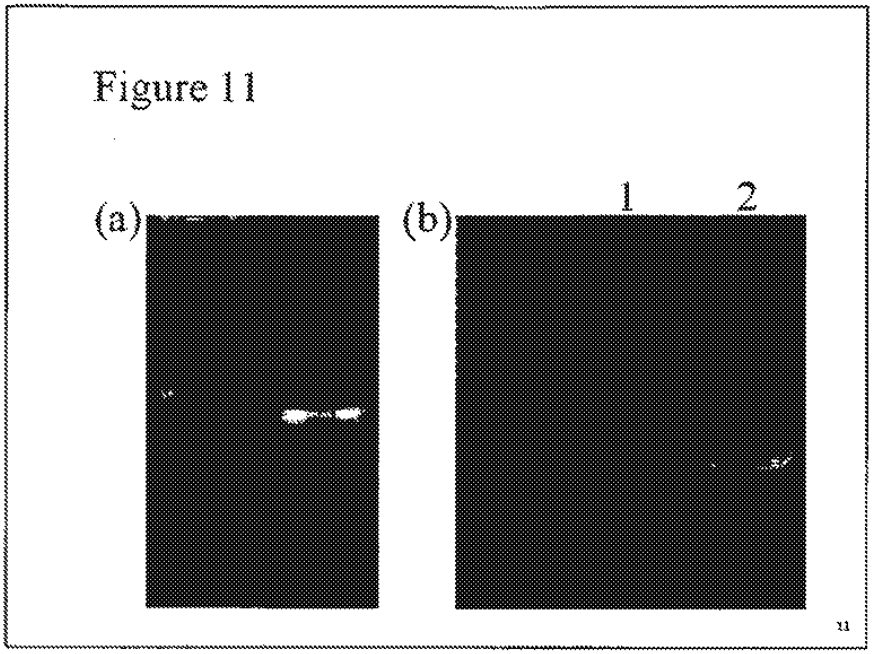
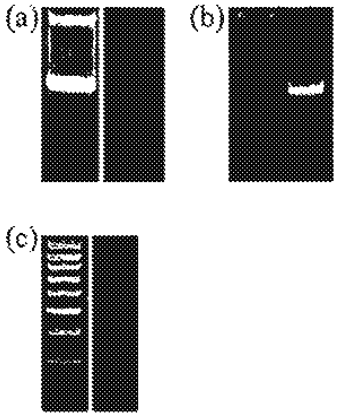
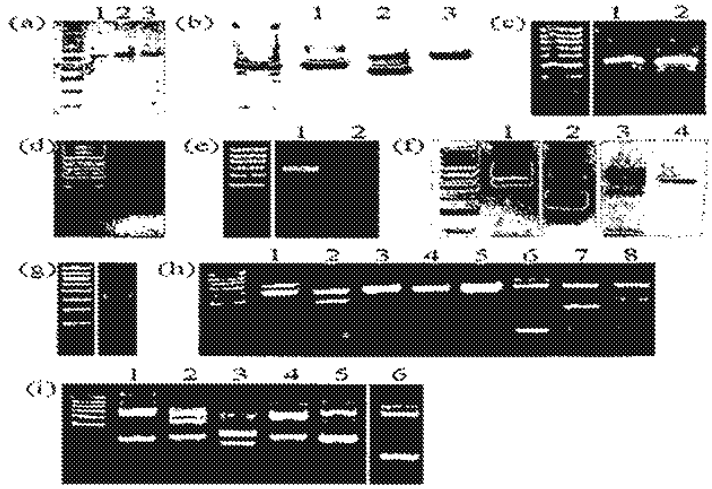


Figure 12



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Figure 13



13

Figure 14

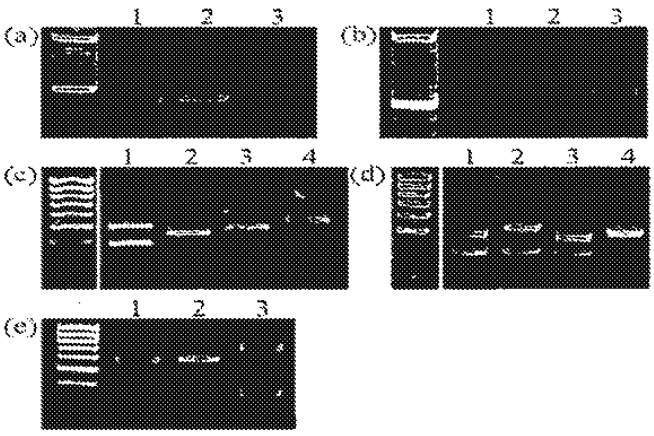


Figure 15

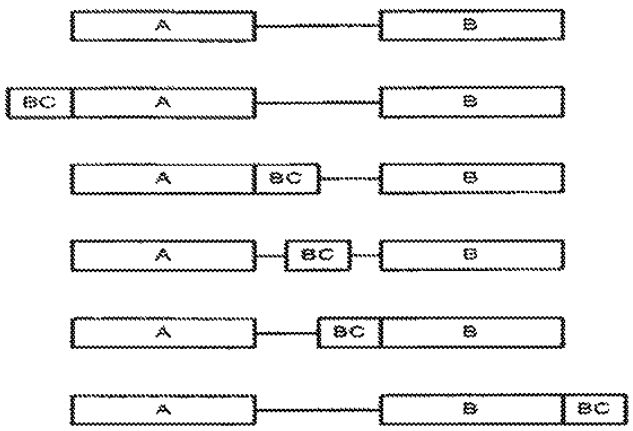


Figure 16

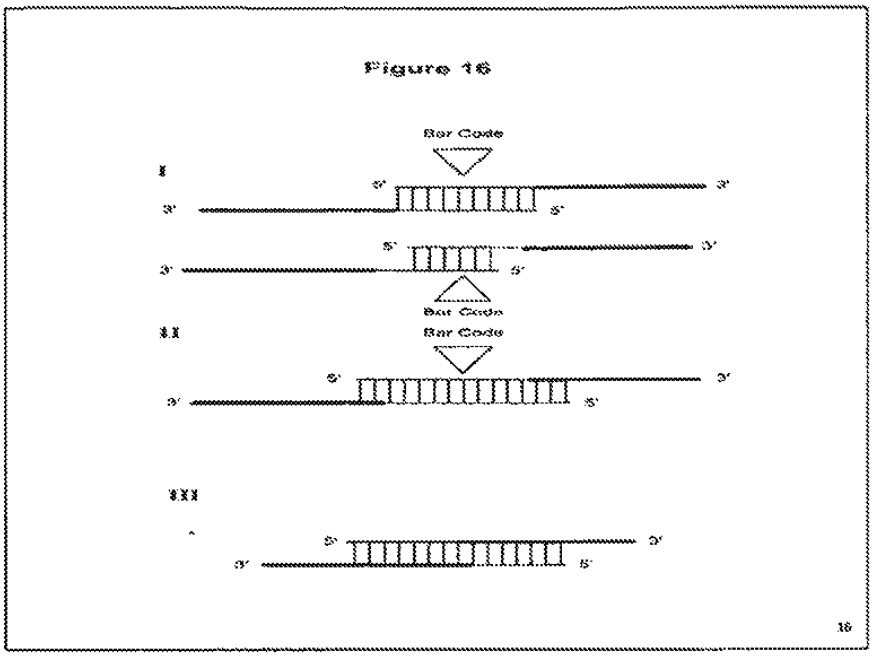
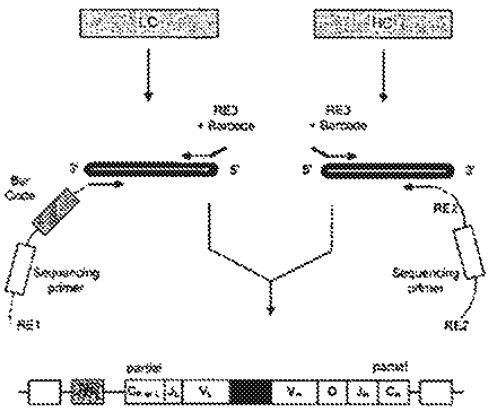


Figure 17


RE3
+ Barcode
RE3
+ Barcode
5'
3'
Bar
Code
RE1
RE2
Sequencing
primer
Sequencing
primer
RE1
RE2
partial
partial

17

Figure 18

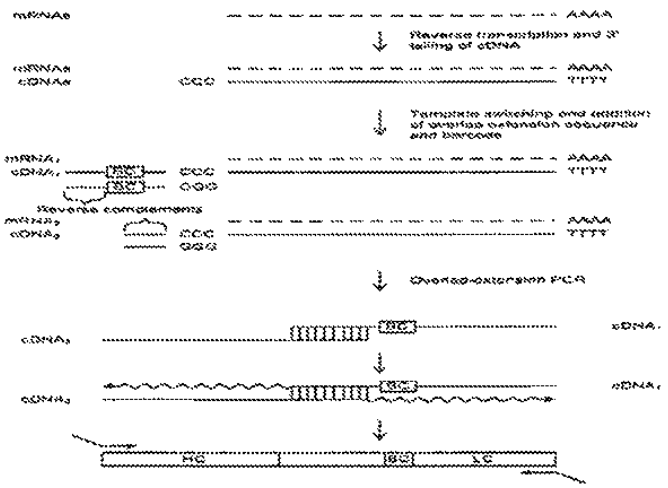

mRNAs
AAAA
Reverse transcription and 3' tailing of cDNA
cRNAs
cDNAs
CCC
TTTT
AAAA
Template switching and addition of universal extension sequence and barcode
mRNA1
cDNA1
SBC
UMI
CCC
GGG
TTTT
Reverse complement
mRNA2
cDNA2
AAAA
Overlap-extension PCR
cDNA1
cDNA2
RC
SBC
1C

Figure 19

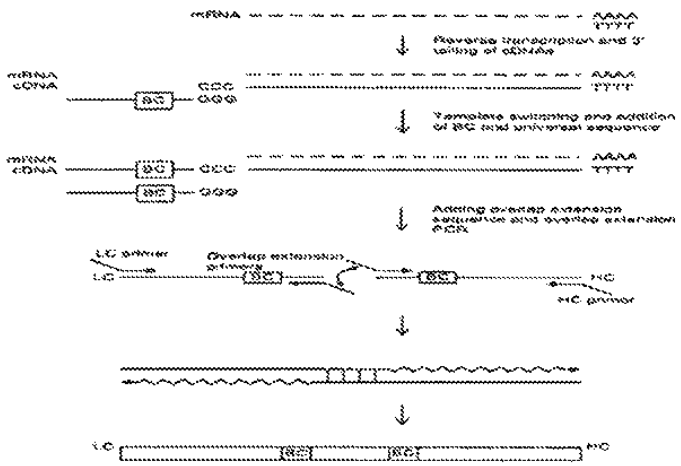


Figure 20

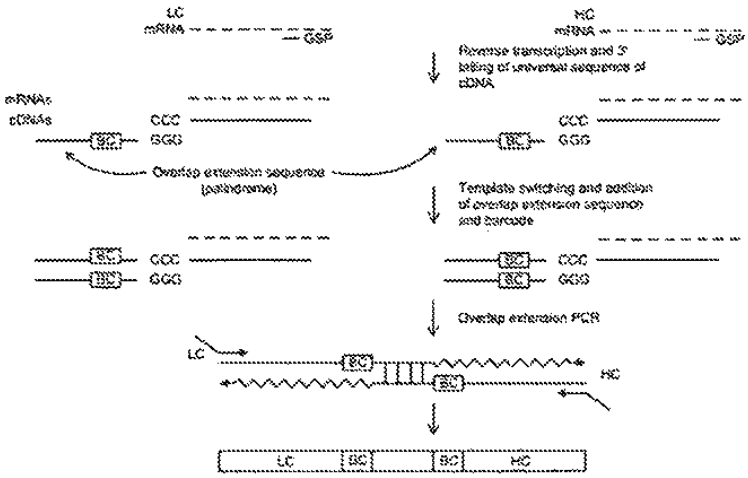

LC
mRNA
GSP
HC
mRNA
GSP
Reverse transcription and 3' tailing of universal sequence at cDNA
mRNA
cDNA
CCC
GGG
BC
Overlap extension sequence (polynucleotide)
Template switching and addition of overlap extension sequence and barcode
Overlap extension PCR
LC
HC
BC

Figure 21

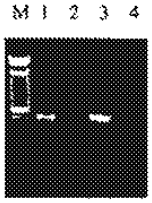


FIGURE 22

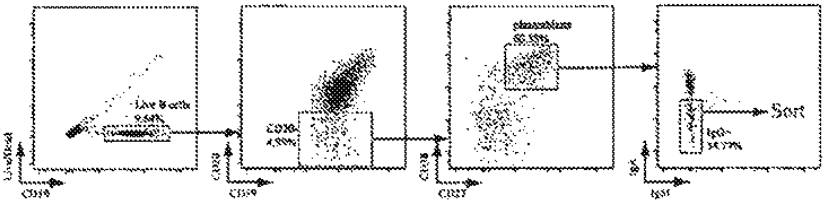


FIGURE 23

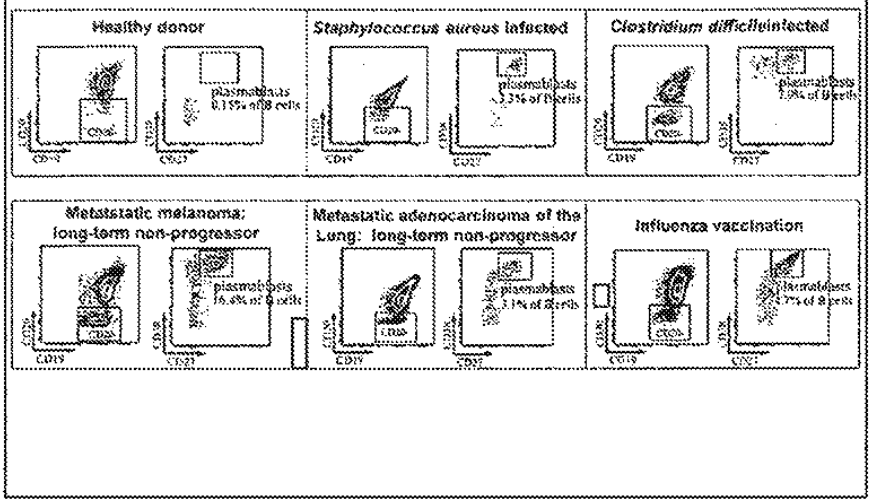

Healthy donor
Staphylococcus aureus infected
Clostridium difficile infected
Metastatic melanoma: long-term non-progressor
Metastatic adenocarcinoma of the Lung: long-term non-progressor
Influenza vaccination

FIGURE 24

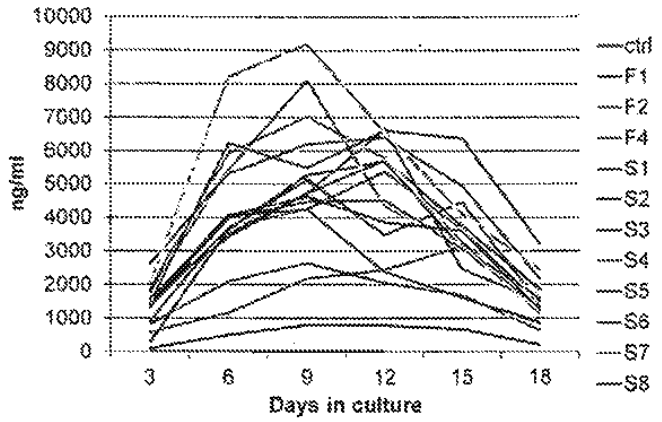
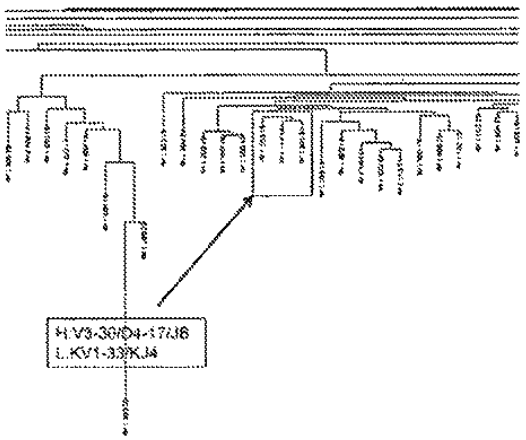

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
ng/ml
3
6
9
12
15
18
Days in culture
ctrl
F1
F2
F4
S1
S2
S3
S4
S5
S6
S7
S8

FIGURE 25A



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FIGURE 25B

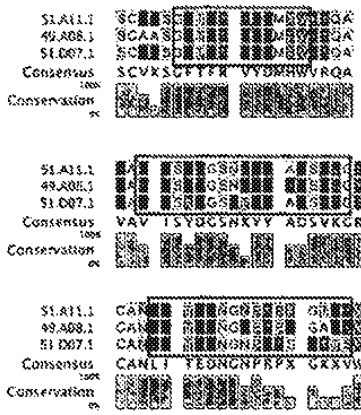


FIGURE 25C

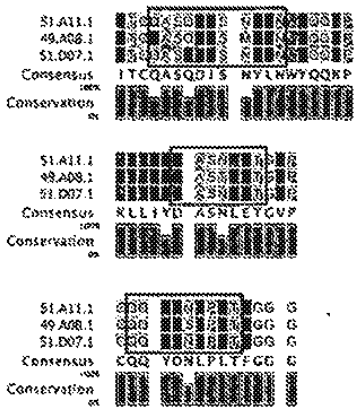


FIGURE 26

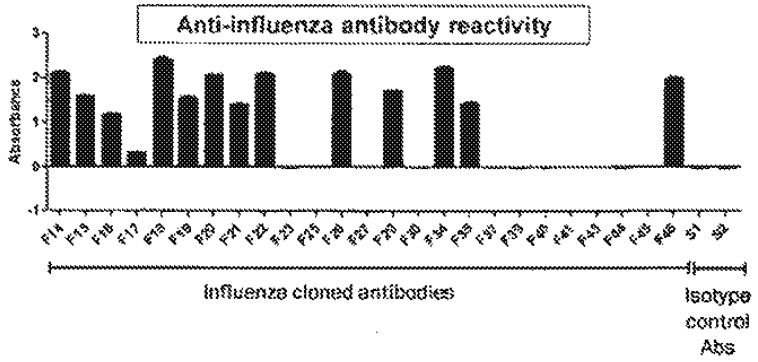

Anti-influenza antibody reactivity
Absorbance
3
2
1
0
-1
F14
F13
F16
F17
F18
F19
F20
F21
F22
F23
F25
F26
F27
F28
F30
F34
F36
F41
F39
F40
F42
F43
F44
F45
F46
S1
S2
Influenza cloned antibodies
Isotype control Abs

FIGURE 27

Antibody	Bound H3 Perth?	Bound H1 California?	k_D	k_d	K_D
FLU 24	Y	N	2.12E+05	1.31E-03	6.16E-09
FLU 25	Y	N	5.35E+05	5.88E-03	1.09E-08
FLU 28	Y	N	2.99E+05	1.60E-03	5.30E-09
FLU 29	Y	N	1.96E+05	5.08E-02	2.59E-07
FLU 30	Y	N	3.77E+05	6.28E-02	1.66E-07
FLU 32	Y	N	1.38E+05	6.75E-03	5.38E-08
FLU 26	Y	N	5.34E+05	1.52E-04	2.85E-10
FLU 34	Y	N	1.46E+05	1.36E-02	9.29E-08
FLU 35	Y	N	1.80E+04	2.35E-03	1.29E-08
FLU 46	Y	N	2.38E+04	4.13E-03	1.74E-08
FLU 21	N	Y	2.23E+05	5.29E-02	2.38E-07
FLU 15	N	N	--	--	--
FLU 17	N	N	--	--	--
FLU 29	N	N	--	--	--

FIGURE 28

Antibody tested	Neutralization of infectivity	
	H1N1- A/California/07/2009	H3N2 A/Perth/16/2009
mAb F15	No	No
mAb F16	No	Yes, Neutralizes at ≤1.5 µg/ml
mAb F18	No	Yes, Neutralizes at ≤1.5 µg/ml
mAb F19	No	Yes, Neutralizes at 3.1 µg/ml
mAb F20	No	Yes, Neutralizes at 12.5 µg/ml
mAb F21	Yes, Neutralizes at ≥ 1.5 µg/ml	Yes, Neutralizes at 100 µg/ml
Polyclonal Control	Yes, Neutralizes at ≥1/128 dil	Yes, Neutralizes at ≥1/128 dil

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FIGURE 29

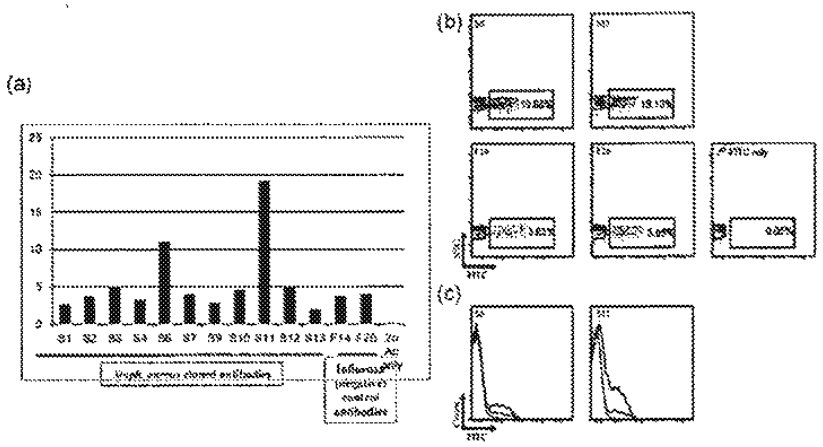


FIGURE 30

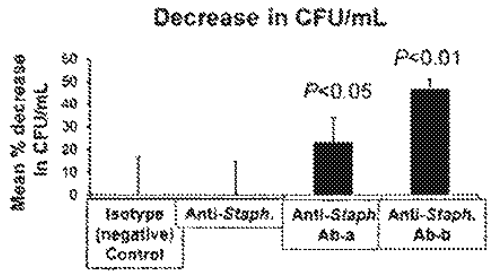

Decrease in CFU/mL
Mean % decrease in CFU/mL
0
10
20
30
40
50
60
P<0.05
P<0.01
Isotype (negative) Control
Anti-Staph.
Anti-Staph. Ab-a
Anti-Staph. Ab-b

FIGURE 31

Antibody	Target Accession #	Protein Name
S04	Q6G7S2	Phenol-soluble modulin alpha 1 peptide
S04	P0C7Y6	Delta-hemolysin

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FIGURE 32

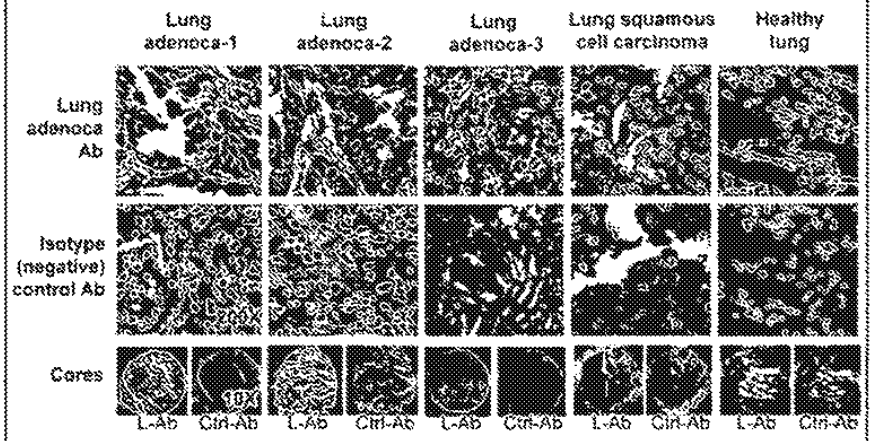

Lung adenoca-1
Lung adenoca-2
Lung adenoca-3
Lung squamous cell carcinoma
Healthy lung
Lung adenoca Ab
Isotype (negative) control Ab
Cores
10X
L-Ab
Ctrl-Ab

FIGURE 33

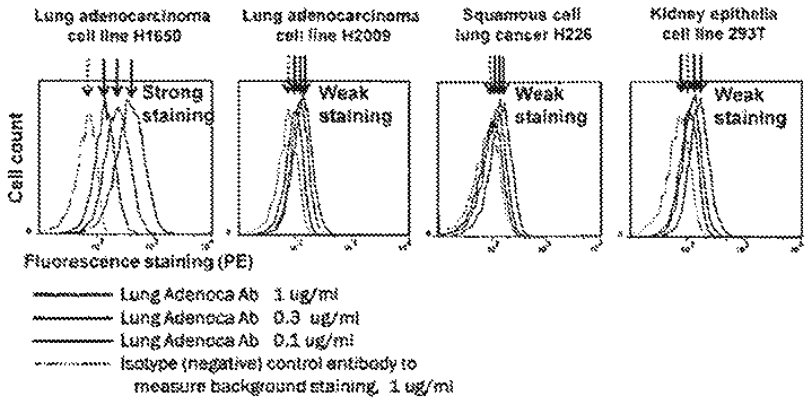

Lung adenocarcinoma cell line H1650
Lung adenocarcinoma cell line H2009
Squamous cell lung cancer H226
Kidney epithelia cell line 293T
Strong staining
Weak staining
Weak staining
Weak staining
Cell count
Fluorescence staining (PE)
Lung Adenoca Ab 1 ug/ml
Lung Adenoca Ab 0.3 ug/ml
Lung Adenoca Ab 0.1 ug/ml
Isotype (negative) control antibody to measure background staining, 1 ug/ml

FIGURE 34

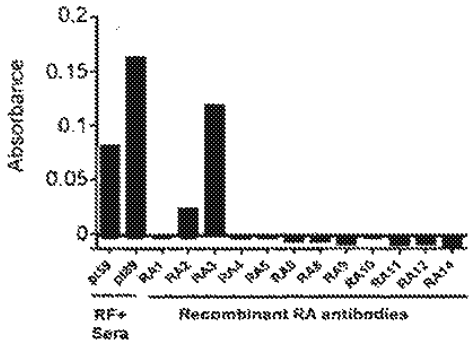

0.2
0.15
0.1
0.05
0
Absorbance
pt59
pt69
RA1
RA2
RA3
RA4
RA5
RA6
RA8
RA9
RA10
RA11
RA12
RA14
RF+ Sera
Recombinant RA antibodies

FIGURE 35

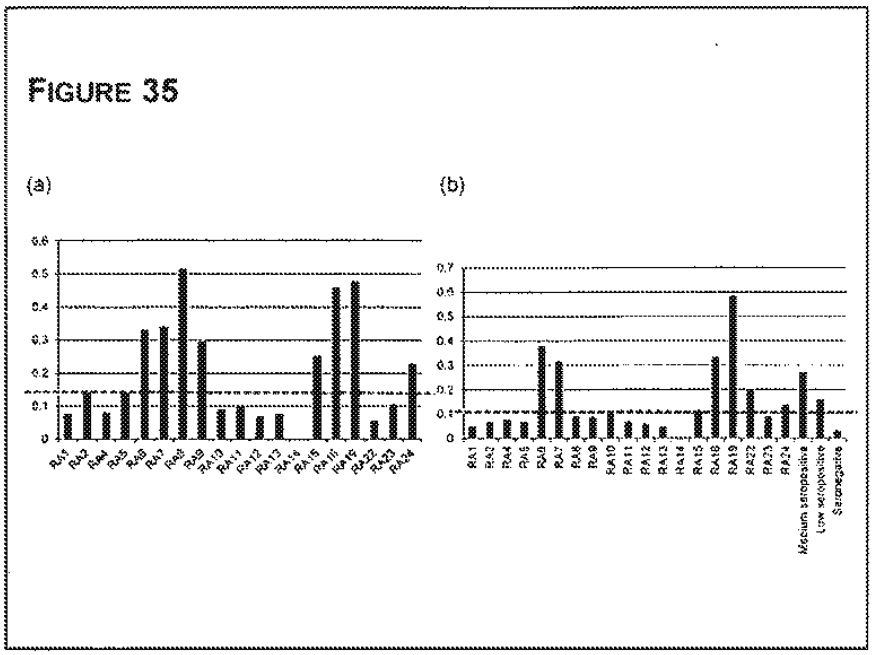

(a)
(b)
RA1
RA2
RA4
RA5
RA6
RA7
RA8
RA9
RA10
RA11
RA12
RA13
RA14
RA15
RA16
RA17
RA18
RA19
RA22
RA23
RA24
Medium seropositive
Low seropositive
Seronegative

FIGURE 36

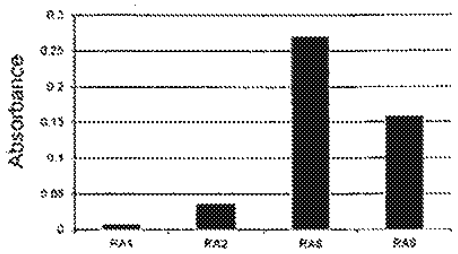


FIGURE 37

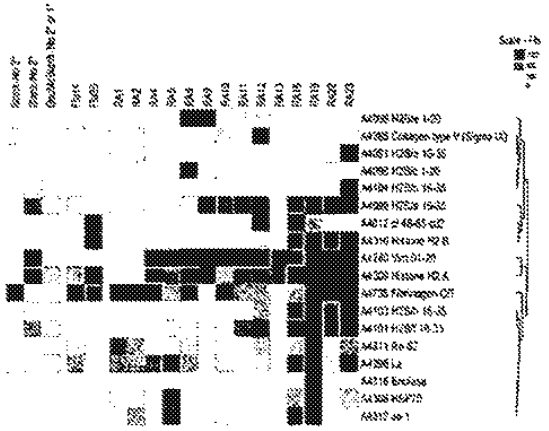


FIGURE 38

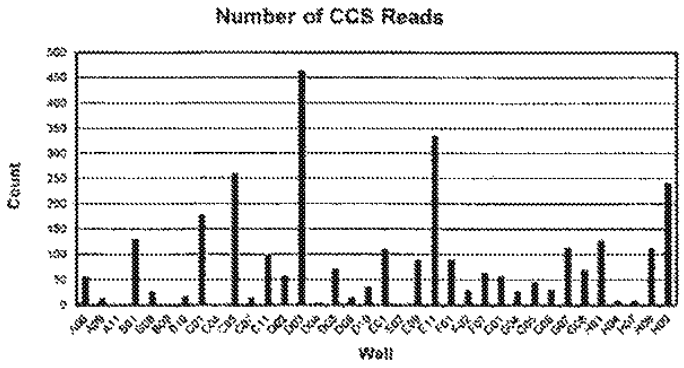


FIGURE 39

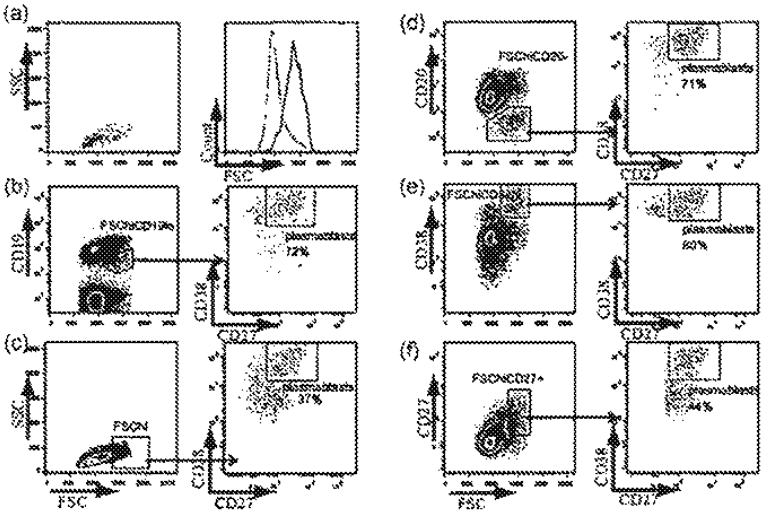

(a)
SSC
FSC
Count
(b)
CD19
CD38
CD27
(c)
SSC
FSC
CD38
CD27
(d)
CD20
CD38
CD27
(e)
CD38
CD38
CD27
(f)
CD27
FSC
CD38
CD27

FIGURE 40

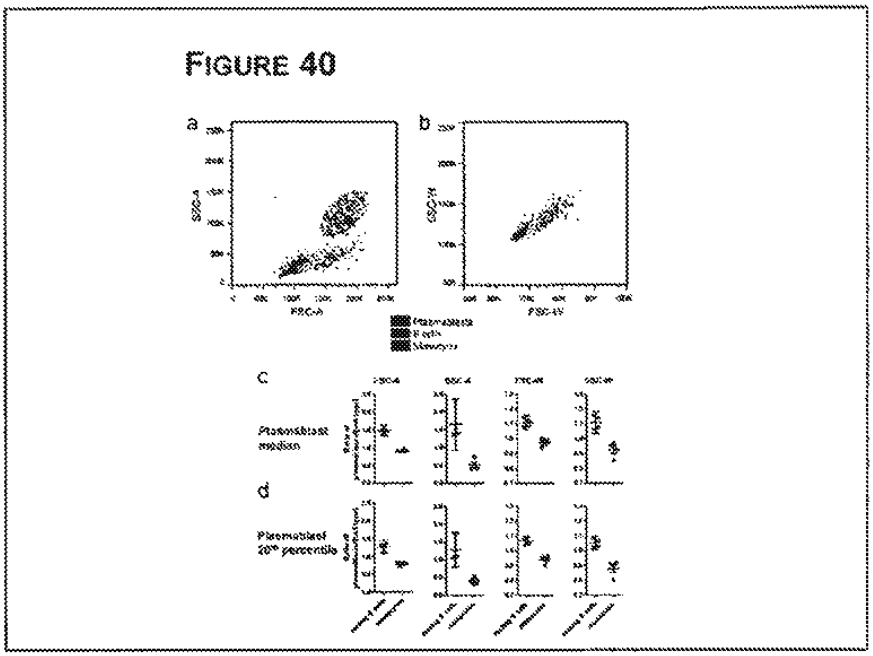


FIGURE 41

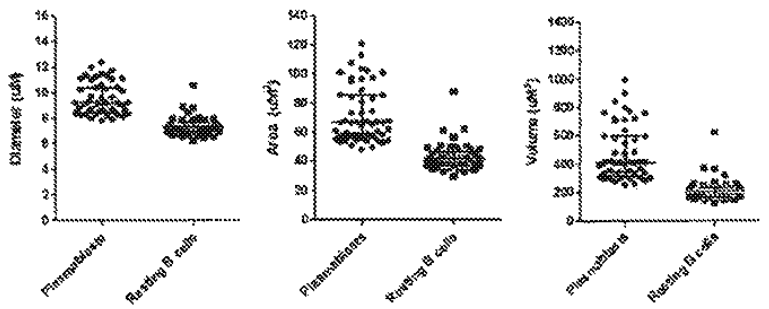

Diameter (um)
Area (um²)
Volume (um³)
Plasmablasts
Resting B cells

FIGURE 42

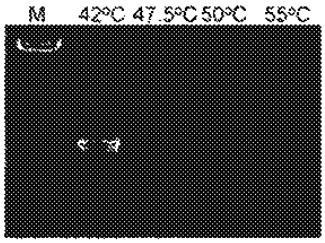
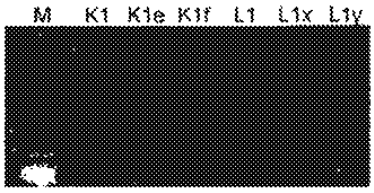
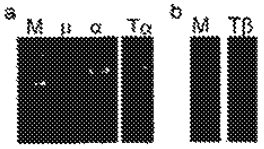


FIGURE 43



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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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FIGURE 44



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FIGURE 45

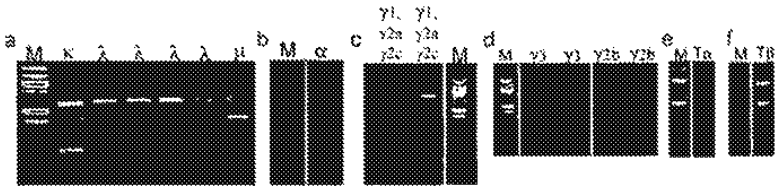
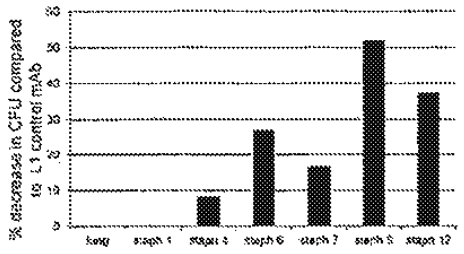


FIGURE 46



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Page 00598

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Attorney Docket Number	CGLC-020CON5

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	9290808		2016-03-22	FODOR, Stephen P.	

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	1					

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	WO2012148497	WO		2012-11-01	ROBINSON, et al.		

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

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IPR2023-00876
Page 00599

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Attorney Docket Number	CGLC-020CON5

	1		

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14792094
	Filing Date	2015-07-06
	First Named Inventor	BRENNER, Sydney
	Art Unit	1636
	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Attorney Docket Number	CGLC-020CON5

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/James S. Keddie, Reg. No. 48,920/	Date (YYYY-MM-DD)	2016-06-29
Name/Print	James S. Keddie	Registration Number	48920

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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IPR2023-00876
Page 00602

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 = *		X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 = *		X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	06/29/2016	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 16	Minus	** 20	= 0	X $40 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $210 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/STELLA LITTLE/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronically Filed

REQUEST TO CHANGE THE APPLICANT UNDER 37 C.F.R. § 1.46(c) Address to: Mail Stop Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket No.	CGLC-020CON5
	Confirmation No.	9697
	First Named Inventor	Brenner, Sydney
	Application Number	14/792,094
	Filing Date	July 6, 2015
	Group Art Unit	1634
	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Title: *"Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"*	

Dear Sir:

Applicants respectfully request that the above-referenced application be amended to change the Applicant.

Please remove [[**Population Genetics Technologies Ltd.**]] and add **10X Genomics, Inc.** as the sole Applicant. A Supplemental Application Data Sheet in compliance with 37 CFR 1.76 is submitted herewith along with a statement under 37 CFR 3.37(c) to show chain of title to the new applicant.

Applicants also request that a Corrected Filing Receipt be issued for the above-identified application.

If for any reason a fee is found to be necessary, the Commissioner is authorized to charge such fee to Deposit Account No. 50-0815 order number CGLC-020CON5.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: July 13, 2016

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie
Registration No. 48,920

Bozicevic, Field & Francis LLP
1900 University Avenue, Suite 200
East Palo Alto, California 94303
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

PTO/AIA/96 (08-12)
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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: **10X Genomics, Inc.**

Application No./Patent No.: **14/792,094** Filed/Issue Date: **July 6, 2015**

Titled: **Compositions and Methods for Intramolecular Nucleic Acid Rearrangement**

10X Genomics, Inc., a **corporation**
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (chose **one** of options 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), or an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: **the inventors** To: **Population Genetics Technologies Ltd**

   The document was recorded in the United States Patent and Trademark Office at

   Reel **036049**, Frame **0472**, or for which a copy thereof is attached.

2. From: **Population Genetics Technologies Ltd** To: **10X Genomics, Inc.**

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

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4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

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5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

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6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/James S. Keddie, Reg. No. 48,920/	**July 12, 2016**
Signature	Date
James S. Keddie	**48,920**
Printed or Typed Name	Title or Registration Number

INTELLECTUAL PROPERTY ASSIGNMENT AGREEMENT

This **INTELLECTUAL PROPERTY ASSIGNMENT AGREEMENT** ("**IP Assignment**"), dated as of June 7, 2016, is made by Population Genetics Technologies Ltd, a corporation existing under the laws of England and Wales with company number 05116842 and registered office at Salisbury House, Station Road, Cambridge, CB1 2LA. United Kingdom ("**PGT**") and 10X Genomics, Inc., with offices at 7068 Koll Center Pkwy #401, Pleasanton, CA 94566 ("**10X**"), the purchaser of certain assets of PGT pursuant to the Intellectual Property Purchase and License Agreement between PGT and 10X, dated as of October 21, 2015 (the "**IP Purchase Agreement**").

WHEREAS, under the terms of the IP Purchase Agreement, PGT has conveyed, transferred and assigned to 10X, among other assets, certain intellectual property of PGT, and has agreed to execute and deliver this IP Assignment, for recording with governmental authorities including, but not limited to, the US Patent and Trademark Office;

NOW THEREFORE, the parties agree as follows:

1. Assignment. In consideration for the receipt by PGT of the Purchase Price stipulated in the IP Purchase Agreement and other good and valuable consideration, the receipt and sufficiency are hereby acknowledged, PGT hereby irrevocably conveys, transfers and assigns to 10X, and 10X hereby accepts, all of PGT's right, title and interest in and to the patents and patent applications set forth in Schedule 1 hereto and all issuances, divisions, continuations, continuations-in-part, reissues, extensions, reexaminations and renewals thereof (the "**Patents**").

2. Recordation and Further Actions. PGT authorizes the Commissioner for Patents and any other governmental officials to record and register this IP Assignment upon request by 10X. PGT shall, at 10X's cost, take such steps and actions following the date hereof, including the execution of any documents, files, registrations, or other similar items, to ensure that the Assigned IP is properly assigned to 10X, or any assignee or successor thereto.

3. Terms of the IP Purchase Agreement. The terms of the IP Purchase Agreement, including, but not limited to, the representations, warranties, covenants, agreements and indemnities relating to the Assigned IP are incorporated herein by this reference. The parties hereto acknowledge and agree that the representations, warranties, covenants and agreements contained in the IP Purchase Agreement shall not be superseded hereby but shall remain in full force and effect to the full extent provided therein. In the event of any conflict or inconsistency between the terms of the IP Purchase Agreement and the terms hereof, the terms of the IP Purchase Agreement shall govern.

4. Counterparts. This IP Assignment may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this IP Assignment delivered by facsimile, e-mail or other means

of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this IP Assignment.

5. Successors and Assigns. This IP Assignment shall be binding upon and shall inure to the benefit of the parties hereto and their respective successors and assigns.

6. Governing Law. This IP Assignment and any dispute, claim or matter arising from or in connection with it or its subject matter or formation (including non-contractual disputes or claims) shall be governed by and construed in accordance with the law of England and Wales. The parties agree that the courts of England and Wales will have non-exclusive jurisdiction to settle any such dispute or claim and irrevocably agree to submit to the jurisdiction of the courts of England and Wales for these purposes.

[SIGNATURE PAGE FOLLOWS]

IN WITNESS WHEREOF, PGT has duly executed and delivered this Intellectual Property Assignment Agreement as of the date first above written.

POPULATION GENETICS TECHNOLOGIES LTD

By: _____
Name: Mel N. Kronick
Title: Director

Address for Notices:

Population Genetics Technologies Ltd
Salisbury House
Station Road
Cambridge
CB1 2LA
United Kingdom

AGREED TO AND ACCEPTED:

10X GENOMICS, INC.

By: _____
Name: Matthew Murphy
Title: Vice President and General Counsel

Address for Notices:

7068 Koll Center Pkwy #401
Pleasanton
CA 94566
United States of America

SCHEDULE 1

ASSIGNED PATENTS AND PATENT APPLICATIONS

PGT Name	Entity	App No	Date Filed	Pat No	Date Issued	Status
CGLC-020PRV	US	61/235,595	8/20/2009			Expired
CGLC-020PRV2	US	61/288,792	12/21/2009			Expired
CGLC-020WO	WIPO	IB2010/002243	8/13/2010			Expired
CGLC-020EP	EP	10768061.3	8/13/2010	2467479		Registered in DE, ES, FR, GB
CGLC-020EPDIV	EP	15193733.1	8/13/2010			Pending
CGLC-020	US	13/387,343	2/15/2012	8,298,767	10/30/2012	Issued
GCLC020CON	US	13/622,872	9/19/2012			Abandoned
CGLC-020CON2	US	13/859,450	4/09/2013	8,563,274	10/22/2013	Issued
CGLC-020CON3	US	14/021,790	9/09/2013	8,679,756	03/25/2014	Issued
CGLC-020CON4	US	14/172,694	2/04/2014	9,102,980	08/11/2015	Issued
CGLC-020CON5	US	14/792,094	7/06/2015			Pending
CGLC-020CON6	US	14/935,255	11/6/2015			Pending
CGLC-020CON7	US	14/939,728	11/12/2015			Pending

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

CORRECTED ADS FORM

Application Number	14792094
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Inventor Information

****If no data is shown, no data has been corrected****

	Data of Record	Updated Data
Order Number		
Name		
Residence Information		
Residency		
City		
State		
Country of Residence		
Mailing Address of Inventor		
Address 1		
Address 2		
City,State/Province, Postal Code		
Country		

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Page 00611

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

Application Information

	Data of Record	Updated Data
Title of Invention	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	
Attorney Docket Number	CGLC-020CON5	
Entity Type	Small	

Domestic Benefit/National Stage Information

****If no data is shown, no data has been corrected****

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121,365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S. C. 119(e) or 120, and 37 CFR 1.78(a).

		Data of Record	Updated Data
	Prior Application Status		
	Application Number		
	Continuity Type		
	Prior Application Number		
	Filing Date (YYYY-MM-DD)		
	Patent Number		
	Issue Date (YYYY-MM-DD)		

Foreign Priority Information

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IPR2023-00876
Page 00612

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

****If no data is shown, no data has been corrected****

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

		Data of Record	Updated Data
	Application Number		
	Country		
	Filing Date		
	Access Code		

Applicant Information

****If no data is shown, no data has been corrected****
Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

	Data of Record	Updated Data
Applicant Type	ASG	
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is		
Name of the Deceased or Legally Incapacitated Inventor		
Applicant is an Organization	Yes	
Name		
Organization Name	~~Population Genetics Technologies LTD.~~	10X Genomics, Inc.
Address 1		7068 Koll Center Pkwy
Address 2		#401

Corrected ADS 1.0

City,State/Province,Postal Code	~~Cambridge~~	Pleasanton CA 94566
Country	~~GB~~	US
Phone Number		
Fax Number		
Email Address		

Assignee Information including Non-Applicant Assignee Information

****If no data is shown, no data has been corrected****
Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office

	Data of Record	Updated Data
Order		
Applicant is an Organization		
Name		
Organization Name		

Mailing Address

Address 1		
Address 2		
City,State/Province,Postal Code		
Country		
Phone Number		
Fax Number		
Email Address		

Corrected ADS 1.0

Signature

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b).

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/James S. Keddie, Reg. No. 48,920/	Registration Number	48920
First Name	James S.	Last Name	Keddie

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Electronic Acknowledgement Receipt

EFS ID:	26331044
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	13-JUL-2016
Filing Date:	06-JUL-2015
Time Stamp:	20:51:56
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	CGLC-020CON5-Req_Corr_Applcnt.pdf	24756 60d397c6e133acc1fae7fd25623dea9400 2f6c	no	1

Warnings:

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	CGLC-020CON5-373C_Statement.pdf	32443 a600230a6dd805cadaa6c5d140d223e30c038ca8	no	2
Warnings:					
Information:					
3	Assignee showing of ownership per 37 CFR 3.73	2016-06-07-PGT-10X-Patent-Assignment-Agreement.pdf	126517 620c9f335799a56ce7ce2af33565a8d61c09e939	no	4
Warnings:					
Information:					
4	Application Data Sheet to update/ correct info	CorrectedADS.pdf	65647 0c0ed1e75ac6cf3e1e1147283f3ed2ac7f2ac2ec	no	5
Warnings:					
Information:					
		Total Files Size (in bytes):	249363		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	CGLC-020CON5	9697

93726 7590 07/25/2016
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
07/25/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

docket@bozpat.com

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Examiner-Initiated Interview Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Examiner: Bhat*. (3)_____.

(2) *Representative: Mr. Keddie*. (4)_____.

Date of Interview: *11 July 2016*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☒102 ☒103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *21,35 and 37*.

Identification of prior art discussed: *Arts of the record*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

See Continuation Sheet.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

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Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

For compact prosecution the examiner contacted the representative Mr. Keddie on July 11, 2016 to discuss further amendments to place the pending claims in better condition for the allowance. The examiner discussed that the claim amendments of June 29, 2016 requiring same multiplex identifier for the sample and the plurality of segments of the nucleic acids still read on the teachings over Braverman and further amendments and terminal disclaimers are needed to place the pending claims in condition for the allowance.

It was agreed that inclusion of an additional step '(b) iii', of "wherein the multiplex identifier associated with a plurality of nucleic acid molecules of the sample is different from a multiplex identifier associated with a plurality of segments from a particular nucleic acid molecule of the sample", fixing the claim language for any minor informalities, 112 second and fourth paragraphs issues and filing the terminal disclaimers over claims of US 8,298,767, 8,563,274, 8,679,756 and 9,102,980 and over claims of 14/935,255 and 14/939,728 copending applications and their acceptance by the office and identifying the support for the amendments in the specification would overcome the rejections on the record and thus allowable.

The representative informed that the amendments require approval from the applicant. On July 13, 2016 the representative requested to issue an office action in view of not hearing a response from the applicant.

The communication between the examiner and the representative is made of record.

Office Action Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *6/29/2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *21, 24 and 26-39* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *21,24 and 26-39* is/are rejected.

8)☒ Claim(s) *28* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *7/6/15* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☒ Interview Summary (PTO-413) Paper No(s)/Mail Date. *7/11/16*.

4) ☒ Other: *IMIS*.

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Final Action

1. The present application is being examined under the pre-AIA first to invent provisions. This office action is in response to the papers filed on June 29, 2016. The petition to make the instant application "special" has been granted by the office on December 22, 2015 and thus has been examined on expedited basis.

The instant application is a continuation of 14/172,694, now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450, now U.S. Patent 8,563,274, which is a continuation of 13/622,872, now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, claiming priority from provisional application 61288792, filed on 12/21/2009 and from provisional application 61235595, filed on 08/20/2009.

The amendments to independent claims 21, 35 and 37 changing the scope of the previously rejected said claims necessitated the new grounds of rejections as set forth in this office action. Accordingly ***this action has been made final***.

It is noted that this office action has been issued due to time constraint to move the instant application in allotted time and not able to receive the discussed and agreed amendments on time to place the pending claims in better condition for the allowance.

Interview Summary

2. For compact prosecution the examiner contacted the representative Mr. Keddie on July 11, 2016 to discuss further amendments to place the pending claims in better condition for the allowance. The examiner discussed that the claim amendments of June 29, 2016 requiring same multiplex identifier for the sample and the plurality of segments of the nucleic acids still read on the teachings over Braverman and further amendments and terminal disclaimers are needed to place the pending claims in condition for the allowance.

It was agreed that inclusion of an additional step '(b) iii', of "wherein the multiplex identifier associated with a plurality of nucleic acid molecules of the sample is different from a multiplex identifier associated with a plurality of segments from a particular nucleic acid molecule of the sample", fixing the claim language for any minor informalities, 112 second and fourth paragraphs issues and filing the terminal disclaimers over claims of US 8,298,767, 8,563,274, 8,679,756 and 9,102,980 and over claims of 14/935,255 and 14/939,728 copending applications and their acceptance by the office and identifying the support for the amendments in the specification would overcome the rejections on the record and thus allowable.

The representative informed that the amendments require approval from the applicant. On July 13, 2016 the representative requested to issue an office action in view of not hearing a response from the applicant.

The communication between the examiner and the representative is made of record.

Claim Status

3. In the claim listing of June 29, 2016 claims 21, 24 and 26-39 are pending in this application and are under prosecution. Claims 21, 24, 28, 29, 31 and 35-37 are amended. Claims 1-20, 22 and 23 are canceled. No new matter has been introduced by the claim amendments as applicant has identified the support for the amendments in the instant specification (Remarks, pg. 7).

Withdrawn Objection and Rejections and Response to the Remarks

4. The previous objection to the abstract has been withdrawn in view of amending the abstract in line with the claims (Remarks, pg. 7). The amendments do not introduce the new matter.

The previous objection to the specification not listing the header "Brief Description of the Drawings" was an oversight on the part of the examiner. It is noted that said header is listed in page 1 of the instant specification.

5. The previous ODP rejections of instant claims 21-39 over claims of 7,544,473, 8,685,678, 8,722,368 and over claims of 14/273,197 copending application in view of Braverman has been withdrawn in view of the claim amendments. The arguments regarding said ODP rejections have been fully considered (Remarks, pgs. 7 and 8). They are moot in view of withdrawn rejections necessitated by the claim amendments.

6. The previous rejection of claims 21-25 and 28-39 under pre-AIA 102(b) as being anticipated by Brenner has been withdrawn necessitated by the claim amendments.

The arguments regarding said rejection have been fully considered (Remarks, pg. 8). They are moot in view of withdrawn rejections necessitated by the claim amendments.

Maintained Rejections and Response to the Remarks

7. The previous rejection of claims 21-25 and 28-39 under pre-AIA 35 USC 102(b) as being anticipated by Braverman has been maintained. The arguments regarding said rejection have been fully considered (Remarks, pg. 8). They are not persuasive because claims as amended only require single multiplex identifier that identifies the sample and the segments of nucleic acids recited in generality without distinguishing the segment of the nucleic acid molecule and the segments of the particular nucleic acid molecule. This feature is taught by Braverman.

8. The previous rejection of claims 21, 24 and 26-39 under pre AIA 35 USC 103(a) as being unpatentable over Braverman in view of Bosnes has been maintained. The arguments regarding the said rejection have been fully considered (Remarks, pg. 9). They are not persuasive for the following reasons.

The arguments are directed to Braverman is deficient because it fails to teach or suggest a method for tagging nucleic acid molecules in a way that results in the segments of a particular nucleic acid molecule being tagged with the same tag sequence and Bosnes, which is cited solely to provide a way to analyze mRNA, fails to meet Braverman's deficiency and, as such, the cited references, taken in any combination, fail to provide each and every element of the rejected claims (Remarks, pg. 9).

The above arguments are not persuasive because claims 21, 35 and 37 as amended requiring the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence has been interpreted as to require only one type of MID because the MID sequence associated with a segment of a nucleic acid molecule identifying the sample has been interpreted as to be present in all of the plurality of tagged segments because the claims as recited do not recite any distinguishing features of the nucleic acid molecule of step '(b)i.' and step '(b)ii.'.

Braverman teaches the claimed steps and is not deficient as allegedly asserted by the applicants. Furthermore as discussed in the interview summary, said claims have been further amended to make the pending claims non-obvious improvements over the arts of record. Therefore rejection over Braverman in view of Bosnes has been maintained.

Claim interpretation

9. Instant independent claims 21, 35 and 37 as amended requiring the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence has been interpreted as to require only one type of MID because the MID sequence associated with a segment of a nucleic acid molecule identifying the sample has been interpreted

as to be present in all of the plurality of tagged segments because the claims as recited do not recite any distinguishing features of the nucleic acid molecule of step '(b)i.' and step '(b)ii.'.

The following Objection and rejections are necessitated by the claim amendments.

Double Patenting

10. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

11. Claims 21, 24 and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,168,385 in view of Braverman et al (US 2009/0105959, cited in the previous office action) and Bosnes (WO 03/062462, cited in the previous office action) for the following reasons.

Regarding instant independent claims 21, 35 and 37, claim 1 of '385 patent is drawn to a method of sample analysis, comprising: a) separately ligating an oligonucleotide tag to each of a plurality of samples that comprise fragmented genomic DNA to produce a plurality of adaptor-ligated samples, wherein: i.) each of said samples is made from a different source; ii.) each of said samples is ligated to a different oligonucleotide tag; iii.) each oligonucleotide tag comprises at least one word comprising a unique nucleotide sequence that distinguishes said oligonucleotide tag from other oligonucleotide tags; and iv.) each adaptor-ligated sample comprises adaptor-ligated genomic fragments that contain an adaptor and a genomic fragment; b) pooling said plurality of adaptor-ligated samples to make a pooled sample; c) isolating from said pooled sample adaptor-ligated genomic fragments that correspond to one or more genomic loci, thereby producing isolated fragments; and d) sequencing an isolated fragment of (c) to obtain: i.) the unique nucleotide sequence of an oligonucleotide tag; and ii.) the nucleotide sequence of at least part of a genomic fragment; wherein the unique nucleotide sequence of said isolated fragment identifies from which of said plurality of different polynucleotide samples said isolated fragment is derived.

One having ordinary skill in the art would recognize that the oligonucleotide tag specific to each sample meets the limitation of the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence. Claims of '385 patent is not drawn to the nucleic acid samples are from a single isolated cell, which is taught by Braverman (Figs. 1 and 2 and paragraphs 0077-0084) for enriching the concentration of nucleic acids from the single cell and to determine the error rate in the sequencing run (paragraph 0082).

With regard to the limitations of dependent claims 24, 26-34, 36, 38 and 39 claims 1-12 of '385 patent in view of Braverman and Bosnes are drawn to the limitations of said dependent claims and therefore are not patentably significant.

12. Claims 21, 24 and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 8,318,433 in view of Braverman et al (US 2009/0105959, cited in the previous office action) and Bosnes (WO 03/062462, cited in the previous office action) for the following reasons.

Regarding instant independent claims 21, 35 and 37 claim 1 of '433 patent is drawn to method of sequencing comprising: sequencing a plurality of genomic fragments, wherein the genomic fragments are obtained from a plurality of different sources, and wherein: i.) each of said genomic fragments comprises an oligonucleotide tag that comprises a unique nucleotide sequence that indicates from which source a

genomic fragment is derived; and, separate from said unique nucleotide sequence, a binding site for a sequencing primer; ii.) said sequencing of the plurality of genomic fragments is done using said sequencing primer; and iii.) said genomic fragments correspond to one or more selected portions of a genome that have been isolated from a pooled genomic sample by specific hybridization; to obtain, for each of the sequenced fragments, the unique nucleotide sequence of an oligonucleotide tag and the nucleotide sequence of at least a portion of a genomic fragment.

Claims of '433 patent is not drawn to the nucleic acid samples are from a single isolated cell, which is taught by Braverman (Figs. 1 and 2 and paragraphs 0077-0084) for enriching the concentration of nucleic acids from the single cell and to determine the error rate in the sequencing run (paragraph 0082).

With regard to the limitations of dependent claims 24, 26-34, 36, 38 and 39 claims 1-13 of '433 patent in view of Braverman and Bosnes are drawn to the limitations of said dependent claims and therefore are not patentably significant.

In response to this office action and depending on the claim amendments, the ODP issue will be revisited at the time of the allowance.

Claim Objections

13. Claim 28 is objected to because of it is dependent from canceled claim 23. For compact prosecution, claim 28 presumed to be dependent from claim 21. If applicant disagree with claim 28 dependency, applicant is suggested to correct its dependency. Appropriate correction is required.

Claim Rejections - 35 USC § 112

14. The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

15. Claims 21, 24 and 26-39 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

16. Claim 21 recites the limitation "the nucleic acid molecules" in step (b) line 1. There is insufficient antecedent basis for "the" nucleic acid molecules limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" nucleic acid molecules limitation in the claim.

17. Claims 24 and 26-34 are indefinite because they are dependent from claim 21.

18. Claim 24 recites the limitation "the product" in line 1. There is insufficient antecedent basis for "the" product limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" product limitation in the claim

19. Claim 35 recites the limitation "the nucleic acid molecules" in line 4. There is insufficient antecedent basis for "the" nucleic acid molecules limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" nucleic acid molecules limitation in the claim.

20. Claim 36 is indefinite because it is dependent from claim 35.

21. Claim 37 recites the limitation "the nucleic acid molecules" in line 4. There is insufficient antecedent basis for "the" nucleic acid molecules limitation in the claim. Applicant is suggested to provide proper antecedent basis for "the" nucleic acid molecules limitation in the claim.

22. Claims 38 and 39 are indefinite because they are dependent from claim 37.

For compact prosecution, applicant is suggested to carefully review the claims for any minor informalities and 112 second and fourth paragraph issues and further clarify the steps of distinguishing the multiplex identifier (MID) sequence associated with a segment of a nucleic acid molecule of (a) uniquely identifies a sample and all of the plurality of tagged segments of a particular nucleic acid molecule of (a) are associated with the same MID sequence because of lack of distinguishing features of "a nucleic acid molecule" of step '(b)i.' from "a particular nucleic acid molecule" from step '(b)ii.'.

Claim Rejections - 35 USC § 102

23. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

The following rejection is based on the interpretation of claims 21, 35 and 37 requiring only one multiplex identifier based on the interpretation of amended claim as discussed in section 9.

24. Claims 21, 24 and 28-39 are rejected under pre-AIA 35 U.S.C. 102(a) and 102(e) as being anticipated by Braverman et al (US 2009/0105959, cited in the previous office action).

Regarding claims 21 and 25, Braverman method of analyzing nucleic acids, comprising providing a plurality of nucleic acids from a single cell source (paragraph 0077) and tagging (i.e., associating) the plurality of nucleic acids from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 24, Braverman teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claim 28, Braverman teaches identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within a single cell source from a number of unique MID sequences identified (Fig. 1 and paragraphs 78-83).

Regarding claim 29, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claim 30, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claims 31 and 32, Braverman teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Fig. 2 and paragraphs 0085-0088).

Regarding claims 33 and 34, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claim 35-37, Braverman teaches a method of analyzing nucleic acids, comprising: providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with a unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003). Braverman further teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a

plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 38, Braverman teaches that the amplifying step comprises performing a polymerase chain reaction amplification (paragraph 0086).

Regarding claim 39, Braverman teaches that the amplifying step comprises a linear amplification (i.e., isothermal amplification) step (paragraph 0007).

Claim Rejections - 35 USC § 103

25. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

26. Claims 21, 24 and 26-39 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Braverman et al (US 2009/0105959, cited in the previous office action) in view of Bosnes (WO 03/062462, cited in the previous office action).

Braverman and Bosnes teach a method for analyzing nucleic acid from a sample and therefore are analogous arts. The teachings of Bosnes are specifically applied for the limitations of claims 26 and 27 of nucleic acid is DNA and mRNA for correlating information relating to mRNA to genomic information.

Regarding claims 21 and 25, Braverman method of analyzing nucleic acids, comprising providing a plurality of nucleic acids from a single cell source (paragraph

0077) and tagging (i.e., associating) the plurality of nucleic acids from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 24, Braverman teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claims 26 and 27, Braverman teaches nucleic acids (0018). However, Braverman does not specifically teach that the nucleic acid is DNA and mRNA.

Regarding claim 28, Braverman teaches identifying a number of the plurality of nucleic acids tagged with a unique MID, and determining a number of the plurality of nucleic acid molecules that were within a single cell source from a number of unique MID sequences identified (Fig. 1 and paragraphs 78-83).

Regarding claim 29, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claim 30, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claims 31 and 32, Braverman teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Fig. 2 and paragraphs 0085-0088).

Regarding claims 33 and 34, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claim 35-37, Braverman teaches a method of analyzing nucleic acids, comprising: providing a plurality of nucleic acids from a single cell source (paragraph 0077). Braverman also teaches tagging (i.e., associating) the plurality of nucleic acids from the single cell source with a unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003). Braverman further teaches an amplification of plurality of nucleic acid attached to the UID sequence before determining the nucleic acid sequence (paragraph 0088) and determining a nucleotide sequence of the plurality of nucleic acids and attached MID sequences, wherein the MID sequences uniquely identify the single cell source, wherein the MID sequences uniquely identify each of a

plurality of nucleic acid molecules from the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Regarding claim 38, Braverman teaches that the amplifying step comprises performing a polymerase chain reaction amplification (paragraph 0086).

Regarding claim 39, Braverman teaches that the amplifying step comprises a linear amplification (i.e., isothermal amplification) step (paragraph 0007).

As discussed above regarding claims 26 and 27, Braverman does not specifically teach that the nucleic acid is DNA and mRNA. However, the DNA and mRNA from the same sample were known in the art at the time the claimed invention was made as taught by Bosnes, who teaches a method for isolating DNA and mRNA from the same sample and further teaches having both DNA and mRNA from the same sample allows for correlating information relating to mRNA to genomic information (Abstract).

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the DNA and mRNA from the single cell source of Bosnes in the method of Braverman with a reasonable expectation of success with the expected benefit of correlating information relating to mRNA to genomic information thus understanding the interrelationship of genotype and phenotype as taught by Bosnes (Abstract). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the mRNA from

the single cell source because isolation of DNA, mRNA and protein are is routinely practiced in the art as exemplified by Bosnes.

It is further noted that in KSR, the Supreme Court particularly emphasized "the need for caution in granting a patent based on the combination of elements found in the prior art," (USPQ2d at 1395), and reaffirmed principles based on its precedent that "[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."

In the instant case, the steps of claims 21, 24 and 26-39 are a combination of known steps which are well understood, routine, conventional steps already engaged in by the scientific community for improving the sensitivity, specificity of the nucleic acid analysis and were known in the art at the time the claimed invention was made. As discussed above, Braverman in view of Bosnes teaches the claimed steps and combined as suggested above would arrive at the claimed invention. Also, one having ordinary skill in the art would recognize that choosing both DNA and mRNA template would be a mere design choice because steps of analyzing mRNA templates would not be different as the steps for converting mRNA to cDNA are routine, conventional and well established in the art. Thus the steps of pending claims when viewed as whole, add nothing significant beyond the sum of the steps taken from Braverman and Bosnes. Therefore the steps of claims 21, 24 and 26-39 do not amount to "significantly more" and thus obvious improvement over Braverman.

27. Claims 21, 24 and 26-39 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Brenner (US 2006/0177832 published Aug. 10, 2006) in view of Braverman et al (US 2009/0105959, cited in the previous office action) and further in view of Bosnes (WO 03/062462, cited in the previous office action).

Brenner, Braverman and Bosnes teach a method for analyzing nucleic acid from a sample and therefore are analogous arts. The teachings of Braverman are specifically applied for the nucleic acid sample from a single cell and the teachings of Bosnes are specifically applied for the limitations of claims 26 and 27 of nucleic acid is DNA and mRNA for correlating information relating to mRNA to genomic information.

Regarding claims 21, 24 and 26-39, Brenner teaches a method comprising providing fragments from a population of genomes, wherein the fragments from each genome has the same unique oligonucleotide tag attached. In this aspect, the invention includes a method of determining a frequency of a nucleotide at a predetermined locus in a population of genomes, such method comprising the following steps: (i) separately generating fragments of each genome of the population; (ii) attaching a unique oligonucleotide tag to each genome; (iii) selecting fragments from each genome that contains the predetermined locus; (iv) generating a labeled oligonucleotide tag from each unique oligonucleotide tag, the labeled oligonucleotide tag generating a signal indicative of the nucleotide at the predetermined locus; and (v) determining the frequency of the nucleotide at the predetermined locus by detecting the signals generated by the labeled oligonucleotide tags specifically hybridized with their respective tag complements, the respective tag complements being attached in spatially

discrete regions on the one or more solid phase supports (paragraph 0008). Brenner also teaches the amplification and sequencing of the nucleic acid fragments (paragraphs 0031 and 0050). As discussed in the claim interpretations section, since there are no distinguishing features between "a nucleic acid molecule" of 'step (b)i' and 'a particular nucleic acid molecule of 'step (b)ii' is encompassed by the MID identifier of the sample.

Brenner does not specifically teach that the nucleic acids are from a single cell, and comprise mRNA. Braverman teaches a method of analyzing nucleic acids, providing a plurality of nucleic acids from a single cell source (paragraph 0077).

Brenner in view of Braverman does not specifically teach that the nucleic acid is DNA and mRNA. However, the DNA and mRNA from the same sample were known in the art at the time the claimed invention was made as taught by Bosnes, who teaches a method for isolating DNA and mRNA from the same sample and further teaches having both DNA and mRNA from the same sample allows for correlating information relating to mRNA to genomic information (Abstract).

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the DNA and mRNA from the single cell source of Bosnes in the method of Brenner in view of Braverman with a reasonable expectation of success with the expected benefit of correlating information relating to mRNA to genomic information thus understanding the interrelationship of genotype and phenotype as taught by Bosnes (Abstract). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the

mRNA from the single cell source because isolation of DNA, mRNA and protein are is routinely practiced in the art as exemplified by Bosnes.

Conclusion

28. No claims are allowed.

29. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 7 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

Examiner-Initiated Interview Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Examiner: Bhat*. (3)_____.

(2) *Representative: Mr. Keddie*. (4)_____.

Date of Interview: *11 July 2016*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☒102 ☒103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *21,35 and 37*.

Identification of prior art discussed: *Arts of the record*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

See Continuation Sheet.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

10x Genomics, Inc. Exhibit 2004
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Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

For compact prosecution the examiner contacted the representative Mr. Keddie on July 11, 2016 to discuss further amendments to place the pending claims in better condition for the allowance. The examiner discussed that the claim amendments of June 29, 2016 requiring same multiplex identifier for the sample and the plurality of segments of the nucleic acids still read on the teachings over Braverman and further amendments and terminal disclaimers are needed to place the pending claims in condition for the allowance.

It was agreed that inclusion of an additional step '(b) iii', of "wherein the multiplex identifier associated with a plurality of nucleic acid molecules of the sample is different from a multiplex identifier associated with a plurality of segments from a particular nucleic acid molecule of the sample", fixing the claim language for any minor informalities, 112 second and fourth paragraphs issues and filing the terminal disclaimers over claims of US 8,298,767, 8,563,274, 8,679,756 and 9,102,980 and over claims of 14/935,255 and 14/939,728 copending applications and their acceptance by the office and identifying the support for the amendments in the specification would overcome the rejections on the record and thus allowable.

The representative informed that the amendments require approval from the applicant. On July 13, 2016 the representative requested to issue an office action in view of not hearing a response from the applicant.

The communication between the examiner and the representative is made of record.

Notice of References Cited	Application/Control No. 14/792,094	Applicant(s)/Patent Under Reexamination BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-2006/0177832 A1	08-2006	Brenner; Sydney	C12P19/34	435/6.12
*	B	US-8,168,385 B2	05-2012	Brenner; Sydney	C12N15/1065	435/6.11
*	C	US-8,318,433 B2	11-2012	Brenner; Sydney	C12N15/1065	435/6.1
*	D	US-8,298,767 B2	10-2012	Brenner; Sydney	C12Q1/6855	435/287.2
*	E	US-8,563,274 B2	10-2013	Brenner; Sydney	C12Q1/6855	435/287.2
*	F	US-8,679,756 B1	03-2014	Brenner; Sydney	C12Q1/6855	435/287.2
*	G	US-9,102,980 B2	08-2015	Brenner; Sydney	C12Q1/6855	1/1
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

10x Genomics, Inc. Exhibit 2004
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	28875	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or ("POPULATION GENETICS TECHNOLOGIES").as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:09
L2	79	1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code")same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:11
L3	51	2 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code")same sample.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:12
L4	18	3 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code")same sequencing.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:13
L5	11	4 and ("nucleotide sequence") same ("multiplex identifier" or mid or barcode or zipcode or token or identifier or tag or uniqu$3 or "bar-code" or "bar code").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/07/16 18:14
S1	28662	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or population.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:41
S2	232	S1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:45
S3	12	S2 and ("multiplex identifier" or mid or	US-PGPUB;	OR	ON	2016/01/05

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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		barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna).clm.	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			15:47
S4	0	S3 and (single or one) near3 (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:53
S5	10	S3 and (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 15:53
S6	65	S1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:00
S7	55	S6 and (single or one) same(source or cell) same (nucleic or dna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:01
S8	8	S1 and "reflex sequence".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:08
S9	174	("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:11
S10	25	S9 and (single or one) same (source or cell) same (nucleic or dna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:11
S11	69	("20040224331" | "20060008799" | "20070020640" | "20070238113" | "20070259357" | "20090155780" | "20100086914" | "20100113296" | "20100120098" | "20110287435" | "20120208724" | "20120316074" |	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2016/01/05 16:30

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		"20130059310" | "6723513" | "7544473" | "7700325" | "8101346" | "8298767" | "8318460").PN.	IBM_TDB			
S12	8	S11 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:31
S13	138	"7393665"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:36
S14	2	"7393665".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:36
S15	599	("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:37
S16	147	S15 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (determin$3 or obtain$3) near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:38
S17	697052	435/$.ccls. or 506/$.ccls. or 422/$.ccls. or 536/$.ccls. or (c12q1/68 or c07h21/02 or c07h21/04 or c12p19/34 or c12m1/34 or g01n33/52 or c40b30/04 or c40b40/04 or c40b50/04 or g01n31/22).cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:50
S18	481	S17 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) near5 (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:51
S19	131	S18 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) near5 (determin$3 or obtain$3) near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:51
S20	45	S19 and (single or one) near5 (source or cell) near5 (nucleic or dna or	US-PGPUB; USPAT;	OR	ON	2016/01/05 16:52

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		polynucleotide)	USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S21	17	S1 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (insert$4 or attach$3 or coupl$3 or conjugat$3 or connect$3 or tagg$3) same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:55
S22	3	S21 and (single or one or indivudual) same (source or cell) same (nucleic or dna or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:56
S23	3	S21 and ("multiplex identifier" or mid or barcode or zipcode or token or identifier) same (determin$3 or obtain$3) same sequence.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/05 16:58
S24	101206	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:01
S25	101649	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:01
S26	196	S25 and (dna and rna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:02
S27	72	S26 and (dna and rna) near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:03
S28	29	S27 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)near5 primer	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:04

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S29	23047	extract$3 near5 (dna or nucleic) near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:06
S30	1272	S29 and extract$3 near5 (ribonucleic or mrna)near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:07
S31	6	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)near5 primer	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:07
S32	199	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:08
S33	1	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) near5 track$3 near5 (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:09
S34	11	S30 and ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) same (track$3 or origin or identif$3) same (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:10
S35	31772	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) same (track$3 or origin or identif$3) same (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:11
S36	505	S35 and amplif$3 same (dna or nucleic) same ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:12
S37	91	S36 and amplif$3 same (cdna or mrna) same ("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2016/01/06 18:12

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			DERWENT; IBM_TDB			
S38	5559	("multiplex identifier" or mid or barcode or zipcode or token or "unique identifier" or uid) near5 (track$3 or origin or identif$3) near5 (sample or source)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:22
S39	41	S38 and extract$3 near5 (ribonucleic or mrna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:22
S40	70439	genotype and phenotype	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:26
S41	1534	S40 and phenotype near5 mrna	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:27
S42	75	S41 and extract$3 near5 (ribonucleic or mrna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:27
S43	0	S42 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:28
S44	2218	S40 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:28
S45	1788	S44 and (ribonucleic or mrna)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:29
S46	63	S45 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier") same mrna	US-PGPUB; USPAT; USOCR;	OR	ON	2016/01/06 18:29

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			FPRS; EPO; JPO; DERWENT; IBM_TDB			
S47	153	S40 and ("multiplex identifier" or barcode or zipcode or token or "unique identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:31
S48	10	S47 and (ribonucleic or mrna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:31
S49	60223	("multiplex identifier" or barcode or zipcode or token or "unique identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:33
S50	75	S49 and (ribonucleic or mrna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/06 18:33
S51	2	(bhat.xa. or bhat.xp.) and (simultan$4 near5 isolat$4)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/07 10:54
S52	103	(bhat.xa. or bhat.xp.) and isolat$4.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/01/07 10:54

7/ 16/ 2016 6:34:16 PM
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From:	James S. Keddie
To:	Bhat, Narayan
Subject:	Re: 14/792,094 -status update
Date:	Wednesday, July 13, 2016 1:46:44 PM

Nope. Please go ahead and send an office action. Thx. James

On Jul 14, 2016, at 1:25 AM, Bhat, Narayan <Narayan.Bhat@USPTO.GOV> wrote:

> Mr. Keddie,
> Good morning. Did you get a response from your client yet? For reducing the number of actions per disposal, I can wait for two more days. Any update from your end is greatly appreciated. You have a good day.
>
> Narayan K Bhat
> Primary Examiner, AU 1634,
> Remsen 3C09, TC 1600, USPTO, DOC, Alexandria, VA-22313
> T: 571-272-5540
> Fax: 571-273-5540
>
> **From:** James S. Keddie [mailto:keddie@bozpat.com]
> **Sent:** Monday, July 11, 2016 4:50 PM
> **To:** Bhat, Narayan <Narayan.Bhat@USPTO.GOV>
> **Subject:** 14/792,094
>
> Dear Exr. Bhat,
>
> I forwarded the proposed amendment to the client earlier today but I haven't heard back. If you don't hear from me in time, please just send out an office action.
>
> Thanks,
>
> James
>
> James Keddie Ph.D.
> Senior Patent Agent
> BOZICEVIC, FIELD & FRANCIS LLP
> 1900 University Ave Suite 200
> East Palo Alto, Ca 94303
> Main: (650) 327-3400; Fax: (650) 327-3231
> Direct: (650) 833-7723; Email: keddie@bozpat.com
>
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	14792094
Filing Date	2015-07-06
First Named Inventor	BRENNER, Sydney
Art Unit	1636
Examiner Name	BHAT, NARAYAN KAMESHWAR
Attorney Docket Number	CGLC-020CON5

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	1	WO2012148497	WO		2012-11-01	ROBINSON, et al.		

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/NARAYAN K BHAT/ 07/16/2016

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.K.B/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

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Filing Date	2015-07-06
First Named Inventor	BRENNER, Sydney
Art Unit	1636
Examiner Name	BHAT, NARAYAN KAMESHWAR
Attorney Docket Number	CGLC-020CON5

	1	

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EXAMINER SIGNATURE

Examiner Signature	/NARAYAN K BHAT/	Date Considered	07/16/2016

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Receipt date: 06/29/2016 14792094 - GAU: 1634

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	14792094
Filing Date	2015-07-06
First Named Inventor	BRENNER, Sydney
Art Unit	1636
Examiner Name	BHAT, NARAYAN KAMESHWAR
Attorney Docket Number	CGLC-020CON5

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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/James S. Keddie, Reg. No. 48,920/	Date (YYYY-MM-DD)	2016-06-29
Name/Print	James S. Keddie	Registration Number	48920

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00660

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/792,094	07/06/2015	1634	800	CGLC-020CON5	19	3

CONFIRMATION NO. 9697

CORRECTED FILING RECEIPT

93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

Date Mailed: 07/27/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Sydney Brenner, Ely, UNITED KINGDOM;
Gi Mikawa, Great Shelford, UNITED KINGDOM;
Robert Osborne, Great Chesterford, UNITED KINGDOM;
Andrew Slatter, London, UNITED KINGDOM;

Applicant(s)

10X Genomics, Inc., Pleasanton, CA;

Power of Attorney: The patent practitioners associated with Customer Number 93726

Domestic Priority data as claimed by applicant

This application is a CON of 14/172,694 02/04/2014 PAT 9102980 *
which is a CON of 14/021,790 09/09/2013 PAT 8679756
which is a CON of 13/859,450 04/09/2013 PAT 8563274
which is a CON of 13/622,872 09/19/2012 ABN
which is a CON of 13/387,343 02/15/2012 PAT 8298767
which is a 371 of PCT/IB2010/002243 08/13/2010
which claims benefit of 61/288,792 12/21/2009
and claims benefit of 61/235,595 08/20/2009
(*)Data provided by applicant is not consistent with PTO records.

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00661

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: No

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 07/20/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/792,094**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No

** **SMALL ENTITY** **

Title

Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00662

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00664

Filed Electronically

CHANGE OF CORRESPONDENCE ADDRESS		
	Attorney Docket	CGLC-020CON5
	First Named Inventor	Brenner, Sydney
	Application Number	14/792,094
	Confirmation No.	9697
	Filing Date	July 6, 2015
	Group Art Unit	1634
Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Examiner Name	BHAT, NARAYAN KAMESHWAR
	Title: "Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"	

Please change the Correspondence Address for the above-identified application to:

☒ **The address associated with Customer Number 43850**

Firm Name	Morgan, Lewis & Bockius LLP		
Individual Name			
Address	One Market, Spear Street Tower		
City, State, Zip	San Francisco, California 94105		
Country	U.S.A.		
Telephone	(415) 442-1000	Facsimile	(415) 442-1001

SIGNATURE OF ATTORNEY/AGENT OF RECORD

Name/Reg. No.	James S. Keddie, 48,920		
Signature	/James S. Keddie, Reg. No. 48,920/	Date	07-27-2016

Electronic Acknowledgement Receipt

EFS ID:	26472517
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020CON5
Receipt Date:	27-JUL-2016
Filing Date:	06-JUL-2015
Time Stamp:	19:27:54
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	CGLC-020CON5-Change_Corresp.pdf	20439 dfcd776d085e442d1f3dafe2707ad202e51a286a	no	1

Warnings:

Information:	
Total Files Size (in bytes):	20439

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/82 (07-13)
Approved for use through 11/30/2014. OMB 0651-0035
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

☒ Practitioners associated with Customer Number: 43850

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

☒ The address associated with Customer Number: 43850

OR

☐	Firm or Individual Name			
	Address			
	City		State	Zip
	Country			
	Telephone		Email	

Assignee Name and Address: 10X Genomics, Inc.
7068 Koll Center Parkway, Suite 401
Pleasanton, CA 94566

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be Filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of The practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record
The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature		Date	8/13/2015
Name	Matthew Murphy	Telephone	(925) 401-7495
Title	Vice President, General Counsel		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: 10X Genomics, Inc.

Application No./Patent No.: 14/792,094 Filed/Issue Date: July 6, 2015

Titled: Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

10X Genomics, Inc., a corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☑ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☑ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 039159, Frame 0296, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

   1. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

   2. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Gargi Talukder — Signature

Aug 18, 2016 — Date

Gargi Talukder — Printed or Typed Name

61,368 — Title or Registration Number

Electronic Acknowledgement Receipt	
EFS ID:	26680070
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	18-AUG-2016
Filing Date:	06-JUL-2015
Time Stamp:	14:29:20
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	POA.pdf	635597 7:x73:x5671866Qx3761359cafQ:xd3d65987f20/5	no	2
Warnings:					

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	Stmt.pdf	120223 4e577ffd7c80a10cb20f92645e4d2ce34de7d7d3	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	755820	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05

CONFIRMATION NO. 9697

43850
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

POWER OF ATTORNEY NOTICE

OC000000085406701

Date Mailed: 08/30/2016

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/18/2016.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/khoang/

10x Genomics, Inc. Exhibit 2004
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05

CONFIRMATION NO. 9697

43850
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

POA ACCEPTANCE LETTER

OC000000085406703

Date Mailed: 08/30/2016

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/18/2016.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The applicant, 10X Genomics, Inc., owner of 100 percent interest in the instant application, hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior **U.S. Patent No. 8,168,385**, as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later: expires for failure to pay a maintenance fee; is held unenforceable; is found invalid by a court of competent jurisdiction; is

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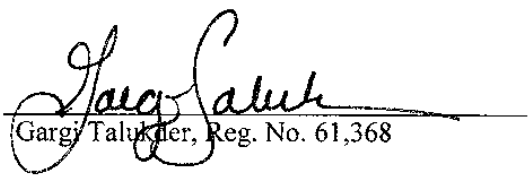
statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321; has all claims canceled by a reexamination certificate; is reissued; or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer.

Additionally, in the event that the patent term of any patent granted on the instant application is extended based on patent term adjustment under 35 U.S.C. § 154, applicant does not disclaim the extension of the patent term based on the patent term adjustment, as the term of any patent granted on the instant application has not been disclaimed beyond a specific date.

Included herewith is the terminal disclaimer fee required under 37 C.F.R. § 120(d).

The undersigned is an attorney or agent of record.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: December 22, 2016 By: 
Gargi Talukder, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
Telephone: (415) 442-1000
Facsimile: (415) 442-1001

DB2/ 30936205.1

PTO/SB/30 (07-14)
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Request for Continued Examination (RCE) Transmittal

Address to:
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Commissioner for Patents
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Alexandria, VA 22313-1450

Application Number	14/792,094
Filing Date	July 6, 2015
First Named Inventor	Sidney Brenner
Art Unit	1634
Examiner Name	Narayan, Kameshwar Bhat
Attorney Docket Number	104371-5016-US05

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not compy with the requirements of 35 U.S.C 371, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO on page 2.)

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. ☐ Other _____

   b. ☑ Enclosed

      i. ☑ Amendment/Reply

      ii. ☐ Affidavit(s)/ Declaration(s)

      iii. ☐ Information Disclosure Statement (IDS)

      iv. ☐ Other _____

2. Miscellaneous

   a. ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

   a. ☑ The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 50-0310 .

      i. ☑ RCE fee required under 37 CFR 1.17(e)

      ii. ☑ Extension of time fee (37 CFR 1.136 and 1.17)

      iii. ☐ Other _____

   b. ☐ Check in the amount of $ _____ enclosed

   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Gargi Talukder/	Date	December 22, 2016
Name (Print/Type)	Gargi Talukder, Ph.D.	Registration No.	61,368

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature	/Leonor Rivera-Huerta/		
Name (Print/Type)	Leonor Rivera-Huerta	Date	December 22, 2016

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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Instruction Sheet for RCEs

(not to be submitted to the USPTO)

NOTES:

An RCE is not a new application, and filing an RCE will not result in an application being accorded a new filing date.

Filing Qualifications:

The application must be a utility or plant application filed on or after June 8, 1995. The application cannot be a provisional application, a utility or plant application filed before June 8, 1995, an international application that does not comply with the requirements of 35 U.S.C. 371, a design application, or a patent under reexamination. See 37 CFR 1.114(e). An international application does not comply with the requirements of 35 U.S.C. 371 until the requirements under 35 U.S.C. 371(c), including the requirement for the inventor's oath or declaration under 35 U.S.C. 371(c)(4), have been complied with.

Filing Requirements:

Prosecution in the application must be closed. Prosecution is closed if the application is under appeal, or the last Office action is a final action, a notice of allowance, or an action that otherwise closes prosecution in the application (e.g., an Office action under *Ex parte Quayle*). See 37 CFR 1.114(b).

A submission and a fee are required at the time the RCE is filed. If reply to an Office action under 35 U.S.C. 132 is outstanding (e.g., the application is under final rejection), the submission must meet the reply requirements of 37 CFR 1.111. If there is no outstanding Office action, the submission can be an information disclosure statement, an amendment, new arguments, or new evidence. See 37 CFR 1.114(c). The submission may be a previously filed amendment (e.g., an amendment after final rejection).

WARNINGS:

Request for Suspension of Action:

All RCE filing requirements must be met before suspension of action is granted. A request for a suspension of action under 37 C FR 1.103(c) does not satisfy the submission requirement and does not permit the filing of the required submission to be suspended.

Improper RCE will NOT toll Any Time Period:

Before Appeal - If the RCE is improper (e.g., prosecution in the application is not closed or the submission or fee has not been filed) and the application is not under appeal, the time period set forth in the last Office action will continue to run and the application will be abandoned after the statutory time period has expired if a reply to the Office action is not timely filed. No additional time will be given to correct the improper RCE.

Under Appeal - If the RCE is improper (e.g., the submission or the fee has not been filed) and the application is under appeal, the improper RCE is effective to withdraw the appeal. Withdrawal of the appeal results in the allowance or abandonment of the application depending on the status of the claims. If there are no allowed claims, the application is abandoned. If there is at least one allowed claim, the application will be passed to issue on the allowed claim(s). See MPEP 1215.01.

See MPEP 706.07(h) for further information on the RCE practice.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 104371-5016-US05
Application Number 14/792,094	Filed July 6, 2015
For Compositions and Methods for Intramolecular Nucleic Acid Rearrangement	
Art Unit 1634	Examiner BHAT, NARAYAN KAMESHWAR

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☑	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$ 300
☐	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☑ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 50-0310.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☑ attorney or agent of record. Registration number 61,368.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.



Signature

December 22, 2016
Date

Gargi Talukder, Ph.D.
Typed or printed name

415-442-1000
Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☑ * Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce. P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	RESPONSE TO FINAL OFFICE ACTION

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Final Office Action mailed July 25, 2016, please enter the following amendments and remarks:

Amendments to the Claims are reflected in the listing of claims which begins on page **2** of this paper.

Remarks/Arguments begin on page **5** of this paper.

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Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-39 (Cancelled)

40. (New) A method of analyzing nucleic acids, the method comprising:

(a) providing a first plurality of segments of nucleic acid molecules from a contiguous region of a genome from a first single isolated cell, wherein each of the first plurality of segments is appended to a first multiplex identifier (MID) sequence to form first appended segments;

(b) providing a second plurality of segments of nucleic acid molecules from a contiguous region of a genome from a second single isolated cell, wherein each of the second plurality of segments is appended to a second MID sequence to produce second appended segments, and the first MID sequence and the second MID sequence are different from each other;

(c) sequencing the first appended segments and the second appended segments to provide a plurality of sequences,

wherein each sequence comprises (i) nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, and wherein the MID sequences uniquely identify the segments with a single isolated cell.

41. (New) The method of claim 40, wherein the method further comprises amplifying the first appended segments and the second appended segments before the sequencing step (c).

42. (New) The method of claim 40, wherein the nucleic acid molecules are selected from DNA and RNA.

43. (New) The method of claim 42, wherein the nucleic acid molecules comprise mRNA.

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44. (New) The method of claim 40, wherein the MID sequences are appended to the segments through ligation.

45. (New) The method of claim 40, wherein the MID sequences are appended to the segments by performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

46. (New) The method of claim 40, wherein the sequences of the segments are attributed to different isolated cells based on the associated MID sequence.

47. (New) The method of claim 40, wherein the MID sequences further comprise a sequencing primer sequence.

48. (New) The method of claim 40, wherein the MID sequences are from 2 to 100 bases in length.

49. (New) The method of claim 40, wherein the segments of (a) and (b) are in the range of 100 to 5,000 bp in length.

50. (New) The method of claim 40, wherein the segments of (a) and (b) are 400 bp or less in length.

51. (New) The method of claim 40, wherein the method further comprises providing a third plurality of segments of nucleic acid molecules from a contiguous region of a genome from a third single isolated cell, wherein each of the third plurality of segments is appended to a third MID sequence to produce third appended segments, and the third MID sequence is different from the first MID sequence and the second MID sequence.

52. (New) The method of claim 40, wherein the contiguous region is greater than 2 kb in length.

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53. (New) A method of analyzing a plurality of contiguous genomic sequences, comprising:

(a) providing a sample comprising the plurality of contiguous genomic sequences, wherein the plurality of contiguous genomic sequences are from a plurality of single isolated cells;

(b) tagging a plurality of fragments of the contiguous genomic sequences with a multiplex identifier (MID) sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence; and

(c) sequencing the plurality of fragments and MID sequences to identify fragments of the contiguous sequence.

54. (New) The method of claim 53, wherein the first contiguous nucleic acid sequence is from a first single isolated cell and the second contiguous nucleic acid sequence is from a second single isolated cell.

55. (New) The method of claim 53, wherein sequences of the fragments are attributed to different single isolated cells based on the associated MID sequence.

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Remarks/Arguments

Applicants have not dedicated or abandoned any unclaimed subject matter and have not acquiesced to any rejections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

Status of the Claims

Claims 40-55 are newly added and under consideration in this application. The newly added claims are supported throughout the specification as filed, for example, see page 7 and pages 16-20 of the application as filed.

Applicants respectfully request entry of the claims into the record.

Response to Office Action

Claim Objections

Claim 28 is objected to because it is dependent from canceled claim 23. Claim 28 is canceled in the currently pending claims, thus rendering this objection moot.

Claim Rejections – 35 USC § 102 and § 103

The previous rejections of the claims under § 102 as being allegedly anticipated by Braverman et al. (US 2009/0105959 (hereinafter "Braverman") and under § 103 as allegedly being obvious over Braverman in view of Bosnes, WO 03/062462 (hereinafter "Bosnes") have been maintained. Applicants respectfully traverse these rejections.

In order to clarify the claimed invention, Applicants have prepared a new set of claims. Applicants submit that Braverman fails to disclose all elements of the claimed invention as clarified in this set of claims. In particular, Braverman fails to disclose a method in which each of a first plurality of segments from a first isolated cell is appended to a first multiplex identifier (MID) and each of a second plurality of segments from a second isolated cell are appended to a

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second MID, as is recited in independent claim 40. In addition, Braverman fails to provide a method in which a plurality of fragments of contiguous genomic sequences are tagged with a MID sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence, as is recited in independent claim 53. Furthermore, Braverman fails to disclose methods in which segments appended to or tagged with MIDs can be attributed to their single cell of origin. As such, Braverman fails to disclose or suggest all elements of the claimed invention and thus cannot support a rejection for anticipation.

Bosnes is cited solely for its description of methods of analyzing mRNA and thus fails to rectify the above-discussed deficiencies in Braverman. As such, the combined teachings of Braverman and Bosnes fail to render the presently claimed invention obvious.

For at least the foregoing reasons, Applicants respectfully request that all rejections under § 102 and § 103 be withdrawn.

Claim Rejections – Double Patenting

Claims 21, 24, and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,168,385 in view of Braverman et al., and Bosnes.

Claims 21, 24, and 26-39 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 8,318,433 in view of Braverman and Bosnes.

Without conceding to the propriety of these rejections and solely to move this application toward allowance, Applicants herewith file terminal disclaimers over the above-referenced patents.

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CONCLUSION

All the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action, and as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided below.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any necessary fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17, which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310 (Order No.: 104371-5016-US05).

Prompt and favorable consideration of this Amendment and Response is respectfully requested.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: December 22, 2016 By: _____
Gargi Talukder, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
Telephone: (415) 442-1000
Facsimile: (415) 442-1001

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No. 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The applicant, 10X Genomics, Inc., owner of 100 percent interest in the instant application, hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior **U.S. Patent No. 8,318,433**, as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later: expires for failure to pay a maintenance fee; is held unenforceable; is found invalid by a court of competent jurisdiction; is

10x Genomics, Inc. Exhibit 2004
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Page 00689

statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321; has all claims canceled by a reexamination certificate; is reissued; or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer.

Additionally, in the event that the patent term of any patent granted on the instant application is extended based on patent term adjustment under 35 U.S.C. § 154, applicant does not disclaim the extension of the patent term based on the patent term adjustment, as the term of any patent granted on the instant application has not been disclaimed beyond a specific date.

Included herewith is the terminal disclaimer fee required under 37 C.F.R. § 120(d).

The undersigned is an attorney or agent of record.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: December 22, 2016 By: _____
Gargi Talukdar, Reg. No. 61,368

Customer No.: 43850
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower
San Francisco, CA 94105
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Facsimile: (415) 442-1001

DB2/ 30936273.1

Electronic Patent Application Fee Transmittal

Application Number:	14792094
Filing Date:	06-Jul-2015
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	Gargi Talukder/Leonor Rivera-Huerta
Attorney Docket Number:	104371-5016-US05

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
REQUEST FOR PRIORITIZED EXAMINATION	2817	1	2000	2000
Pages:				
Claims:				
Miscellaneous-Filing:				
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	2830	1	70	70
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
RCE- 1st Request	2801	1	600	600
STATUTORY OR TERMINAL DISCLAIMER	2814	2	160	320
		Total in USD ($)		**3290**

Electronic Acknowledgement Receipt	
EFS ID:	27885111
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Leonor Rivera-Huerta
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	22-DEC-2016
Filing Date:	06-JUL-2015
Time Stamp:	19:07:50
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$3290
RAM confirmation Number	122316INTEFSW00006963500310
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	TrackOne Request	104371_5016US05_Track_1_Request.pdf	335899 ecec8522ea727fe7a46dad5e0a234e6964e43e0e	no	1
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	104371_5016US05_RCE.pdf	839149 e3a8280ea3decc0093016d7e91ea427a37552ecf	no	3
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
3	Extension of Time	104371_5016US05_EOT.pdf	288822 521001170a8290695a1283ea6769e68425e	no	1
Warnings:					
Information:					
4		104371_5016US05_Response_to_Final_Office_Action.pdf	842216 55da1f86582dafea3e4e46a4e16a41ae8ac0869e	yes	7

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	7

Warnings:
Information:

5	Terminal Disclaimer Filed	104371_5016US05_TD_over_US8168385.pdf	270993 866d132181277ac9cdf342a8d9c0922018a04859	no	2
Warnings:					
Information:					
6	Terminal Disclaimer Filed	104371_5016US05_TD_over_US8318433.pdf	262359 7854a<a81ea2653c0f33acd95a72758d<0f92a1	no	2
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	41171 e5dc1eca5f57083cc48cda0913ce039a26d890	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	2880609		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Sidney Brenner	Nonprovisional Application Number (if known):	14/792,094
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.

2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.

3. The applicable box is checked below:

I. ☐ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. ☑ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature Gargi Talukder	Date December 22, 2016
Name (Print/Typed) Gargi Talukder, Ph.D.	Practitioner Registration Number 61,368

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

☑ *Total of 1 forms are submitted.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014 OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 = *		X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 = *		X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 12/22/2016	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 16	Minus	** 20	= 0	X $40 =	0
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
William Phillips

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco CA 94105

MAILED
JAN 10 2017
OFFICE OF PETITIONS

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 14/792,094

1. THE REQUEST FILED December 22, 2016 IS **GRANTED.**

The above-identified application has met the requirements for prioritized examination

A. ☐ for an original nonprovisional application (Track I).
B. ☒ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;
B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
C. filing a **request for continued examination**;
D. filing a notice of appeal;
E. filing a request for suspension of action;
F. mailing of a notice of allowance;
G. mailing of a final Office action;
H. completion of examination as defined in 37 CFR 41.102; or
I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Brian W. Brown at 571-272-5338.

/Brian W. Brown/
[*Signature*]

Petitions Examiner, Office of Petitions
(Title)

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Application Number	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed: 12/22/2016	**This patent is subject to a Terminal Disclaimer**	

Approved/Disapproved by:	
2 TD's	/T.J./ Art Unit OCRU

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 02/01/2017
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
02/01/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

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Office Action Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12/22/16*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *40-55* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *40-55* is/are rejected.

8)☒ Claim(s) *53* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *7/6/15* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.

4) ☐ Other: _____.

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Continued Examination under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on December 22, 2016 has been entered.

Track1 Request – Granted

2. Track1 request filed by the applicant on December 22, 2016 has been granted by the office on January 10, 2017. Therefore instant application has been examined on a priority basis. To maintain its status applicant is required to follow the requirements listed in the Track1 grant document.

3. The present application is being examined under the pre-AIA first to invent provisions. The instant application is a continuation of 14/172,694, now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450, now U.S. Patent 8,563,274, which is a continuation of 13/622,872, now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, claiming priority from provisional application 61288792, filed on 12/21/2009 and from provisional application 61235595, filed on 08/20/2009.

This office action is in response to the papers filed on December 22, 2016.

Claim Status

3. In the claim listing of December 22, 2016 claims 40-55 are pending in this application and are under prosecution. Claims 40-55 are new. Claims 1-39 are canceled. Claims have been reviewed and entered. As discussed below, the limitation of claim 53 of "a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of second contiguous nucleic acid sequence" introduces a new matter (new matter is underlined by the examiner).

Withdrawn Objection, Rejections and Response to the Remarks

4. The previous objection of claim 28 has been withdrawn in view of cancellation of said claim. (Remarks, pg. 5).

5. The previous rejection under pre-AIA 35 USC 102(b) as being anticipated by Braverman and rejection under pre-AIA 35USC 103(a) as being unpatentable over Braverman in view of Bosnes have been withdrawn in view of cancellation of said claims.

The arguments regarding the teachings of Braverman as they pertain to the rejection made in this office action have been fully considered (Remarks, pgs. 5 and 6). They are not persuasive for the following reasons.

The arguments are directed to Braverman fails to disclose a method in which each of a first plurality of segments from a first isolated cell is appended to a first multiplex identifier (MID) and each of a second plurality of segments from a second isolated cell are appended to a second MID, as is recited in independent claim 40. In

addition, Braverman fails to provide a method in which a plurality of fragments of contiguous genomic sequences are tagged with a MID sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence, as is recited in independent claim 53. Furthermore, Braverman fails to disclose methods in which segments appended to or tagged with MIDs can be attributed to their single cell of origin. As such, Braverman fails to disclose or suggest all elements of the claimed invention and thus cannot support a rejection for anticipation (Remarks, pg. 6).

The arguments regarding the subject matter of claims 40 and 53 as being anticipated by Braverman are moot because of withdrawn rejection necessitated by cancellation of previous claims and addition of new claims.

With regard to claimed invention being obvious over Braverman in view of Drmanac (new art, see below) are not persuasive because new claim 40 as recited require providing two single isolated cells, which could be the same but processed independently, wherein nucleic acid template (i.e., a contiguous region of the genome) subjected to an amplification thereby producing a plurality of segments of nucleic acids, wherein said segments from each single cells are appended with a single cell specific unique identifier (UID) or referred also as multiplex identifier (MID), pooling them and sequencing them and sequence data for segments from single cells de-convoluted to identify the sequence of the MID sequence and their association with each of the two cells. Claim 53 has additional "common sequence" in MID.

Braverman teaches the steps of claims 40 and 53 including the analyzing nucleic acid from a single cell and also teaches analyzing nucleic acid template from multiple samples and sequencing the segments of the nucleic acid template (i.e., contiguous region of the genome) from each sample and identifying the segments from each sample associated with specific multiplex identifier from each sample that includes a cell. Braverman does not specifically teach that the nucleic acid segments from at least two isolated single cells. The teachings of Drmanac are specifically applied for the teachings of analyzing nucleic acids using the tagging (i.e., using multiplex identifier) method similar to the Braverman, wherein the nucleic acids are derived from two aliquots (i.e., samples) wherein each aliquot comprises a single isolated cell, thereby meeting the limitations of two isolated single cells.

The unique identifier element (also referred as multiplex identifier) tagged to each nucleic template comprising plurality of PCR amplified segments from each sample and identifying each sample origin associated with the unique multiplex identifier sequence of Braverman meets the limitations of the multiplex identifier. Furthermore, Braverman teaches key element 207 associated with each multiplex identifier, which meets the limitation of sharing common sequence associated with different multiplex identifiers (limitation of claim 53).

The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (WO 2008/061193; Fig. 1 and pg. 17, paragraph 3).

Therefore it maintained that the limitations of claims 40-55 are obvious over Braverman in view of Drmanac as further evidenced by Gillevet.

7. The previous ODP rejection of claims 21, 24 and 26-39 over claims 1-12 of '385 patent or over claims of '433 patent has been withdrawn in view of filing the terminal disclaimer on 12/22/16 and its acceptance by the office on 1/22/17.

Claim Objections

8. Claim 53 is objected to because of the following informalities. The recitation of "an MID sequence" in step 'b' lines 3 and 4 has been objected because of minor typographical error. It is suggested to use the phrase "a MID sequence" instead for consistency. Appropriate correction is required.

Claim Rejections - 35 USC § 112-first paragraph

8. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

9. The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

10. Claims 40-55 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention. eMPEP 2163.06 notes, "If new matter is added to the claims, the examiner should reject the claims under 35 U.S.C. 112, first paragraph written description requirement (In re Rasmussen, 650 F.2d 1212, 211 USPQ 323 (CCPA 1981)."

In the instant case, the recitations of "a genome from a first single isolated cell" and "a genome from a second single isolated cell" in claim 40 and "a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence" in claim 53 represents a new matter (New matter is underlined by the Examiner). Applicant has cited support for the new claims in the specification pages 7 and 16-20 (Remarks, pg. 5, paragraph 2). However, review of the cited pages in the instant specification does not support the "isolated single cell" and "common MID sequence". Also, review of the instant figure 12 shows the presence of a separate "common" sequence among three different nucleic acid segment and thus appears to be the "same" common sequence rather than different "common sequence". Pending further clarification, the "common MID sequence" introduces the new matter.

Also, "the written description requirement prevents an applicant from claiming subject matter that was not adequately described in the specification as filed. New or amended claims which introduce elements or limitations which are not supported by the as-filed disclosure violate the written description requirement. See, e.g., In re Lukach, 442 F.2d 967, 169 USPQ 795 (CCPA 1971) (subgenus range was not supported by generic disclosure and specific example within the subgenus range); In re Smith, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily described by a genus encompassing it and a species upon which it reads)." (MPEP § 2163).

Since Applicant has not identified specific citations of support in the instant specification or in claims as originally filed for the new matter, Applicant is required to cancel the new matter in the reply to this Office Action or identify the new matter in the specification as originally filed.

11. Claims 41-52 and 54-55 are rejected under 35 USC first paragraph because they are dependent from claims 1 and 53.

Claim Rejections - 35 USC § 112-second paragraph

12. The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

13. The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

14. Claims 53-55 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

15. Claim 53 is indefinite over the recitation of "a first contiguous nucleic acid sequence share a common MID sequence that is different from a MID sequence of fragments of second contiguous nucleic acid sequence" in step 'b' it is unclear how sharing sequences is different. Further amendment or clarification is required.

16. Claims 54 and 55 are indefinite because they are dependent from claim 53.

Claim Rejections - 35 USC § 103

17. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

18. Claims 40-55 are rejected under pre-AIA 35 U.S.C. 102(a) and 102(e) as being anticipated by Braverman et al (US 2009/0105959, cited in the previous office action) in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007). The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (WO 2008/061193 published May 22, 2008).

Claim interpretation: The steps of claim 40 has been interpreted as providing multiple samples that includes a first single isolated cell and a second single isolated

cell, wherein nucleic acid template (i.e., a contiguous nucleic acid from genome) produced by PCR amplification from each sample (i.e., plurality of segments) is appended with a sample specific unique identifier (UID) as also referred as multiplex identifier (MID), pooling the appended segments and sequencing them and sequence data for each template nucleic acid (i.e., plurality of segments) is de-convoluted to identify the sequence composition of coupled UID or MID sequences and its association with the sample of origin.

The steps of claims 53-55 has been interpreted to encompass the steps of claim 40 except for sharing a common sequence.

It is noted that the recitation of the first single isolated cell and the second single isolated cell does not exclude the first and the second being the same cell but are appended with different multiplex identifier.

Braverman and Drmanac teach a method for analyzing nucleic acid from a sample and therefore are analogous arts. Braverman teaches the steps of claims 40 and 53 including the analyzing nucleic acid from a single cell and also teaches analyzing nucleic acid template from multiple samples and sequencing the segments of the nucleic acid template (i.e., contiguous region of the genome) from each sample and identifying the segments from each sample associated with specific multiplex identifier from each sample that includes a cell. Braverman does not specifically teach that the nucleic acid segments from at least two isolated single cells. The teachings of Drmanac are specifically applied for the teachings of analyzing nucleic acids using the tagging method similar to the Braverman, wherein the nucleic acids are derived from two

aliquots (i.e., samples) wherein each aliquot comprises a single isolated cell, which are useful for more accurate pathogen diagnostic for health and biodefense applications.

Regarding claims 40 and 53-55, Braverman method of analyzing nucleic acids, comprising providing a plurality of nucleic acid segments from a nucleic acid template (i.e., contiguous region of the genome) from a single cell source (paragraph 0077) and tagging (i.e., associating) the plurality of segments from the single cell source with an unique identifier (UID) element also referred as multiplex identifier (MID; paragraph 0003) and determining a nucleotide sequence of the segments and attached MID sequences, wherein the MID sequences uniquely identify the single cell source (Fig. 2 and paragraphs 0003, 0077, 0082-0088). Braverman also teaches that the nucleic acid templates (i.e., the contiguous region of the genome) is amplified by PCR amplification from each sample, thereby generating plurality of segments of nucleic acids and appended with a sample specific unique identifier (i.e., UID or MID), pooling the appended segments and sequencing them and the sequence data for each template nucleic acid is de-convoluted to identify the sequence composition of coupled UID element and its association with sample of origin (paragraphs 0046, 0047 and 0077).

As discussed above, though Braverman teaches that the DNA is from single cell but does not specifically teach that the nucleic acid segments are derived from two isolated single cells. However, providing the nucleic acid segments from genomic DNA from two isolated single cells were known in the art at the time the claimed invention was made as taught by Drmanac, who teaches a method for analyzing mixtures of bacterial cells genomes, wherein each aliquot comprises a single cell analyzing plurality

of single cells using the tagging method (Fig. 1 and paragraphs 0009 and 0043) similar to Braverman and further teaches the step for tracking the nucleic acid using oligonucleotide tag (i.e., identifier) for each polynucleotide (paragraph 0105). Drmanac also teaches analyzing plurality of single cell nucleic acid DNA separately allows for more accurate pathogen diagnostic for health or biodefense applications including the changes associated with the single cell (paragraph 0043), thus providing motivation to one of ordinary skill in the art to include the step of analyzing nucleic acid from a plurality of individual single cells of Drmanac in the method of Braverman. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (Fig. 1 and pg. 17, paragraph 3).

One having ordinary skill in the art would also recognize that while combining the steps of Braverman with the step of Drmanac some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Drmanac and not gleaned from the instant specification.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid from a plurality of individual single cells of Drmanac in the method of Braverman with a reasonable expectation of success with the expected benefit of more accurate pathogen diagnosis and changes associated with each single cell. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves

including the two individual single cells for analysis which is routinely practiced in the art and therefore not novel.

The teachings of Braverman in view of Drmanac regarding dependent claims 41-52 are discussed below.

Regarding claim 41, Braverman teaches an amplification of plurality of nucleic acid attached to the MID sequence before determining the nucleic acid sequence (paragraph 0088).

Regarding claims 42 and 43 Braverman teaches that the nucleic acids are DNA or mRNA (paragraph 0026).

Regarding claim 44, Braverman teaches that the tagging step comprises ligating a sequence comprising the MID sequences to the plurality nucleic acids (paragraphs 0097).

Regarding claims 45 and 47, Braverman teaches that the tagging step comprises performing PCR of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer (Fig. 2 and paragraphs 0088 and 0096-0099).

Regarding claim 46, Braverman in view of Drmanac teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the

attaching step and a sequencing primer sequence (Braverman, Fig. 2 and paragraphs 0085-0088).

Regarding claim 48, Braverman teaches that the MID sequences are 10-mer in length, which is within the range of 2 to 100 bases in length and at least 144 different MID sequences (paragraph 0092).

Regarding claims 49 and 50, Braverman teaches that the nucleic acid segments are about 200 to 300 bp in length (paragraph 0047), which is in the claimed range of 100 to 5000 bp.

Regarding claim 51, the step of providing third isolated single cell and the nucleic acid appended with third MID sequence is mere duplication and therefore not novel especially when Braverman teaches multiplexed sample comprise about 384 samples (paragraph 0077), wherein each sample is identified by an unique identifier (paragraph 0077).

Regarding claim 52, Drmanac teaches that nucleic acid template is in the range of 10-30 kb (paragraph 0023), which is greater than 2 kb in length.

It is further noted that in KSR, the Supreme Court particularly emphasized "the need for caution in granting a patent based on the combination of elements found in the prior art," (USPQ2d at 1395), and reaffirmed principles based on its precedent that "[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."

In the instant case, the steps of claims 40-55 are a combination of known steps which are well understood, routine, conventional steps already engaged in by the

scientific community for improving the sensitivity, specificity of the nucleic acid analysis and were known in the art at the time the claimed invention was made. As discussed above, Braverman in view of Drmanac teaches the claimed steps and combined as suggested above would arrive at the claimed invention. Thus the steps of pending claims when viewed as whole, add nothing significant beyond the sum of the steps taken from Braverman and Drmanac. Therefore the steps of claims 40-55 do not amount to "significantly more" and thus obvious improvement over Braverman.

Double Patenting

19. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all

requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

20. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (WO 2008/061193 published May 22, 2008).

Regarding instant independent claims 40 and 53, claims 1, 2 and 11 of '756 patent discloses a method comprising: a) obtaining a composition comprising a mixture of: i. a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and ii. a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprises a unique MID sequence that uniquely identifies each of the nucleic acid molecules; and b) sequencing said first plurality of segments and said a second plurality of segments to provide a plurality of sequences; wherein each

sequence comprises: (i) the nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, wherein said genomic DNA is genomic DNA from a virus or a bacterium, wherein defining a linkage relationship between said sequences using the MID sequence that is associated with each of the sequences, wherein sequences that comprise the first MID sequence are linked and sequences that comprise the second MID sequence are linked (common steps between instant claims 40 and 53 are underlined by the examiner). One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell. One having ordinary skill in the art would also recognize that providing a genome from a second isolated cell would be a duplication of the steps of '756 patent because instant claims as recited merely require analysis of the first and second appended segments and not linking the sequence to the first and the second isolated single cell.

However, claims of '756 patent do not disclose nucleic acid segments are from first and second cell. However, providing the genomic DNA from two bacteria was known in the art at the time the claimed invention was made as taught by Drmanac, who teaches a method for analyzing mixtures of bacterial genomes, aliquots may be made so that each aliquot contains one cell type, or a maximum of one cell (paragraph 0043) and further teaches method for tracking the nucleic acid using oligonucleotide tag (i.e., identifier) for tracking each polynucleotide (paragraph 0105). Drmanac also teaches analyzing single cell nucleic acid DNA allows for more accurate pathogen diagnostic for health or biodefense applications (paragraph 0043), thus providing motivation to one of

ordinary skill in the art to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '756 patent. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (Fig. 1 and pg. 17 and paragraph 3).

One having ordinary skill in the art would also recognize that while combining the steps of '756 patent with the step of Drmanac some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Drmanac.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '756 patent with a reasonable expectation of success with the expected benefit of more accurate pathogen diagnosis. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '756 patent in view of Drmanac.

With regard to instant dependent claims 41-52, 54 and 55, claims of '756 patent in view of Drmanac disclose the steps and therefore are not patentably distinct.

21. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons. The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (as cited above).

Regarding instant claims 40 and 53, claims 1 and 10 of '274 patent is drawn to a composition comprising a mixture of: a) a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and b) a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprise a unique MID sequence that uniquely identifies each of the nucleic acid molecules, wherein said genomic DNA is genomic DNA from a virus or a bacterium.

One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell. One having ordinary skill in

the art would also recognize that providing a genome from a second isolated cell would be a duplication of the steps of '274 patent because instant claims as recited merely require analysis of the first and second appended segments and not linking the sequence to the first and the second isolated single cell.

However, claims of '274 patent do not disclose nucleic acid segments are from first and second cell and not using the composition for performing the method. However, providing genomic DNA from two different bacteria was known in the art at the time the claimed invention was made as taught by Drmanac, who teaches a method for analyzing mixtures of bacterial genomes, aliquots may be made so that each aliquot contains one cell type, or a maximum of one cell (paragraph 0043) and further teaches method for tracking the nucleic acid using oligonucleotide tag (i.e., identifier) for tracking each polynucleotide (paragraph 0105). Drmanac also teaches analyzing single cell nucleic acid DNA allows for more accurate pathogen diagnostic for health or biodefense applications (paragraph 0043), thus providing motivation to one of ordinary skill in the art to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '274 patent. One having ordinary skill in the art would recognize that the composition of claims 1-11 of '274 patent used to carry out the method because claimed method merely requires providing the plurality of segments of nucleic acid molecules, which is also taught by Drmanac.

The routine, well established and conventional method of identifying the polynucleotides in each sample from the pooled samples using unique tags for each

sample nucleic acids as applied for the rejection of claims 40 and 53 are further evidenced by Gillevet (Fig. 1 and pg. 17 and paragraph 3).

One having ordinary skill in the art would also recognize that while combining the composition of '274 patent with the step of Drmanac some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Drmanac.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid from a plurality of individual single cell of Drmanac in the method of '274 patent with a reasonable expectation of success with the expected benefit of more accurate pathogen diagnosis. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '274 patent in view of Drmanac.

With regard to instant dependent claims 41-52, 54 and 55, claims of '274 patent in view of Drmanac disclose the steps and therefore are not patentably distinct.

22. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 9,410,149 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons.

The subject matter of claims 1-13 of '149 patent is similar to claims 1-11 of '756 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-13 of '149 patent in view of Drmanac.

23. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of Drmanac (US 2007 /0072208 published Mar. 29, 2007) for the following reasons.

The subject matter of claims 1-19 of '150 patent is similar to claims 1-11 of '274 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-19 of '150 patent in view of Drmanac.

Conclusion

24. No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 7 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

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Format: Summary Sort by: Publication Date

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(54) Title: MULTITAG SEQUENCING AND ECOGENOMICS ANALYSIS

(57) Abstract: Embodiments of the invention herein described relate to multiplex polynucleotide sequence analysis without the use of size separation methods or blotting. In certain particulars the invention relates to multiplex sequencing using massively parallel sequencing methods, such as pyrosequencing methods and sequencing by synthesis. The invention provides increased throughput and the ability to analyze greater numbers of samples simultaneously or serially on presently available systems, as well as others yet to be developed. In certain of its embodiments the invention relates to the analysis of complex microbial communities, particularly to in-depth analysis thereof in large numbers of samples.

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MULTITAG SEQUENCING AND ECOGENOMICS ANALYSIS

Related Applications

This application is a continuation in part and claims full benefit of priority of US provisional application number 60/858,948, filed on 15 November 2006 by Patrick Gillevet for Multitag Sequencing and Ecogenomics Analysis, which is incorporated herein by reference in its entirety.

Field of the Invention

The invention relates to the determination of polynucleotide sequences. It also relates to determining sequences in multiple samples, in some particulars in multiple environmental samples and in multiple clinical samples.

Statement of Government Rights

Work described herein was done partly with Government support under Grant No. 1R43DK074275-01A2 awarded by the U.S. National Institute of Diabetes and Digestive and Kidney Diseases, and the US Government therefore may have certain rights in the invention.

Background

Sequence determination technologies for proteins, RNAs and DNAs, have been pivotal in the development of modern molecular biology. During the past fifteen years, DNA sequencing in particular has been the core technology in an on-going revolution in the scope and the depth of understanding of genomic organization and function. The on-going development of sequencing technology is, perhaps, best symbolized by the determination of the complete sequence of a human genome.

The human genome sequencing project served a number of purposes. It served as a platform for programmatic development of improved sequencing technologies and of genome sequencing efforts. It also served to provide a framework for the production and distribution of sequencing information from increasingly large scale sequencing projects. These projects provided complete genome sequences for a succession of model organisms of increasingly large genetic complements. These accomplishments, culminating in the completion of a human genome sequence, highlight the very considerable power and throughput of contemporary sequencing technology.

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At the same time, however, they highlight the limitations of current technology and the need for considerable improvements in speed, accuracy, and cost before sequencing can be fully exploited in research and medicine. Among the areas that can be seen most readily to require advances in sequencing technology are clinical sequencing applications that require whole genome information, environmental applications involving multiple organisms in mixtures, and applications that require processing of many samples. These are, of course, just a few among a great many areas that either require or will benefit greatly from more capable and less expensive sequencing methods.

To date, virtually all sequencing has been done by Sanger chain elongation methods. All Sanger methods require separating the elongation products with single base resolution. Currently, while PAGE still is used for this purpose in some commercial sequencers, capillary electrophoresis is the method of choice for high throughput DNA sequencers. Both gel-based and capillary-based separation methods are time consuming, costly, and limit throughput. Chip based methods, such as Affymetrix GeneChips and HySeq's sequencing by hybridization methods, require chips that can be produced only by capital intensive and complex manufacturing processes. These limitations pose obstacles to the utilization of sequencing for many purposes, such as those described above. Partly to overcome the limitations imposed by the necessity for powerful separation techniques in chain termination sequencing methods and the manufacturing requirements of chip-based methods, a number of technologies are currently being developed that do not require the separation of elongation products with integer resolution and do not require chips.

A lead technology of this type is a bead, emulsion amplification, and pyrosequencing-based method developed by 454 Life Sciences. (See Margulies, et al. (2005) Nature 437: 376, which is incorporated herein by reference in its entirety, particularly as to the afore-mentioned methods. The method utilizes a series of steps to deposit single, amplified DNA molecules in individual wells of a plate containing several million picoliter wells. The steps ensure that each well of the plate either contains no DNA or the amplified DNA from a single original molecule. Pyrosequencing is carried out in the wells by elongation of a primer template in much the same way as Sanger sequencing. Pyrosequencing does not involve chain termination and does not require separation of elongation products. Instead sequencing proceeds stepwise by single base addition cycles. In each cycle one of the four bases – A, T, G, or C – is included in the elongation reaction. The other three bases are omitted. A base is added to the growing chain if it is complementary to the next position on the template. Light

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is produced whenever a base is incorporated into the growing complimentary sequence. By interrogating with each of A, C, G, or T in succession, the identity of the base at each position can be determined. Sequencing reactions are carried out in many wells simultaneously. Signals are collected from all the wells at once using an imaging detector. Thus, a multitude of sequences can be determined at the same time

In principle, each well containing a DNA will emit a signal for only one of the four bases for each position. In practice, runs of the same base at two or more positions in succession lead to the emission of proportionally stronger signals for the first position in the run. Consequently, reading out the sequence from a given well is a bit more complicated then simply noting, for each position, which of the four bases is added. Nevertheless, because signals are proportional to the number of incorporations, sequences can be accurately reconstructed from the signal strength for most runs.

The technology has been shown to read accurately an average of about 250 or so bases per well with acceptable accuracy. A device offered by 454 Life Sciences currently uses a 6.4 cm^2 picoliter well "plate" containing 1,600,000 picoliter sized wells for sequencing about 400,000 different templates. The throughput for a single run using this plate currently is about 100 million bases in four hours. Even though this is a first generation device, its throughput is nearly 100 times better than standard Sanger sequencing devices.

Numerous other methods are being developed for ultra high throughput sequencing by other institutions and companies. Sequencing by synthesis methods that rely on target amplification are being developed and/or commercialized by George Church at Harvard University, by Solexa, and by others. Ligation sequencing methods have been developed and/or are being commercialized by Applied Biosystems and Solexa, among others. Array and hybridization sequencing methods are commercially available and/or are being developed by Affymetrix, Hyseq, Biotrove, Nimblegen, Illumina, and others. Methods of sequencing single molecules are being pursued by Helicos based on sequencing by synthesis and U.S. Genomics (among others) based on poration.

These methods represent a considerable improvement in throughput over past methods, in some regards. And they promise considerable improvement in economy as well. However, currently they are expensive to implement and use, they are limited to relatively short reads and, although massively parallel, they have limitations that must be overcome to realize their full potential.

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One particular disadvantage of these methods, for example, is that samples must be processed serially, reducing throughput and increasing cost. This is a particularly great disadvantage when large numbers of samples are being processed, such as may be the case in clinical studies and environmental sampling, to name just two applications.

The incorporation of indexing sequences by ligation to random shotgun libraries has been disclosed in US patent numbers: 7264929, 7244559, and 7211390, but the direct ligation methods therein disclosed distort the distribution of the components within the samples (as illustrated in Figure 4 herein) and therefore are inappropriate for enumerating components within each sample.

Accordingly, there is a need to improve sample throughput, to lower the costs of sequencing polynucleotides from many samples at a time, and to accurately enumerate the components of samples analyzed by high throughput, parallelized and multiplex techniques. .

Summary

It is therefore an object of the present invention to provide sequencing methods with improved sample throughput. The following paragraphs describe a few illustrative embodiments of the invention that exemplify some of its aspects and features. They are not exhaustive in illustrating its many aspects and embodiments, and thus are not in any way limitative of the invention. Many other aspects, features, and embodiments of the invention are described herein. Many other aspects and embodiments will be readily apparent to those skilled in the art upon reading the application and giving it due consideration in the full light of the prior art and knowledge in the field.

Embodiments provide multiplex methods for the quantitative determination of polynucleotides in two or more samples, comprising:

hybridizing a first primer to polynucleotides in a first sample, said first primer comprising a first tag sequence and a first probe sequence specific for a first target sequence, wherein said first target sequence is 3' to a variable genetic region;

elongating primer templates formed thereby to form a first population of tagged polynucleotides comprising: said first primer including said first tag sequence; and sequences of said variable genetic region;

hybridizing a second primer to polynucleotides in a second sample, said second primer comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein said second target sequence is 3' to the same variable genetic region as said

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first target sequence, wherein further said second probe sequence may be the same as or different from said first probe sequence;

elongating primer templates formed thereby to form a second population of tagged polynucleotides comprising: said second primer including said second tag sequence; and sequences of said variable genetic region;

mixing said first and second populations together;

determining sequences of polynucleotides comprising tag sequences and the sequences of the variable genetic element in said mixture;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

from this information determining the number of time one or more given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples;

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide multiplex methods for the quantitative determination of polynucleotides in two or more samples, comprising:

hybridizing a first primer pair to polynucleotides in a first sample, the first primer of said first primer pair comprising a first tag sequence and a first probe sequences specific for a first target sequence and the second primer of said first primer pair comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein the first and the second probe sequences flank and hybridize to opposite strands of a variable genetic region;

elongating primer templates formed thereby to from a first population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said first primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said first primer pair or (b) a sequence complementary to the sequence of said first primer of said first primer pair, a sequence of said variable genetic region and the sequence of said second primer of said first primer pair;

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hybridizing a second primer pair to polynucleotides in a second sample, the first primer of said second primer pair comprising a third tag sequence and said first probe sequences specific for said first target sequence and the second primer of said second primer pair comprising a fourth tag sequence and said second probe sequence specific for said second target sequence;

elongating primer templates formed thereby to from a second population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said second primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said second primer pair or (b) a sequence complementary to the sequence of said first primer of said second primer pair, a sequence of said variable genetic region and the sequence of said second primer of said second primer pair;

mixing said first and second populations together;

determining sequences of polynucleotides in said mixture, comprising the tag sequences and the variable genetic element;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

from this information determining the number of times given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples.

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide methods in accordance with any of the foregoing or the following wherein given polynucleotide sequences in a sample is quantified by a method comprising normalizing the number occurrences determined for the given sequence. In embodiments the number of occurrences is normalized by dividing the number of occurrences determined for the given polynucleotide sequence by the total number of occurrences of polynucleotide sequences in the sample. In embodiments the given polynucleotide sequences is that of a given variant of a variable genetic region and, in embodiments, the quantity of the given variant in the sample is normalized by dividing the number of occurrences of that

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variant by the total number of occurrences of all variants of the variable genetic region in the sample.

Embodiments provide a multiplex method for determining polynucleotide sequences in two or more samples, comprising: attaching a first tag sequence to one or more polynucleotides of a first sample; attaching a second tag sequence different from said first tag sequence to one or more polynucleotides of a second sample; mixing the tagged polynucleotides of said first and second samples together; determining sequences of said polynucleotides comprising said first and said second tags; and identifying said first and second tags in said sequences; thereby identifying sequences of said polynucleotides of said first sample and second samples, wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide a multiplex method for determining polynucleotide sequences in two or more samples comprising:

attaching a first tag sequence, t_1, to $P_{1\text{-}1}$ through $P_{1\text{-}n1}$ polynucleotides in a first sample, thereby to provide a first plurality of polynucleotides tagged with said first tag, $t_1P_{1\text{-}1}$ through $t_1P_{1\text{-}n1}$;

attaching a second tag sequence, t_2, to $P_{2\text{-}1}$ through $P_{2\text{-}n2}$ polynucleotides in a second sample, thereby to provide a second plurality of polynucleotides tagged with said second tag, $t_2P_{2\text{-}1}$ through $t_2P_{2\text{-}n2}$;

mixing together said polynucleotides tagged with said first and said second tags;

determining sequences of polynucleotides comprising said tags in said mixture;

identifying said first and second tags in said sequences and;

by said first tag identifying polynucleotide sequences of said first sample and by said second tag identifying polynucleotide sequences of said second sample;

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

Embodiments provide a method according to any of the foregoing or the following, wherein the number of said polynucleotides in said first sample, n_1, is any of 2, 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500, 5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more, and the number of said polynucleotides in said second sample, n_2, is any of 2, 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500,

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5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more.

Embodiments provide a method according to any of the foregoing or the following, wherein the number of said samples and of said different tags therefor is 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 500, 1,000, 2,500, 5,000, 10,000 or more.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are nucleotide sequences that are 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 nucleotides long or any combination thereof.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are incorporated into said polynucleotides by a step of ligation, provided that the step of ligation does not result in biasing.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are incorporated into said polynucleotides by a step of ligation and/or by a step of amplification.

Embodiments provide a method according to any of the foregoing or the following, wherein said tags are comprised in primers for amplification and are incorporated into said polynucleotides by amplification using said primers.

Embodiments provide a method according to any of the foregoing or the following, wherein said tags are incorporated into said polynucleotides by a process comprising a step of cloning into a vector.

Embodiments provide a method according to any of the foregoing or the following, wherein the tags are comprised in adapters for amplification and said adapters are ligated to polynucleotides in said samples. Embodiments provide a method in this regard, wherein further, said polynucleotides ligated thereby to said tags are amplified via said adapters. Embodiments provide a method in this regard, wherein further, said adapters comprise a moiety for immobilization. In embodiments said moiety is a ligand; in embodiments it is biotin. Embodiments provide a method in this regard, wherein further, said tags are comprised on adapters for bead emulsion amplification. In embodiments the adapters are suitable for use in a sequencing system of 454 Life Sciences or other sequencing system in which bead emulsion amplification is carried out.

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Embodiments provide a method according to any of the foregoing or the following, wherein the primer for amplification comprises a sequence for PCR amplification, linear amplification, transcriptional amplification, rolling circle replication, or QB replication.

Embodiments provide a method according to any of the foregoing or the following, wherein the primer for amplification comprises a sequence for PCR amplification.

Embodiments provide a method according to any of the foregoing or the following, wherein each of said polynucleotides is disposed individually on a bead isolated from other polynucleotides.

Embodiments provide a method according to any of the foregoing or the following, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, and the amplification products thereof also are disposed on said bead.

Embodiments provide a method according to any of the foregoing or the following, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, the amplification products thereof also are disposed on said bead, and each said bead is disposed individually in a well isolated from other said beads.

Embodiments provide a method according to any of the foregoing or the following, wherein the sequences are determined by pyrosequencing.

Embodiments provide a method according to any of the foregoing or the following, wherein said samples are biological samples, each comprising one or more species.

Embodiments provide a method according to any of the foregoing or the following, wherein at least one sequence of said polynucleotides is specific to a particular organism.

Embodiments provide a method according to any of the foregoing or the following, wherein said sequences comprise a variable 16S rRNA sequence.

Embodiments provide a method according to any of the foregoing or the following, wherein said sequences comprise a variable 18S rRNA sequence, a variable rRNA ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, and/or a genetic disease sequence.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community samples for clinical analysis of a patient.

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Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community environmental samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community soil samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are microbial community water samples.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are samples for SNP analysis.

Embodiments provide a method according to any of the foregoing or the following, wherein the samples are samples for genotyping.

Embodiments provide a multiplex method according to any of the foregoing or the following for determining polynucleotide sequences of two or more samples, comprising,

amplifying polynucleotides of a first sample to produce first amplified polynucleotides comprising a first tag sequence;

separately amplifying polynucleotides of a second sample to produce second amplified polynucleotides comprising a second tag sequence different from said first tag sequence;

wherein the amplification products arising from different individual polynucleotides are spatially separated from one another;

mixing together amplicons of said first and second samples;

distributing the amplicons in the mixture into spatially distinct locations; sequencing the amplicons thus distributed using one or more primers that hybridize 5' to said tag sequences;

identifying said tag sequences in the sequences of polynucleotides thus determined; and

identifying by said tags polynucleotides of said first sample and polynucleotides of said second sample.

Embodiments provide a method according to any of the foregoing or the following, comprising,

(a) for each sample separately: isolating polynucleotides to be sequenced, ligating said polynucleotides to a common adaptor comprising a tag sequence, and capturing individual ligated polynucleotides onto individual beads under conditions that provide predominately for the immobilization of 0 or 1 molecule per bead;

(b) thereafter mixing together said beads comprising said polynucleotides.

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Embodiments provide a method according to any of the foregoing or the following, further comprising, amplifying bead-immobilized polynucleotides in droplets of an emulsion thereby to clonally amplify said individual polynucleotides on said beads, wherein amplification comprises amplification of said tag sequence.

Embodiments provide a method according to any of the foregoing or the following, further comprising, distributing individual droplets containing said amplified polynucleotides into wells under conditions that provide predominantly for 0 or 1 droplet per well, determining in individual wells the sequences of polynucleotides comprising said tag sequences, and by said tag sequences identifying polynucleotides of said first and said second samples.

In embodiments the invention provides methods in accordance with any of the foregoing or the following, for any one or more of detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like.

In embodiments the invention provides methods in accordance with any of the foregoing or the following, for analyzing the composition, diversity, stability, dynamics, and/or changes in agricultural, food, biosecurity, veterinary, clinical, ecological, zoological, oceanological, and/or any other sample comprising one or more polynucleotides.

Embodiments provide kits comprising a plurality of two or more primers, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primers the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence and the target sequence is located 3' to a variable genetic region, and

(D) each of said primers is disposed separately from the others in containers in said kit.

Embodiments provide kits in accordance with any of the foregoing or the following, wherein each of said primers further comprises a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, and the priming sequence is the same in all of said primers,

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said kit further comprising a primer complimentary to and effective for polymerization from said priming sequence.

Embodiments provides kits comprising a plurality of two or more primers pairs, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primer the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence,

(D) in each primer pair the probe sequences are specific to target sequences that flank a variable genetic region;

(E) each of said primers is disposed separately from the others in said kit.

Embodiments provides kits in accordance with any of the foregoing or the following, wherein, the primers further comprise a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, the priming sequence either is the same in all the primers, or one member of each pair has the same first priming sequence and the second member of each pair has the same second priming sequence, said kit further comprising disposed separately from one another in one or more containers one or more primers complementary to and effective for elongation from said priming

Embodiments provide a kit useful in methods according to any of the foregoing or the following, comprising a set of primers and/or adapters, wherein each primer and/or adapter in said set comprises a tag sequence and a primer sequence. In embodiments the primers and/or adapters further comprise a moiety for immobilization. In embodiments the primers and/or adapters comprise biotin. In embodiments the primers and/or adapters in the set comprise all tag sequences defined by 2, 3, 4, 5, 6, 7, or 8 base polynucleotide sequences, wherein each of said primers and/or adapters are disposed in containers separate from one another. In embodiments there are 1-5, 3-10, 5-15, 10-25, 20-50, 25-75, 50-100, 50-150, 100-200, 150-500, 250-750, 100-1000, or more different tag sequences disposed separately from one

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another, so as to be useful for uniquely tagging said number of different samples. In embodiments the primers and/or adapters are suitable for use as 454 Life Sciences amplification adapters and/or primers. In embodiments the primers and/or adapters further comprise any one or more of a primer sequence for any one or more of a 16S rRNA sequence, an 18S rRNA sequence, an ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, a genetic disease sequence, and/or any other sequence for amplification or analysis.

In embodiments the invention provides a kit, in accordance with any of the foregoing or the following, comprising a set of primers and/or adapters for use in a method according to any of the foregoing or the following, wherein each primer and/or adapter in said set comprises a tag sequence, the tag sequence of each of said primers and/or adapters is different from that of the other primers and/or adapters in said set, the primers and/or adapters further comprise a priming sequence that is the same in all of the primers and/or adapters in said set, the tag sequences are located 5' to the priming sequence and the different primers and/or adapters comprising each different tag sequence are disposed separately from one another. In embodiments the tags are any number of bases long. In embodiments the tags are 2, 3, 4, 5, 6, 8, 10, 12 bases long. In embodiments the tags are 4 bases long. In embodiments the priming sequence is specific to any target polynucleotide of interest. In embodiments the priming sequence is specific to a sequence in 16S rRNA. In embodiments the tags differ from each other by at least 2 bases. In embodiments the tags do not contain polynucleotide tracts within the tag. In embodiments the tags do not contain homo-polynucleotide tracts within or at the junction of the tag and PCR primer. In embodiments the tags do not contain polynucleotide tracts within or at the junction of the tag and emulsion PCR adapter. In embodiments, the tags are not reverse compliments of each other.

Brief Description of the Figures

Figure 1 is a schematic diagram showing a general embodiment of the invention. A plurality of samples (S_1, S_2, through S_j) is shown topmost in the Figure. Each sample is comprised of a plurality of polynucleotides ($P_{1\text{-}1}$ to $P_{1\text{-}n1}$ in S_1; $P_{2\text{-}1}$ to $P_{2\text{-}n2}$ in S2; through $P_{j\text{-}1}$ to $P_{j\text{-}nj}$). The polynucleotides in each sample are labeled separately with a tag polynucleotide sequence, all the polynucleotides in a given sample being tagged (in this illustration) with a single tag sequence, designated in the figure as T_1 for S_1, T_2 for S_2, through T_j for S_j. The individual tagged polynucleotides are denoted accordingly. The tagged polynucleotides in

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each sample are designated collectively, for each sample, T_1S_1, T_2S_2, through T_jS_j. The tagged polynucleotides from the samples are mixed together to form a mixture, designated M_i. The mixture is sequenced, typically by a massively parallel sequencing method. The tag sequences are identified in the data thus obtained. The sequences are grouped by tag. The sequences from the individual samples are thereby identified.

Figure 2A is a diagram depicting step I in the multitag sequencing of microbial community samples using a tagged 16S forward and reverse primer-linker pairs for PCR amplification. (a) represents the Forward 16S rRNA primer with Tag *I* and Emulsion PCR Linker, (b) represents the 16S rRNA sequence, (c) represents the Reverse 16S rRNA primer with Tag *j* and Emulsion PCR Linker, (d) represents the Amplified 16S rRNA sequence with Forward and Reverse Tags *ij*, (e) represents the Emulsion PCR Bead, (f) represents the pyrosequencing read, (g) represents the well in picoliter plate, (h) represents a Unique tag, (i) represents Amplified Community 1, (j) represents Amplified Community 2, and (k) represents Amplified Community *n*. Step 1 involves the amplification of the microbial community from each sample using uniquely tagged universal primers-linkers. In step 1, different samples are amplified separately, using 16S rRNA specific adapter-tag-primers with a different tag for each sample.

Figure 2B is a diagram depicting the Emulsion PCR reaction beads randomly arrayed into picoliter plate. In step 2 in the process, the PCR products from all the samples are mixed, immobilized on beads, distributed into wells of the picoliter plate, and emulsion PCR amplified.

Figure 2C is a diagram depicting the pyrosequencing process from each outside adapter in each well of the picoliter plate. Each reaction reads sequence from the adapter, through the unique tags and the associated sequence of the tagged sample

Figure 2D is a diagram depicting the algorithmic sorting of the Pyrosequencing reads using the individual tag sequence and a portion of the primer sequence. (l) represents the sequence reads from sample 1, (m) represents the sequence reads from sample 2, and (n) represents the sequence reads from sample n.

Figure 2E is a diagram depicting the identification of microbial taxa by comparing the sequence reads for each sample against the 16S rRNA sequence database and then normalize abundance in each taxa with respect to the total reads in that particular sample. (o) represents the normalized species histogram derived the pyrosequencing reads obtained from sample 1, (p) represents the normalized species histogram derived the pyrosequencing reads obtained

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from sample 2, (q) represents the normalized species histogram derived the pyrosequencing reads obtained from sample n,

Figure 3 is the species distribution in (A) Controls, (B) Crohns, and (C) Ulcerative colitis samples determined by the 454 Life Science pyrosequencing process. Each bar in the histogram is the average normalized abundance of that taxa in each disease state. Each sample was run in a separate well on the picoliter plate using the 454 16 well mask.

Figure 4 is an example of the distortion of the components of a complex mixture caused by ligating the Emulsion PCR adapters onto PCR amplicons. Figure 4A shows the size distribution of PCR amplicons in sample 309 before ligation and Figure 4B shows the size distribution of sample 309 after ligation.

Figure 5 is an example of the normalized taxa abundances in duplicate samples determined by Multitag pyrosequencing after direct ligation of the emulsion PCR adapters.

Figure 6 shows all possible hexameric polynucleotide tags within which there are no dinucleotide repeats and no tag is the reverse complement of any other tag.

Figure 7 shows 96 tagged adaptor primers in which there are no dinucleotide repeats in the tags, no dinucleotide repeats at the junction of the tags and the tags are not reverse complements of one another. In each case 5 bases of the primer also can be used to identify samples. 7A and 7B show the forward primers. 7C and 7D show the reverse primers.

Glossary

The meanings ascribed to various terms and phrases as used herein are illustratively explained below.

"A" or "an" means one or more; at least one.

"About" as used herein means roughly, approximately. Should a precise numerical definition be required, "about" means +/- 25%.

"Adapter" means a polynucleotide sequence used to either attach single polynucleotide fragments to beads and/or to prime the emulsion PCR reaction and/or as a template to prime pyrosequencing reactions.

"ALH" is used herein to mean amplicon length heterogeneity.

"Amplicon" is used herein to refer to the products of an amplification reaction.

"Clonally amplified" is used herein generally to mean amplification of a single starting molecule. Typically it also refers to the clustering together of the amplification products, isolated from other amplification templates or products.

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"dsDNA" means double stranded DNA.

Dysbiosis means a shift in a the species and abundance of species in a microbial community.

"Flanking" generally is used to mean on each side, such as on the 5' and the 3' side of a region of a polynucleotide – with reference to the 5' and the 3' ends of one or the other stand of a double stranded polynucleotide. Forward and reverse primers for amplifying a region of a polynucleotide by PCR, for instance, flank the region to be amplified.

"Microbial community sample" is used herein to refer to a sample, generally of a biological nature, containing two or more different microbes. Microbial community samples include, for instance, environmental samples, as well as biological samples, such as samples for clinical analysis. The term applies as well to preparations, such as DNA preparations, derived from such samples.

"Multiplex sequencing" herein refers to sequencing two or more types or samples of polynucleotides in a single reaction or in a single reaction vessel.

"PCO" means principal coordinates analysis.

"PCA" means principal component analysis.

"Picotiter plate" means a plate having a large number of wells that hold a relatively small volume, typically more wells than a 96-well microtiter plate, and smaller volumes than those of a typical 96-well microtiter plate well.

"Primer" means a polynucleotide sequence that is used to amplify PCR products and/or to prime sequencing reactions.

"ssDNA" means single stranded DNA.

"Tag," "Tag sequence," etc. means typically a heterologous sequence, such as a polynucleotide sequence that identifies another sequence with which it is associated as being of a given type or belonging to a given group.

"Variable genetic region" as used herein means a genetic region that varies, such as between individuals of a species and between species. The phrase does not denote a specific length, but, rather is used to denote a region comprising a variation the exact length of which may vary and may differ in different contexts. As to a double stranded polynucleotide, the term includes one or the other and both stands of the region, and may be used to refer to one, the other, or to both strands, and it will generally be clear from the context which is meant. A specific example of a genetic region that varies between individuals, provided for illustration only, is a genetic region that contains an SNP (single nucleotide polymorphism) site. By

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variable genetic region in this regard is meant a region containing the SNP site. Different sequences of the SNP in this regard constitute the variants of the variable genetic region. A specific example of a variable genetic region that differs between species is the genes for 16S RNA which vary characteristically between microbes and can be used to identify microbes in mixed community samples as described in greater detail in some of the examples herein.

Description of the Invention

In certain aspects and embodiments the invention relates to multiplex sequencing analysis using tags. In various aspects and embodiments of the invention in this regard the invention provides methods for sequencing two or more samples simultaneously in a mixture with one another, wherein each sample is first linked to a sample-specific sequence tag, the tagged samples are mixed and sequenced, and the sequences from each sample then are identified by their respective sample-specific sequence tags.

Figure 1 provides a general depiction of various aspects and embodiments of the invention in this regard, and the figure is discussed by way of illustration below with reference to sequencing DNA from different samples. A plurality of samples (S_1, S_2, through S_j) is shown topmost in the Figure. Each sample is comprised of a plurality of polynucleotides ($P_{1\text{-}1}$ to $P_{1\text{-}n1}$ in S_1; $P_{2\text{-}1}$ to$P_{2\text{-}n2}$ in S2; through $P_{j\text{-}1}$ to $P_{j\text{-}nj}$). The polynucleotides in each sample are labeled separately with a tag polynucleotide sequence, all the polynucleotides in a given sample being tagged (in this illustration) with a single tag sequence, designated in the figure as T_1 for S_1, T_2 for S_2, through T_j for S_j. The individual tagged polynucleotides are denoted accordingly. The tagged polynucleotides in each sample are designated collectively, for each sample, T_1S_1, T_2S_2 through T_jS_j. The tagged polynucleotides from the samples are mixed together to form a mixture, designated M_i. The mixture is sequenced typically by a parallel sequencing method. The tag sequences are identified in the data thus obtained. The sequences are grouped by tag. The sequences from the individual samples are thereby identified.

In embodiments tags are 3 to 30, 4 to 25, 4 to 20 base long sequences. In embodiments the tags are 2, 3 , 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 nucleotides long or any combination thereof.

In embodiments there are 1 – 6, 6 – 12, 10 – 15, 10 – 20, 15 – 25, 20 – 40, 25 – 50, 25 – 75, 50 – 100, 50 – 150, 100 – 200, 100 – 250, 50 – 250, 100 – 500, 500 – 1,000, 100 –

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1,000, 500 – 5,000, 100 – 10,000, 1,000 – 25,000, 500 – 50,000, 100 – 100,000, 1 – 1,000,000 or more samples, tagged, respectively, with 1 – 6, 6 – 12, 10 – 15, 10 – 20, 15 – 25, 20 – 40, 25 – 50, 25 – 75, 50 – 100, 50 – 150, 100 – 200, 100 – 250, 50 – 250, 100 – 500, 500 – 1,000, 100 – 1,000, 500 – 5,000, 100 – 10,000, 1,000 – 25,000, 500 – 50,000, 100 – 100,000, 1 – 1,000,000 or more different tags.

In embodiments the sequences are determined without the use of gel electrophoresis. In embodiments the sequences are determined without the use of transfer of sequences from a gel onto a membrane or a filter for hybridization. In embodiments, sequences are determined by a parallel sequencing method. In embodiments the sequences are determined by pyrosequencing, sequencing by synthesis, hybridization sequencing, subtractive sequencing, pore sequencing or direct read sequencing.

In embodiments the tags are incorporated into polynucleotides in samples for sequencing by a step of ligation and/or by a step of amplification.

In embodiments the tags are comprised in primers for amplification.

In embodiments the tags are comprised in primers for PCR amplification, transcription amplification, rolling circle amplification, or amplification by Qβ replicase.

In embodiments the tags are comprised in emulsion PCR adapters and primers for amplification.

In embodiments the tags are incorporated by a step of cloning into a vector.

In embodiments the samples are microbial community samples. In embodiments the samples are clinical samples. In embodiments the samples are environmental samples. In embodiments the samples are samples for SNP analysis. In embodiments the samples are samples for genotyping. In embodiments the sequences are determined in one or more picotiter plates.

In embodiments the samples are fragmented genomic DNAs. In embodiments the samples are fragmented Bacterial genomic DNA, Archae genomic DNA, Fungal genomic DNA, Eukaryotic genomic DNA, chloroplast DNA, and/or mitochondrial DNA. In embodiments the samples are cDNAs. In embodiments the samples are Eukaryotic cDNA, Bacterial cDNA, Archae cDNA, and/or Fungal cDNA. In embodiments the tags are incorporated by a step of ligation and/or by a step of amplification.

In embodiments the samples are for any one or more of detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like.

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In embodiments the samples are for analyzing the composition, diversity, stability, dynamics, and/or changes in agricultural, food, biosecurity, veterinary, clinical, ecological, zoological, oceanological, and/or any other sample comprising one or more polynucleotides.

In embodiments the sequences are determined in wells of a titer plate. In embodiments the sequences are determined in one or more picotiter plates having a mask. In embodiments the sequences are determined in one more picotiter plates having a mask, wherein the mask defines 2, 4, 8, 16, 32, 64 or more compartments.

By way of illustration to a 454 picotiter plate, in embodiments there are about 120,000 templates/plate and the read length averages about 250 bases per template. In embodiments relating thereto there are 10 tags of 4 bases per 1/16 plate, 160 tags total, an average of about 750 templates per tag (and per sample), and about 187,500 bases sequenced per tag (and per sample).

In embodiments there are about 260,000 templates/plate and the read length averages about 250 bases per template. In embodiments relating thereto, there are 12 tags of 4 bases per 1/8 plate, 96 samples total, an average of about 2,708 templates per tag (and per sample) and about 677,083 bases of sequence per tag (and per sample).

In embodiments there are about 400,000 templates/plate and the read length averages about 250 bases per template. In embodiments relating thereto, there are 96 tags of 6 bases for 96 samples per plate, about 4,166 templates per tag (and per sample) and about 1,041,666 bases of sequence per tag (and per sample).

In embodiments the tags are 10 base long sequences, there are 192 different tags, and the samples are analyzed in microtiter plate format.

In embodiments the invention provides algorithms for deconvolving, from a mixture of sequences from two or more samples, the sequences of the samples in the mixture by identifying sample-specific tags in the sequences, grouping the sequences by the tags thus identified, thereby grouping together the sequence from each of said samples, apart from one another.

In embodiments the invention provides algorithms for deconvolving, from a mixture of sequences from two or more samples, the sequences of the samples in the mixture by identifying sample-specific tags in sequences, as follows:

1. Read all sequence reads into an array;
2. Search the beginning of each sequence read and identify the tag;
3. Build an associative array linking tag with sequence read;

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4. Sort the keys for the associate array;
5. Associate each key with the corresponding sample;
6. Pool all sequence reads for each sample;
7. Analyze each sample separately.
8. Normalize the abundance of each component within each samples with respect to the total reads within that sample.

In embodiments the algorithm can be implemented in any programming language. In embodiments the algorithm is implemented in C, C++, JAVA, Fortran, or Basic. In embodiments the algorithm is implemented as a PERL script.

In embodiments the invention provides kits for multiplex sequencing as described herein, comprising a set of primers and/or adapters, wherein each primer and/or adapter in said set comprises a tag sequence, a primer sequence and/or an emulsion PCR adapter. In embodiments the primers and/or adapters further comprise a moiety for immobilization. In embodiments the primers and/or adapters comprise biotin. In embodiments the primers and/or adapters in the set comprise all tag sequences defined by 2, 3, 4, 5, 6, 7, or 8 base polynucleotide sequences, wherein said primers and/or adapters comprising different tag sequences are disposed in containers separate from one another. In embodiments there are 1-5, 3-10, 5-15, 10-25, 20-50, 25-75, 50-100, 50-150, 100-200, 150-500, 250-750, 100-1000, or more different tag sequences disposed separately from one another, so as to be useful for uniquely tagging said number of different samples. In embodiments the primers and/or adapters are suitable for use as 454 Life Sciences amplification adapters and/or primers. In embodiments the primers and/or adapters further comprise any one or more of a primer sequence for any one or more of a 16S rRNA sequence, an 18S rRNA sequence, an ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, a genetic disease sequence, and/or any other sequence for amplification or analysis.

Examples

The present invention is additionally described by way of the following illustrative, non-limiting examples.

EXAMPLE 1: Sequencing Using the 454 Pyrosequencing System

454 Life Sciences, a subsidiary of Roche Diagnostics, provides a device for pyrosequencing approximately 100,000,000 bases of about 400,000 different templates in a single run on a single picotiter plate. The company also provides masks that allows for the

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processing 2, 4, 8, or 16 different samples on one plate. At maximum capacity using the masked plate, the system provides about 1 million bases of sequence data on about 4,000 templates for each of 16 samples.

The general process of sequencing using the 454 system is generally as follows: isolate DNA; optionally fragment the DNA; optionally render the DNA double stranded; ligate the DNA to adaptors; separate the strands of the dsDNA, bind the ssDNA to beads under conditions that result in a preponderance of beads that have either no DNA molecule bound to them or a single molecule of DNA bound to them; capture the beads in individual droplets of an emulsion of a PCR reaction mix in oil; carry out a PCR reaction on the emulsion-encapsulated bead-DNAs (whereby amplification products are captured on the beads); distribute the amplification products into picoliter wells so that there is either no bead in a well or one bead; and carry out pyrosequencing on all the beads in all the wells in parallel.

EXAMPLE 2: Multiplex Pyrosequencing using 96 tagged adapter-PCR primers.

454 Life Sciences, a subsidiary of Roche Diagnostics, provides a device for pyrosequencing approximately 100,000,000 bases of sequence for about 400,000 different templates in a single run on a single picotiter plate. At maximum capacity using the plate, the system provides about 10 million bases of sequence data for each of about 4,000 templates for each of 96 multitagged samples. In this example the 96 tags are 6 bases in length and are used along with 6 bases of the forward or reverse primer to identify the reads that belong with each of the 96 individual samples (see Figure 2).

EXAMPLE 3: Multtag Pyrosequence Analysis of Microbial Community Samples

Various aspects and embodiments of the invention herein described are illustrated by way of the following general example relating to "ecogenomic" analysis of microbial diversity in biological samples.

The ability to quantify the number and kinds of microorganisms within a community is fundamental to the understanding of the structure and function of an ecosystem, as discussed in, for instance, Pace 1997 and Theron and Cloete 2000. Traditionally, the analysis of microbial communities has been conducted using microbiological techniques, but these techniques are limited. For instance they are not useful for the many organisms that cannot be cultivated (Ritchie, Schutter et al. 2000; Spring, Schulze et al. 2000). Even for those

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organisms that can be cultured, these techniques provide little information with which to identify individual microbes or characterize their physiological traits. (Morris, Bardin et al. 2002).

Recent advances in molecular techniques have overcome some of these disadvantages, and have enabled the identification of many more taxa in microbial communities than traditional microbial techniques. These advances have provided considerable insight into the expression of key functions in species in microbial communities. (Pace 1997; Suzuki 1998; Amann 2000; Frischer, Danforth et al. 2000; Ritchie, Schutter et al. 2000; Spring, Schulze et al. 2000). Among these molecular techniques are Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Temporal Temperature Gradient Gel Electrophoresis (TTGE), Terminal-Restriction Fragment Length Polymorphism (T-RFLP), Single Strand Conformation Polymorphism (SSCP), and Length Heterogeneity PCR (LH-PCR) (Frischer, Danforth et al. 2000; Theron and Cloete 2000; Mills, Fitzgerald et al. 2003; Seviour, Mino et al. 2003; Klaper and Thomas 2004).

Among these, LH-PCR is probably the best technique for fingerprinting. It is inexpensive, fast, and can be used routinely to screen several hundred samples a day. It is useful as a routine survey tool that can be used to monitor the dynamics of natural soil microbial communities, and to quickly identify samples of interest by PCO analysis. LH-PCR has been used to extensively assess natural variation in bacterial communities by profiling the amplified variable regions of 16S rRNA genes in mixed microbial population samples, using PAGE. (See Mills 2000; Litchfield and Gillevet 2002; Lydell, Dowell et al. 2004). The LH-PCR products of the individual species in the population give rise to distinct bands in the gels. The "peak area" of each band is proportional to the abundance of the species in the community. LH-PCR of 16S rRNA variable regions has been used quite successfully to estimate species diversity in bacterioplankton communities, in particular. (See Suzuki, Rappe et al. 1998; Ritchie, Schutter et al. 2000).

Community functionality cannot be determined directly from 16S rRNA clone data, however, it must be inferred from the data by phylogenetic analysis. Furthermore, LH-PCR and other fingerprinting technologies, while powerful tools for monitoring population dynamics, cannot identify individual species in a community. For this, fingerprinting investigations must be followed up by library construction, cloning, sequencing, and phylogenetic analysis. (Fitzgerald 1999; McCraig 1999; Spring, Schulze et al. 2000; Theron and Cloete 2000; Litchfield and Gillevet 2002; Bowman and McCuaig 2003; Kang and Mills

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2004; Eckburg, Bik et al. 2005). Identifying species of a fingerprinting study, thus, is a considerable undertaking that is inconvenient, time-consuming, expensive and subject to technical limitations.

Grouping samples can, to some extent, reduce the cost, time, and expense of such analyses. For instance, PCO analysis of LH-PCR data can be used to group samples with similar profiles for batch cloning and sequencing. Combining the samples this way reduces the time, expense, and work involved in analyzing the samples. Sequencing of at least 300 random clones is required to identify the bacterial components of the pooled sample down to 1% of the total bacterial populations in typical samples. This level of resolution is similar to that of ALH fingerprinting. Originally a novel approach, pooling similar samples prior to cloning and sequencing has proven to be robust and effective.

In classic community studies in the literature (Eckburg, Bik et al. 2005), environmental samples are assayed independently. Then the clone sequence data from specific classes/groups are statistically analyzed usually using some sort of averaging metric. Analyses of this type can be extremely costly, especially if the clone libraries are exhaustively analyzed, something that typically involves sequencing thousands of clones. Moreover, for the "averaging" process to be valid, as required for comparing the mixed populations, the samples must be pooled in equal proportions. While simple in principle, in reality, it is difficult to accomplish and, even if accomplished, impossible to verify. A new technique, based on pyrosequencing, offers advantages that overcome a variety of these drawbacks of the fingerprinting technologies mentioned above. The method is implemented on an instrument sold by 454 Life Sciences, Inc., a subsidiary of Curagen Sciences, Inc., using reagents provided by the same company. In addition, 454 Life Sciences provides a custom service for pyrosequencing.

In this technology, individual DNA molecules are amplified on beads by PCR in individual droplets in an oil-in-water emulsion. Beads then are deposited individually in wells of a picotiter plate. The sequences of all the DNAs in the wells are determined in parallel by pyrosequencing. (See Venter, Levy et al. 2003; Margulies, Egholm et al. 2005; Poinar, Schwarz et al. 2006). In a typical run, there are about 200,000 templates per plate, an average read length of about 100 bases from each template, and a single-plate run generates about 20 million bases of sequence in a single four hour run.

Although the technology greatly increases throughput over previous methods, it is expensive. In particular, the cost per plate is too high for it to be economically practical to

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carry out many analyses. To decrease cost, masks can be used that divide a plate into 16 independent sample zones, so that one plate can be used to process 16 different samples, either at the same time or independently. Each 1/16 zone provides about 1,000,000 bases of sequence data from about 10,000 different templates. While this reduces the cost per sample, the expenses associated with using this technology remain undesirably high.

Various aspects and embodiments of the present invention can be used to further reduce the cost per sample of this technology (as well as other techniques, as described elsewhere herein). The use of multitagging techniques (referred to as, among other things, "Multitag Process") to the genomic analysis of bacterial populations in according with certain aspects and embodiments of the invention, notably high coverage sequencing of bacterial communities, is referred to herein as "Multitag Ecogenomics" and also as "Multitag Ecogenomic Analysis."

(Several publications use the term "Multiplex Pyrosequencing" (Pourmand, Elahi et al. 2002) to refer to generating a composite signal from multiple targets that is read as a signature for a specific sample. The term is not used to refer to tag-based multiplexing in which sequences from different samples in a mixture are determined and then deconvolved from the mixed sequencing data using sample-specific tags incorporated during amplification reactions.)

As described below the Multitag Process in a relatively simple series of steps accomplishes everything that otherwise would require not only community fingerprinting analysis, but also all of the cloning and sequencing processes previously required for high coverage Ecogenomic Analysis using conventional techniques.

By way of illustration, the following example describes the use of Multitag Ecogenomic Analysis of variable regions of common genes using tagged universal primers for high coverage analysis of several microbial community samples all at the same time. The analysis is carried out much as described in general above, and further elaborated on in detail below.

Briefly, short tags are added to the 5' ends of the forward and reverse PCR primers normally used for community analysis. These tags can be placed between the Emulsion PCR adapters and the PCT primers (see Figure 2). A different tag is attached to the primers for each of the samples to be combined. For instance primers that span a variable region of 16S rRNA genes may be used for analysis of bacterial and archael communities. 16S rRNA-specific primers with 4 base tags are set out in the Table 1 below. Likewise primers that span

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a variable region of an ITS gene may be used for analysis of fungal communities. It will be appreciated that the choice of these specific primers is not exclusive, and that a wide variety of other primers suitable to other target regions for amplification may be employed in much the same manner as described herein for the 16S and ITS genes. Thus, any gene of interest can be used that provides conserved primer sites across a community, and sufficient variation in the region between the primers for the desired resolution of individual species. Thus, for example, genes specific to functional pathways such as anaerobic methane oxidation, or sulphur reduction can serve as targets for the amplification reaction, as well as 16S rRNA sequences.

Table 1

Name	Tag	Forward Shared Sequence
		AGCTAGAGTTTGATCMTGGCTCAG
L27FA	AGCT	AGCTAGAGTTTGATCMTGGCTCAG
L27FB	AGTC	AGTCAGAGTTTGATCMTGGCTCAG
L27FC	GATC	GATCAGAGTTTGATCMTGGCTCAG
L27FD	GACT	GACTAGAGTTTGATCMTGGCTCAG
L27FE	CTGC	CTGCAGAGTTTGATCMTGGCTCAG
L27FF	CTAG	CTAGAGAGTTTGATCMTGGCTCAG
L27FG	ATGC	ATGCAGAGTTTGATCMTGGCTCAG
L27FH	ATAG	ATAGAGAGTTTGATCMTGGCTCAG
L27FM	ATCT	ATCTAGAGTTTGATCMTGGCTCAG
L27FO	ATAT	ATATAGAGTTTGATCMTGGCTCAG
Name	**Tag**	**Reverse Shared Sequence**
		AGCTGCTGCCTCCCGTAGGAGT
355RA	AGCT	AGCTGCTGCCTCCCGTAGGAGT
355RB	AGTC	AGTCGCTGCCTCCCGTAGGAGT
355RC	GATC	GATCGCTGCCTCCCGTAGGAGT
355RD	GACT	GACTGCTGCCTCCCGTAGGAGT
355RE	CTGC	CTGCGCTGCCTCCCGTAGGAGT
355RF	CTAT	CTATGCTGCCTCCCGTAGGAGT
355RG	ATGC	ATGCGCTGCCTCCCGTAGGAGT
355RH	ATAT	ATATGCTGCCTCCCGTAGGAGT
355RM	ATCT	ATCTGCTGCCTCCCGTAGGAGT
355RO	ATAC	ATACGCTGCCTCCCGTAGGAGT

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Table 1 shows a 16S rRNA-specific primer with a variety of 4 base tag sequences attached. As described herein such primers are useful for amplifying 16S rRNAs in several samples that can then be sequenced together. The 16S rRNA in each sample is amplified using a different tag, but the same 16S primer sequence. The amplified rRNA sequences from the samples are combined and sequenced together. The rRNA sequences from the different samples then are identified and sorted out by their 4 base tag sequence plus the first 4 bases of each primer. It is to be appreciated that the sequences downstream of the shared 16S primer sequence will differ among the samples, as well as the tag sequence.

In each case, the samples are individually amplified. The resulting amplicons comprise the primer sequences including the tags. Since unique tags are used for each sample, the tags in the amplicons from each sample will be different. The amplified DNAs are then pooled and sequenced by pyrosequencing as described above. The sequence data from a run is analyzed, in part, by grouping together all the sequences having the same tag. In this way, the sequences from each sample are demultiplexed from the sequencing data obtained from the mixture.

The working of the invention in this regard is illustrated by the following simulation, carried out using conventionally obtained population data from cold seep samples. The algorithm for sequence analysis uses a PERL script to extract the first 100 bases of sequence. It then analyzes all the 100 bases sequences using a custom RDP PERL script. The script works as follows:

1. Read all sequence reads into an associate array (Hash 1);
2. Extract 100 base subsequences from the beginning of each sequence read;
3. Create an associate array (Hash 2) of the sequences;
4. Perform a Blast search of the RDP database with Hash 1;
5. Perform a Blast search of the RDP database with Hash 2;
6. Compare the identifications for the original sequence (Hash 1) and the subsequence (Hash 2);
7. Compile a list of similar identifications for Hash 1 and Hash 2;
8. Compile a list of different identifications for Hash 1 and Hash 2;
9. Calculate the percentage of similar identifications.

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As shown below, there is virtually no difference at the class level in the microbial diversity generated by the sequencing simulation and that derived directly from the 16S rRNA sequences in the data base.

Table 2

RDP Class	First 100mer	16S rRNA
ALPHA_SUBDIVISION	3.6%	3.6%
ANAEROBIC_HALOPHILES	3.6%	3.6%
BACILLUS-LACTOBACILLUS-STREPTOCOCCUS_SUBDIVISION	3.6%	3.6%
BACTEROIDES_AND_CYTOPHAGA	7.1%	7.1%
CHLOROFLEXUS_SUBDIVISION	3.6%	3.6%
CY.AURANTIACA_GROUP	7.1%	7.1%
CYANOBACTERIA	7.1%	7.1%
DELTA_SUBDIVISION	14.3%	14.3%
ENVIRONMENTAL_CLONE_WCHB1-41_SUBGROUP	7.1%	7.1%
FLX.LITORALIS_GROUP	3.6%	3.6%
GAMMA_SUBDIVISION	10.7%	10.7%
HIGH_G+C_BACTERIA	7.1%	7.1%
LEPTOSPIRILLUM_GROUP	3.6%	3.6%
MYCOPLASMA_AND_RELATIVES	3.6%	3.6%
PIRELLULA_GROUP	3.6%	3.6%
SPHINGOBACTERIUM_GROUP	3.6%	3.6%
SPIROCHAETA-TREPONEMA-BORRELIA_SUBDIVISION	3.6%	3.6%
THERMOANAEROBACTER_AND_RELATIVES	3.6%	3.6%

EXAMPLE 3: Multitag Pyrosequence Analysis of Dysbiosis in IBD

Inflammatory Bowel Diseases (IBD or IBDs), namely ulcerative colitis (UC) and Crohn's disease (CD), are chronic, lifelong, relapsing illnesses, affecting close to 1 million Americans and costing approximately $2 billion per year to the US healthcare system. IBDs are of unknown cause, have no cure, and are increasing in incidence. The natural course of these diseases is characterized by periods of quiescence (inactive disease) interspersed with flare-ups (active disease). It is now widely accepted that flare-ups of IBD are due to a dysregulated inflammatory reaction to abnormal intestinal microflora dysbiosis), however.

Specific changes in the microflora of IBD patients that might cause these diseases remain unknown. Narrow searches for a single pathogen that causes IBD have been

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unsuccessful. (See Guarner and Malagelada 2003). Studies of small bacterial groups have yielded ambiguous results. (See Schultz and Sartor 2000). Only recently have studies of large sets of bacterial flora been attempted. (See Eckburg, Bik, et al. 2005),. Improving our knowledge about GI tract microflora has the potential to revolutionize IBD treatment. Development of real-time methods to study microfloral changes may lead to diagnostic tools to predict flare-ups, and to targeted, safe treatments for IBD.

The key requirement to understanding dysbiosis in polymicrobial diseases is for a method to interrogate widely the microflora in numerous control and disease samples to identify dynamic trends in species composition associated with health and disease progression. In classic community studies (Eckburg, Bik, et al. 2005) environmental samples are assayed independently and then the clone sequence data from specific classes/groups are statistically analyzed usually using some sort of averaging metric. This can be extremely costly, especially if the clone libraries are exhaustively analyzed (i.e., 10,000 clones per sample).

To improve throughput and reduce cost, Amplicon Length Heterogeneity PCR (ALH-PCR) has been used to study the gut microflora. It offers a rapid way of screening complex microbial communities, allowing for easy fingerprinting of microfloral changes. The LH-PCR fingerprinting is inexpensive and fast, with the ability to screen several hundred samples a day. It can be used as a routine survey tool to monitor the dynamics of natural soil microbial communities or to quickly identify samples of interest using PCO analysis. PCO analysis has been used to group samples with similar profiles, allowing them to be pooled for cloning and sequencing. This greatly reduces the cost of analyzing multiple samples, particularly when the analysis requires sequencing at least 300 random clones to identify bacterial components of the sample down to 1% representation in the total population (which is the resolution limit for ALH fingerprinting). Pooling similar samples before cloning and sequencing has proved to be quite robust. However, equal amounts of the PCR product from each sample must be pooled or the results will be skewed.

Multitag Pyrosequencing is a novel pyrosequencing technology that allows many community samples to be sequenced together at high coverage without the necessity for fingerprinting, cloning, or the purification and separation techniques required by conventional methods for analyzing microbial communities, as described herein above. Multitag sequencing is more efficient, faster, and less costly than other methods.

By way of illustration, Multitag Pyrosequencing can be carried out using a set of specific tags on the end of standard universal small ribosomal sub-unit ("SSU") rRNA

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primers (See Table 1). A different set of the tagged primers is used to amplify the SSU rRNA in each different environmental sample (Figure 2-Step 1). The PCR amplicons from all the samples are pooled. Emulsion PCR is performed and the amplicons arising from each molecule are captured on their respective beads. Following amplification, the beads are distributed into the wells of a picoliter plate (Figure 2-Step 2). The sequences, including the tagged sequences, of the amplicons on each bead are determined by pyrosequencing (Figure 2-Step 3). A PERL script or other suitable program is used to sort the sequence information using the tags and primer sequence as a key. Sequences with the same tags are identified thereby with their respective sample. The bacteria species in each sample then are identified by matching the SSU rRNA sequences to entries in the database of the Ribosomal Database Project (either RDP 8.1 or RDP 9.0). The normalized frequency with which a bacteria is thus identified in a given sample is indicative of its relative representation in the microbial community. Histograms based on these frequency determinations can be used for the non-parametric analysis of dysbiotic shifts involved in disease states.

For example, Figure 3 depicts the results of such an experiment in which six Control, ten Crohns, and eight Ulcerative colitis mucosal samples were analyzed by Multitag Pyrosequencing. E ach of the segments in the stacked histogram bars represents the normalized abundance of that specific taxa in a specific sample. In this experiment, identification of the taxa was performed using BLAST analysis of the RDP 8.1 database. It can be seen that some taxa (i.e. Bacillus fragilis subgroup and Rumanococcus gnavus subgroup) are present in the same abundance in both control and disease states. Other taxa, such as Clostridium leptum are more dominant in Ulcerative colitis, while others (i.e. the Glocothece glococapsa subgroup) are indicators of dysbiosis in the disease state.

However, the standard 454 Life Science process using a ligation step to link the emulsion PCR adapters to the PCR amplicons and produces numerous artifacts in the quantitation of the abundances of each taxa in the samples. In the results displayed in Figure 3, we algorithmically removed chimeras, reverse reads and truncated products and filtered the data to remove all taxa that were represented by less than 5% abundance. Only then were we able to see a correlation with disease state and specific microbial taxa.

EXAMPLE 4: Distortion of the distribution of components of a microbial community by directly ligating Emulsion PCR adapters onto PCR amplicons.

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In one experiment we used tagged PCR primers to amplify the components in duplicate microbial community samples, ligated the Emulsion PCR adapters to these samples, and then subjected these samples to separate pyrosequencing runs. The amplicons are routinely run on an Agilent Bioanalyzer system before and after ligation to quantitate the mixture before emulsion PCR. Figure 4 depicts a sample run on the Bioanalyzer before and after direct ligation and clearly shows that the ligation step has drastically altered the distribution of the amplicons.

Additionally, we compared the normalized abundances of the component taxa identified by the multitag process after direct ligation of the Emulsion PCR adapters. In this experiment, identification of the taxa was performed using a Bayesian analysis of the RDP 9.0 database. We can se in Figure 5 that abundances of the forward and reverse primers for various taxa are different within a sample and between duplicate samples. In several cases, we are missing entire families in the comparison between duplicates. Table 3 summarizes the differences between the forward primers and the reverse primers of the duplicate samples and it is clearly stochastic with no predictable pattern. We hypothesize that this differential ligation efficiency could be due to a number of factors such as internal structure in the amplicons or biases in the terminal nucleotide of either the adapter or amplicon.

Table 3 Duplicate Sample Analysis

RDP 9.0 FAMILY	FORWARD PRIMERS RATIOS	REVERSE PRIMER RATIOS
Acidaminococcaceae	544.6%	195.0%
Actinomycetales	144.0%	116.5%
Bacteroidaceae	119.9%	124.5%
Clostridiaceae	97.5%	99.4%
Comamonadaceae	198.0%	
Coriobacteriales	181.5%	141.5%
Enterobacteriaceae	4.2%	
Eubacteriaceae	88.0%	87.5%
Flavobacteriaceae	34.9%	
Incertae sedis 9	106.4%	143.0%
Lachnospiraceae	176.8%	113.1%
Peptococcaceae		91.0%
Peptostreptococcaceae	94.7%	115.4%
Porphyromonadaceae	99.0%	97.3%
Prevotellaceae	264.0%	88.1%
Rikenellaceae	212.2%	106.1%
Streptococcaceae	74.3%	60.7%

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What is claimed is:

1. A multiplex method for the quantitative determination of polynucleotides in two or more samples, comprising:

hybridizing a first primer to polynucleotides in a first sample, said first primer comprising a first tag sequence and a first probe sequence specific for a first target sequence, wherein said first target sequence is 3' to a variable genetic region;

elongating primer templates formed thereby to form a first population of tagged polynucleotides comprising: said first primer including said first tag sequence; and sequences of said variable genetic region;

hybridizing a second primer to polynucleotides in a second sample, said second primer comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein said second target sequence is 3' to the same variable genetic region as said first target sequence, wherein further said second probe sequence may be the same as or different from said first probe sequence;

elongating primer templates formed thereby to form a second population of tagged polynucleotides comprising: said second primer including said second tag sequence; and sequences of said variable genetic region;

mixing said first and second populations together;

determining sequences of polynucleotides comprising tag sequences and the sequences of the variable genetic element in said mixture;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

from this information determining the number of time one or more given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples;

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

2. A multiplex method for the quantitative determination of polynucleotides in two or more samples, comprising:

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hybridizing a first primer pair to polynucleotides in a first sample, the first primer of said first primer pair comprising a first tag sequence and a first probe sequences specific for a first target sequence and the second primer of said first primer pair comprising a second tag sequence and a second probe sequence specific for a second target sequence, wherein the first and the second probe sequences flank and hybridize to opposite strands of a variable genetic region;

elongating primer templates formed thereby to from a first population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said first primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said first primer pair or (b) a sequence complementary to the sequence of said first primer of said first primer pair, a sequence of said variable genetic region and the sequence of said second primer of said first primer pair;

hybridizing a second primer pair to polynucleotides in a second sample, the first primer of said second primer pair comprising a third tag sequence and said first probe sequences specific for said first target sequence and the second primer of said second primer pair comprising a fourth tag sequence and said second probe sequence specific for said second target sequence;

elongating primer templates formed thereby to from a second population of tagged polynucleotides, each of said polynucleotides comprising: (a) the sequence of said first primer of said second primer pair, a sequence of said variable genetic region, and a sequence complementary to the sequence of said second primer of said second primer pair or (b) a sequence complementary to the sequence of said first primer of said second primer pair, a sequence of said variable genetic region and the sequence of said second primer of said second primer pair;

mixing said first and second populations together;

determining sequences of polynucleotides in said mixture, comprising the tag sequences and the variable genetic element;

from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred;

from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element;

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from this information determining the number of times given variants occur in each sample, and

from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples.

wherein said sequences are determined without Southern blot transfer and/or without size-separating primer extension products and/or without electrophoresis.

3. A method according to claim 2, wherein the number of said polynucleotides in said first sample is any of 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500, 5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more, and the number of said polynucleotides in said second sample is any of 5, 10, 25, 50, 100, 150, 200, 250, 500, 1,000, 1,500, 2,000, 2,500, 5,000, 7,500, 10,000, 12,500, 15,000, 17,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 75,000, 100,000, 150,000, 200,000, 250,000, 500,000, 1,000,000 or more.

4. A method according to claim 2, wherein the number of said samples and of said different tags therefor is 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 500, 1,000, 2,500, 5,000, 10,000 or more.

5. A method according to claim 2, wherein the tags are nucleotide sequences that are 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 nucleotides long or any combination thereof.

6. A method according to claim 2, wherein said tags are comprised in primers for amplification and are incorporated into said polynucleotides by amplification using said primers.

7. A method according to claim 6, wherein the primer for amplification comprises a sequence for PCR amplification, linear amplification, transcriptional amplification, rolling circle replication, or QB replication.

8. A method according to claim 7, wherein the primer for amplification comprises a sequence for PCR amplification.

9. A method according to claim 2, wherein each of said polynucleotides is disposed individually on a bead isolated from other polynucleotides.

10. A method according to claim 2, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, and the amplification products thereof also are disposed on said bead.

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11. A method according to claim 2, wherein each of said polynucleotides is disposed individually on a bead isolated from other said polynucleotides, is amplified while disposed therein, the amplification products thereof also are disposed on said bead, and each said bead is disposed individually in a well isolated from other said beads.

12. A method according to claim 2, wherein the sequences are determined by pyrosequencing.

13. A method according to claim 2, wherein said samples are biological samples, each comprising one or more species.

14. A method according to claim 2, wherein variants of the variable genetic region are specific to particular organisms.

15. A method according to claim 2, wherein said variable genetic region comprises a variable 16S rRNA sequence, a variable 18S rRNA sequence, a variable rRNA ITS sequence, a mitochondrial sequence, a microsatellite sequence, a metabolic enzyme sequence, and/or a genetic disease sequence.

16. A method according to claim 2, wherein the samples are microbial community samples.

17. A method according to claim 2, wherein the samples are microbial community samples for clinical analysis of a patient.

18. A method according to claim 2, wherein the samples are microbial community environmental samples.

19. A method according to claim 2, wherein the samples are microbial community soil samples.

20. A method according to claim 2, wherein the samples are microbial community water samples.

21. A method according to claim 2, wherein the samples are samples for SNP analysis.

22. A method according to claim 2, wherein the samples are samples for genotyping.

23. A kit comprising a plurality of two or more primers, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primers the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

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(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence and the target sequence is located 3' to a variable genetic region, and

(D) each of said primers is disposed separately from the others in containers in said kit.

24. A kit according to claim 23, wherein each of said primers further comprises a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, and the priming sequence is the same in all of said primers, said kit further comprising a primer complimentary to and effective for polymerization from said priming sequence.

25. A kit comprising a plurality of two or more primers pairs, each primer in said plurality comprising a tag sequence and a probe sequence specific to a target sequence, wherein:

(A) in each of said primer the probe sequence is 3' to the tag sequence, but not necessarily adjacent thereto;

(B) in each of said primers: the tag sequence is different from the tag sequence of the other in the plurality; the tag sequence is not the complementary sequence to any other tag sequence in the plurality; the tag sequence does not contain any homodinucleotide sequences; the junction sequences between the tag sequence and the adjacent parts of the primer, if any, is not a homodinucleotide sequence;

(C) in each of said primers the probe sequence is complementary to the target sequence,

(D) in each primer pair the probe sequences are specific to target sequences that flank a variable genetic region;

(E) each of said primers is disposed separately from the others in said kit.

26. A kit according to claim 25, wherein the primers further comprise a priming sequence 5' to the tag sequence but not necessarily adjacent thereto, the priming sequence either is the same in all the primers, or one member of each pair has the same first priming sequence and the second member of each pair has the same second priming sequence, said kit further comprising disposed separately from one another in one or more containers one or

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more primers complementary to and effective for elongation from said priming sequence or sequences.

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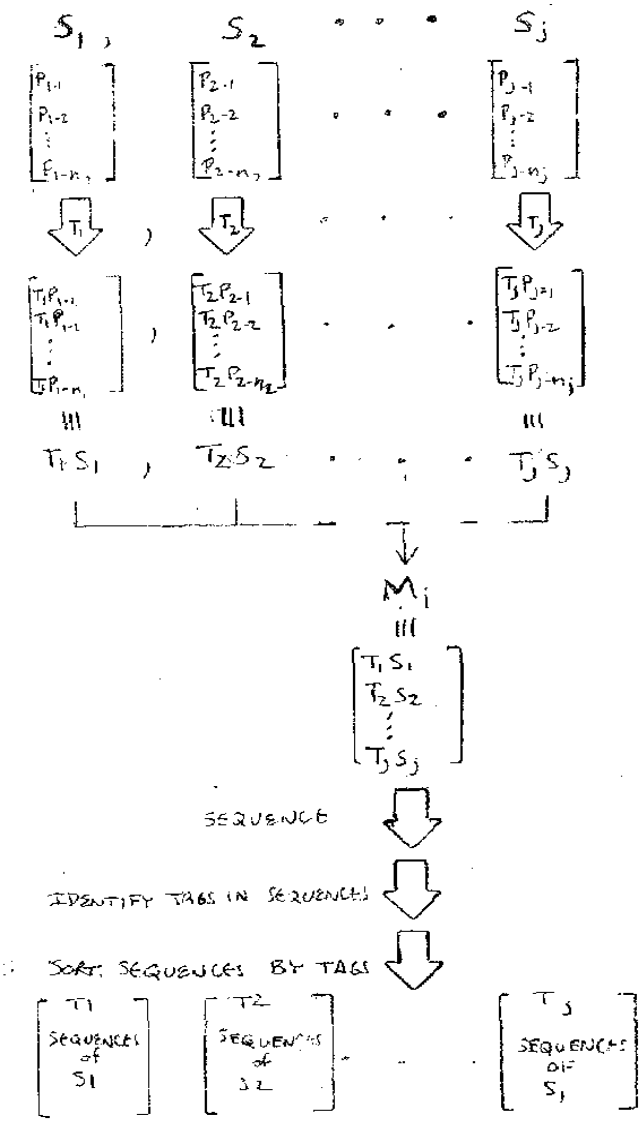

SEQUENCE
IDENTIFY TAGS IN SEQUENCES
SORT SEQUENCES BY TAGS
SEQUENCES of S1
SEQUENCES of S2
SEQUENCES of Sj

Figure 1

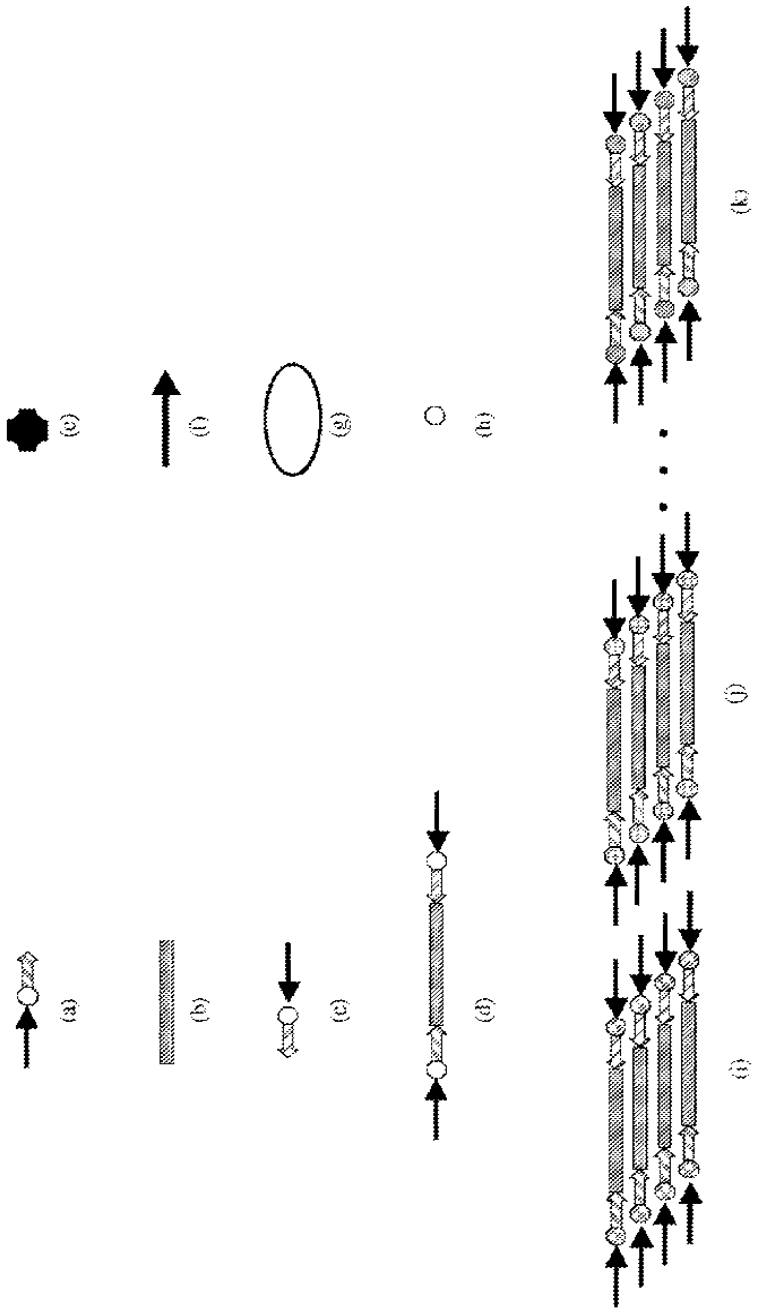


Figure 2A

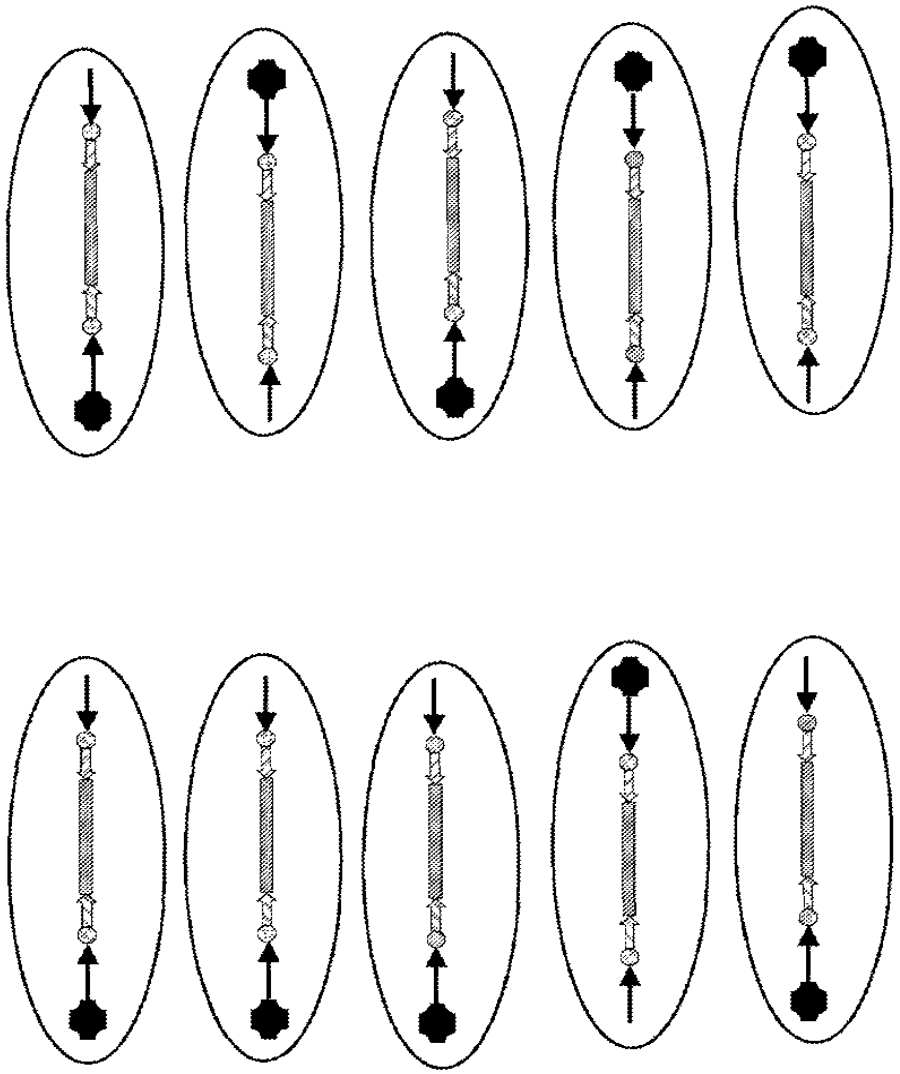


Figure 2 B

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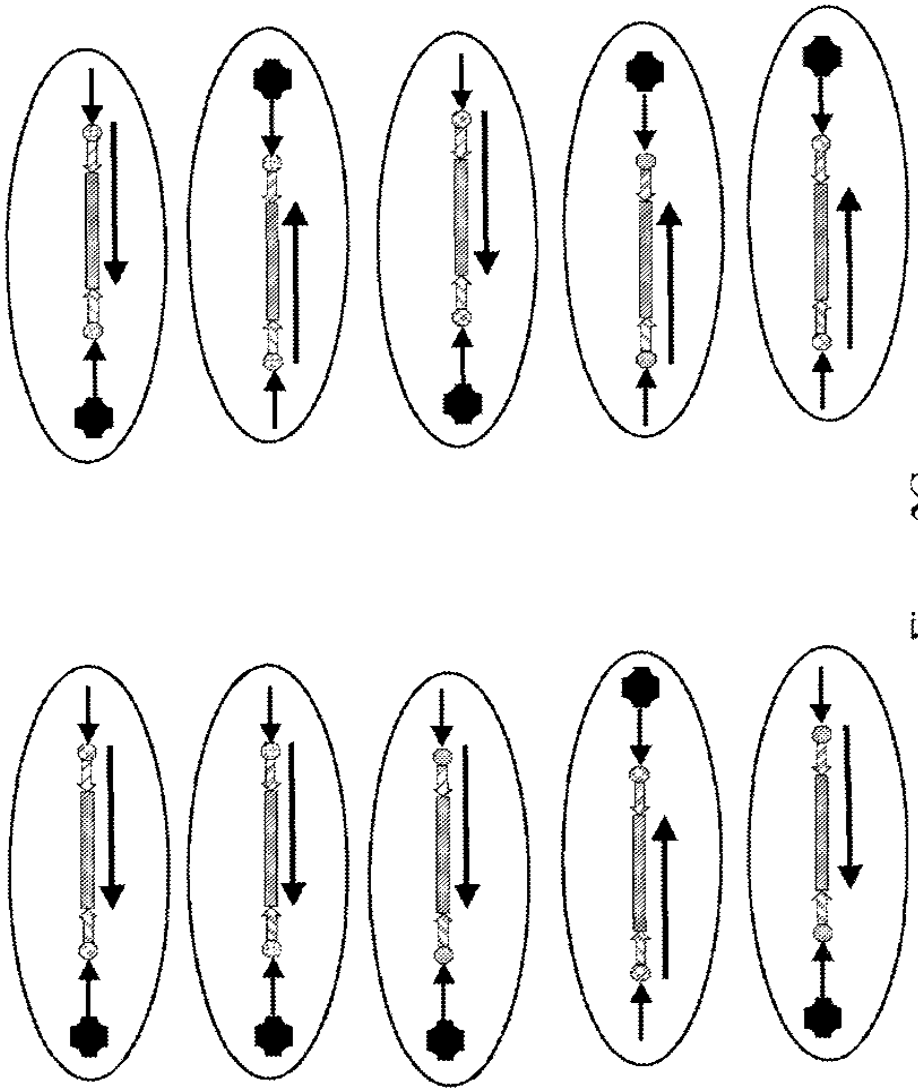


Figure 2C

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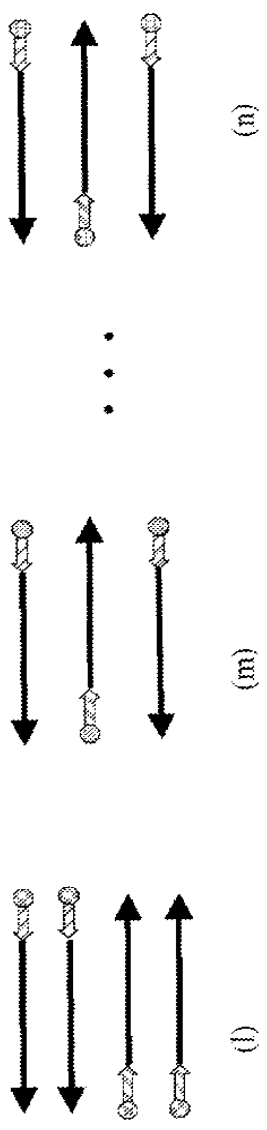


Figure 2D

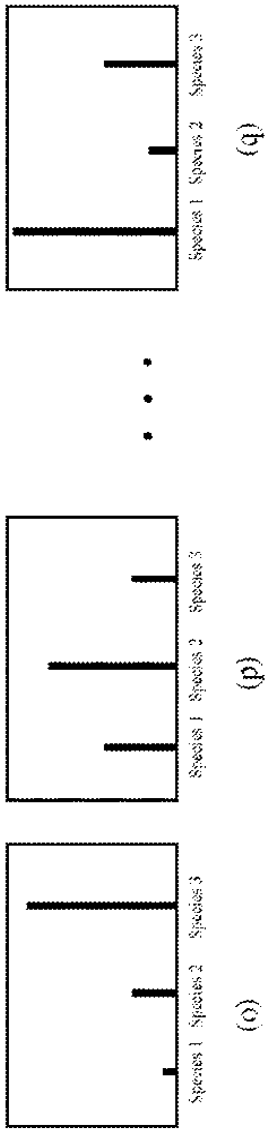


Figure 2E

FIGURE 3

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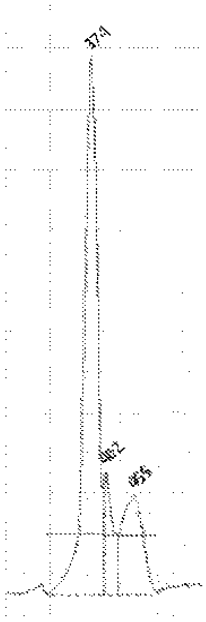


Figure 4A

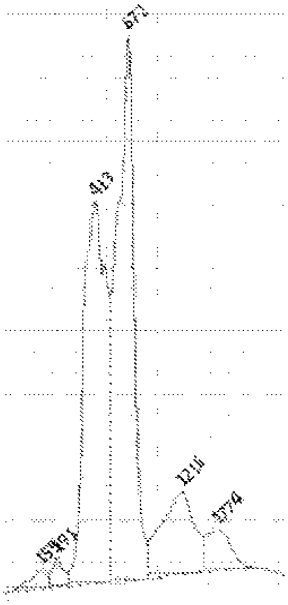


Figure 4B

FAMILY	Forward Primer	Reverse Primer	RATIO	Forward Primer	Reverse Primer	RATIO
	ALD309FO	ALD309RO	Forward/Reverse	ALD309FP	ALD309RP	Forward/Reverse
Acidaminococcaceae	0.8%	0.9%	**88.5%**	0.2%	0.5%	**31.7%**
Actinomycetales	2.4%	2.0%	**120.7%**	1.7%	1.7%	**97.6%**
Bacteroidaceae	58.4%	62.8%	**93.1%**	48.7%	50.5%	**96.6%**
Brucellaceae				7.5%	3.1%	**239.1%**
Burkholderiaceae				0.4%	0.2%	**184.9%**
Clostridiaceae	14.5%	9.6%	**150.7%**	14.9%	9.7%	**153.7%**
Comamonadaceae	0.3%			0.2%	0.3%	**55.5%**
Coriobacteriales	0.8%	0.9%	**94.8%**	0.5%	0.6%	**73.9%**
Enterobacteriaceae	0.2%			3.6%	2.9%	**124.1%**
Eubacteriaceae	1.8%	1.6%	**115.9%**	2.1%	1.8%	**115.2%**
Flavobacteriaceae	0.5%			1.3%	2.1%	**62.9%**
Incertae sedis 9	3.3%	4.2%	**78.6%**	3.1%	2.9%	**105.6%**
Lachnospiraceae	5.7%	8.7%	**65.6%**	3.2%	7.7%	**42.0%**
Lactobacillaceae				1.3%	0.5%	**269.4%**
Peptococcaceae		0.3%		0.2%	0.3%	**83.2%**
Peptostreptococcaceae	3.3%	3.3%	**102.1%**	3.5%	2.8%	**124.4%**
Phyllobacteriaceae				1.6%	6.2%	**26.2%**
Porphyromonadaceae	2.1%	2.0%	**109.0%**	2.2%	2.0%	**107.1%**
Prevotellaceae	3.0%	2.0%	**155.7%**	1.2%	2.2%	**52.0%**
Rikenellaceae	1.1%	0.9%	**129.3%**	0.5%	0.8%	**64.7%**
Streptococcaceae	0.5%	0.3%	**181.1%**	0.6%	0.4%	**147.9%**

FIGURE 5

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1	25	49	73	97	121	145	169	193
AGACAG	AGTGAG	ATGCAG	CATACA	CGCTAG	GCGCAG	GTCACA	TACTAG	TCAGAG
AGACGT	AGTGCA	ATGCGT	CATAGT	CGCTCA	GCGCGT	GTCAGT	TACTCA	TCAGCA
AGACTC	AGTGTC	ATGCTC	CATATC	CGCTGT	GCGCTC	GTCATC	TACTGT	TCAGTC
AGAGAG	ATACAG	ATGTAG	CATCAG	CGTACA	GCGTAG	GTCGAG	TAGACA	TCATAG
AGAGCA	ATACGT	ATGTCA	CATCGT	CGTAGT	GCGTCA	GTCGCA	TAGAGT	TCATCA
AGAGTC	ATACTC	ATGTGT	CATCTC	CGTATC	GCGTGT	GTCGTC	TAGATC	TCATGT
AGATAG	ATAGAG	CACACA	CATGAG	CGTCAG	GCTACA	GTCTAG	TAGCAG	TCGACA
AGATCA	ATAGCA	CACAGT	CATGCA	CGTCGT	GCTAGT	GTCTCA	TAGCGT	TCGAGT
AGATGT	ATAGTC	CACATC	CATGTC	CGTCTC	GCTATC	GTCTGT	TAGCTC	TCGATC
AGCACA	ATATAG	CACGAG	CGACAG	CGTGAG	GCTCAG	GTGACA	TAGTAG	TCGCAG
AGCAGT	ATATCA	CACGCA	CGACGT	CGTGCA	GCTCGT	GTGAGT	TAGTCA	TCGCGT
AGCATC	ATATGT	CACGTC	CGACTC	CGTGTC	GCTCTC	GTGATC	TAGTGT	TCGCTC
AGCGAG	ATCACA	CACTAG	CGAGAG	GCACAG	GCTGAG	GTGCAG	TATACA	TCGTAG
AGCGCA	ATCAGT	CACTCA	CGAGCA	GCACGT	GCTGCA	GTGCGT	TATAGT	TCGTCA
AGCGTC	ATCATC	CACTGT	CGAGTC	GCACTC	GCTGTC	GTGCTC	TATATC	TCGTGT
AGCTAG	ATCGAG	CAGACA	CGATAG	GCAGAG	GTACAG	GTGTAG	TATCAG	TCTACA
AGCTCA	ATCGCA	CAGAGT	CGATCA	GCAGCA	GTACGT	GTGTCA	TATCGT	TCTAGT
AGCTGT	ATCGTC	CAGATC	CGATGT	GCAGTC	GTACTC	GTGTGT	TATCTC	TCTATC
AGTACA	ATCTAG	CAGCAG	CGCACA	GCATAG	GTAGAG	TACACA	TATGAG	TCTCAG
AGTAGT	ATCTCA	CAGCGT	CGCAGT	GCATCA	GTAGCA	TACAGT	TATGCA	TCTCGT
AGTATC	ATCTGT	CAGCTC	CGCATC	GCATGT	GTAGTC	TACATC	TATGTC	TCTCTC
AGTCAG	ATGACA	CAGTAG	CGCGAG	GCGACA	GTATAG	TACGAG	TCACAG	TCTGAG
AGTCGT	ATGAGT	CAGTCA	CGCGCA	GCGAGT	GTATCA	TACGCA	TCACGT	TCTGCA
AGTCTC	ATGATC	CAGTGT	CGCGTC	GCGATC	GTATGT	TACGTC	TCACTC	TCTGTC
24	48	72	96	120	144	168	192	216

FIGURE 6

10x Genomics, Inc. Exhibit 2004
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FIGURE 7A

FORWARD TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER A	TAG	Forward 16S rRNA Primer
1	GCCTCCCTCGCGCCATCAG	AGACGT	AGAGTTTGATCMTGGCTCAG
2	GCCTCCCTCGCGCCATCAG	AGACTC	AGAGTTTGATCMTGGCTCAG
3	GCCTCCCTCGCGCCATCAG	AGAGTC	AGAGTTTGATCMTGGCTCAG
4	GCCTCCCTCGCGCCATCAG	AGATGT	AGAGTTTGATCMTGGCTCAG
5	GCCTCCCTCGCGCCATCAG	AGCAGT	AGAGTTTGATCMTGGCTCAG
6	GCCTCCCTCGCGCCATCAG	AGCATC	AGAGTTTGATCMTGGCTCAG
7	GCCTCCCTCGCGCCATCAG	AGCGTC	AGAGTTTGATCMTGGCTCAG
8	GCCTCCCTCGCGCCATCAG	AGCTGT	AGAGTTTGATCMTGGCTCAG
9	GCCTCCCTCGCGCCATCAG	AGTAGT	AGAGTTTGATCMTGGCTCAG
10	GCCTCCCTCGCGCCATCAG	AGTATC	AGAGTTTGATCMTGGCTCAG
11	GCCTCCCTCGCGCCATCAG	AGTCGT	AGAGTTTGATCMTGGCTCAG
12	GCCTCCCTCGCGCCATCAG	AGTCTC	AGAGTTTGATCMTGGCTCAG
13	GCCTCCCTCGCGCCATCAG	AGTGTC	AGAGTTTGATCMTGGCTCAG
14	GCCTCCCTCGCGCCATCAG	ATACGT	AGAGTTTGATCMTGGCTCAG
15	GCCTCCCTCGCGCCATCAG	ATACTC	AGAGTTTGATCMTGGCTCAG
16	GCCTCCCTCGCGCCATCAG	ATAGTC	AGAGTTTGATCMTGGCTCAG
17	GCCTCCCTCGCGCCATCAG	ATATGT	AGAGTTTGATCMTGGCTCAG
18	GCCTCCCTCGCGCCATCAG	ATCAGT	AGAGTTTGATCMTGGCTCAG
19	GCCTCCCTCGCGCCATCAG	ATCATC	AGAGTTTGATCMTGGCTCAG
20	GCCTCCCTCGCGCCATCAG	ATCGTC	AGAGTTTGATCMTGGCTCAG
21	GCCTCCCTCGCGCCATCAG	ATCTGT	AGAGTTTGATCMTGGCTCAG
22	GCCTCCCTCGCGCCATCAG	ATGAGT	AGAGTTTGATCMTGGCTCAG
23	GCCTCCCTCGCGCCATCAG	ATGATC	AGAGTTTGATCMTGGCTCAG
24	GCCTCCCTCGCGCCATCAG	ATGCGT	AGAGTTTGATCMTGGCTCAG
25	GCCTCCCTCGCGCCATCAG	ATGCTC	AGAGTTTGATCMTGGCTCAG
26	GCCTCCCTCGCGCCATCAG	ATGTGT	AGAGTTTGATCMTGGCTCAG
27	GCCTCCCTCGCGCCATCAG	CACAGT	AGAGTTTGATCMTGGCTCAG
28	GCCTCCCTCGCGCCATCAG	CACATC	AGAGTTTGATCMTGGCTCAG
29	GCCTCCCTCGCGCCATCAG	CACGTC	AGAGTTTGATCMTGGCTCAG
30	GCCTCCCTCGCGCCATCAG	CACTGT	AGAGTTTGATCMTGGCTCAG
31	GCCTCCCTCGCGCCATCAG	CAGAGT	AGAGTTTGATCMTGGCTCAG
32	GCCTCCCTCGCGCCATCAG	CAGATC	AGAGTTTGATCMTGGCTCAG
33	GCCTCCCTCGCGCCATCAG	CAGCGT	AGAGTTTGATCMTGGCTCAG
34	GCCTCCCTCGCGCCATCAG	CAGCTC	AGAGTTTGATCMTGGCTCAG
35	GCCTCCCTCGCGCCATCAG	CAGTGT	AGAGTTTGATCMTGGCTCAG
36	GCCTCCCTCGCGCCATCAG	CATAGT	AGAGTTTGATCMTGGCTCAG
37	GCCTCCCTCGCGCCATCAG	CATATC	AGAGTTTGATCMTGGCTCAG
38	GCCTCCCTCGCGCCATCAG	CATCGT	AGAGTTTGATCMTGGCTCAG
39	GCCTCCCTCGCGCCATCAG	CATCTC	AGAGTTTGATCMTGGCTCAG
40	GCCTCCCTCGCGCCATCAG	CATGTC	AGAGTTTGATCMTGGCTCAG
41	GCCTCCCTCGCGCCATCAG	CGACGT	AGAGTTTGATCMTGGCTCAG
42	GCCTCCCTCGCGCCATCAG	CGACTC	AGAGTTTGATCMTGGCTCAG
43	GCCTCCCTCGCGCCATCAG	CGAGTC	AGAGTTTGATCMTGGCTCAG
44	GCCTCCCTCGCGCCATCAG	CGATGT	AGAGTTTGATCMTGGCTCAG
45	GCCTCCCTCGCGCCATCAG	CGCAGT	AGAGTTTGATCMTGGCTCAG
46	GCCTCCCTCGCGCCATCAG	CGCATC	AGAGTTTGATCMTGGCTCAG
47	GCCTCCCTCGCGCCATCAG	CGCGTC	AGAGTTTGATCMTGGCTCAG
48	GCCTCCCTCGCGCCATCAG	CGCTGT	AGAGTTTGATCMTGGCTCAG
49	GCCTCCCTCGCGCCATCAG	CGTAGT	AGAGTTTGATCMTGGCTCAG
50	GCCTCCCTCGCGCCATCAG	CGTATC	AGAGTTTGATCMTGGCTCAG

FIGURE 7B

FORWARD TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER A	TAG	Forward 16S rRNA Primer
51	GCCTCCCTCGCGCCATCAG	CGTCGT	AGAGTTTGATCMTGGCTCAG
52	GCCTCCCTCGCGCCATCAG	CGTCTC	AGAGTTTGATCMTGGCTCAG
53	GCCTCCCTCGCGCCATCAG	CGTGCA	AGAGTTTGATCMTGGCTCAG
54	GCCTCCCTCGCGCCATCAG	CGTGTC	AGAGTTTGATCMTGGCTCAG
55	GCCTCCCTCGCGCCATCAG	TACAGT	AGAGTTTGATCMTGGCTCAG
56	GCCTCCCTCGCGCCATCAG	TACATC	AGAGTTTGATCMTGGCTCAG
57	GCCTCCCTCGCGCCATCAG	TACGTC	AGAGTTTGATCMTGGCTCAG
58	GCCTCCCTCGCGCCATCAG	TACTGT	AGAGTTTGATCMTGGCTCAG
59	GCCTCCCTCGCGCCATCAG	TAGAGT	AGAGTTTGATCMTGGCTCAG
60	GCCTCCCTCGCGCCATCAG	TAGATC	AGAGTTTGATCMTGGCTCAG
61	GCCTCCCTCGCGCCATCAG	TAGCGT	AGAGTTTGATCMTGGCTCAG
62	GCCTCCCTCGCGCCATCAG	TAGCTC	AGAGTTTGATCMTGGCTCAG
63	GCCTCCCTCGCGCCATCAG	TAGTGT	AGAGTTTGATCMTGGCTCAG
64	GCCTCCCTCGCGCCATCAG	TATAGT	AGAGTTTGATCMTGGCTCAG
65	GCCTCCCTCGCGCCATCAG	TATATC	AGAGTTTGATCMTGGCTCAG
66	GCCTCCCTCGCGCCATCAG	TATCGT	AGAGTTTGATCMTGGCTCAG
67	GCCTCCCTCGCGCCATCAG	TATCTC	AGAGTTTGATCMTGGCTCAG
68	GCCTCCCTCGCGCCATCAG	TATGTC	AGAGTTTGATCMTGGCTCAG
69	GCCTCCCTCGCGCCATCAG	TCACGT	AGAGTTTGATCMTGGCTCAG
70	GCCTCCCTCGCGCCATCAG	TCACTC	AGAGTTTGATCMTGGCTCAG
71	GCCTCCCTCGCGCCATCAG	TCAGTC	AGAGTTTGATCMTGGCTCAG
72	GCCTCCCTCGCGCCATCAG	TCATGT	AGAGTTTGATCMTGGCTCAG
73	GCCTCCCTCGCGCCATCAG	TCGAGT	AGAGTTTGATCMTGGCTCAG
74	GCCTCCCTCGCGCCATCAG	TCGATC	AGAGTTTGATCMTGGCTCAG
75	GCCTCCCTCGCGCCATCAG	TCGCGT	AGAGTTTGATCMTGGCTCAG
76	GCCTCCCTCGCGCCATCAG	TCGCTC	AGAGTTTGATCMTGGCTCAG
77	GCCTCCCTCGCGCCATCAG	TCGTGT	AGAGTTTGATCMTGGCTCAG
78	GCCTCCCTCGCGCCATCAG	TCTAGT	AGAGTTTGATCMTGGCTCAG
79	GCCTCCCTCGCGCCATCAG	TCTATC	AGAGTTTGATCMTGGCTCAG
80	GCCTCCCTCGCGCCATCAG	TCTCGT	AGAGTTTGATCMTGGCTCAG
81	GCCTCCCTCGCGCCATCAG	TCTCTC	AGAGTTTGATCMTGGCTCAG
82	GCCTCCCTCGCGCCATCAG	TCTGTC	AGAGTTTGATCMTGGCTCAG
83	GCCTCCCTCGCGCCATCAG	AGACAG	AGAGTTTGATCMTGGCTCAG
84	GCCTCCCTCGCGCCATCAG	AGAGAG	AGAGTTTGATCMTGGCTCAG
85	GCCTCCCTCGCGCCATCAG	AGATAG	AGAGTTTGATCMTGGCTCAG
86	GCCTCCCTCGCGCCATCAG	AGCGAG	AGAGTTTGATCMTGGCTCAG
87	GCCTCCCTCGCGCCATCAG	AGCTAG	AGAGTTTGATCMTGGCTCAG
88	GCCTCCCTCGCGCCATCAG	AGTCAG	AGAGTTTGATCMTGGCTCAG
89	GCCTCCCTCGCGCCATCAG	AGTGAG	AGAGTTTGATCMTGGCTCAG
90	GCCTCCCTCGCGCCATCAG	ATACAG	AGAGTTTGATCMTGGCTCAG
91	GCCTCCCTCGCGCCATCAG	ATAGAG	AGAGTTTGATCMTGGCTCAG
92	GCCTCCCTCGCGCCATCAG	ATATAG	AGAGTTTGATCMTGGCTCAG
93	GCCTCCCTCGCGCCATCAG	ATCGAG	AGAGTTTGATCMTGGCTCAG
94	GCCTCCCTCGCGCCATCAG	ATCTAG	AGAGTTTGATCMTGGCTCAG
95	GCCTCCCTCGCGCCATCAG	ATGCAG	AGAGTTTGATCMTGGCTCAG
96	GCCTCCCTCGCGCCATCAG	ATGTAG	AGAGTTTGATCMTGGCTCAG

FIGURE 7C

REVERSE TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER B	TAG	Reverse 16S rRNAPrimer
1	GCCTTGCCAGCCCGCTCAG	AGACGT	GCTGCCTCCCGTAGGAGT
2	GCCTTGCCAGCCCGCTCAG	AGACTC	GCTGCCTCCCGTAGGAGT
3	GCCTTGCCAGCCCGCTCAG	AGAGTC	GCTGCCTCCCGTAGGAGT
4	GCCTTGCCAGCCCGCTCAG	AGATGT	GCTGCCTCCCGTAGGAGT
5	GCCTTGCCAGCCCGCTCAG	AGCAGT	GCTGCCTCCCGTAGGAGT
6	GCCTTGCCAGCCCGCTCAG	AGCATC	GCTGCCTCCCGTAGGAGT
7	GCCTTGCCAGCCCGCTCAG	AGCGTC	GCTGCCTCCCGTAGGAGT
8	GCCTTGCCAGCCCGCTCAG	AGCTGT	GCTGCCTCCCGTAGGAGT
9	GCCTTGCCAGCCCGCTCAG	AGTAGT	GCTGCCTCCCGTAGGAGT
10	GCCTTGCCAGCCCGCTCAG	AGTATC	GCTGCCTCCCGTAGGAGT
11	GCCTTGCCAGCCCGCTCAG	AGTCGT	GCTGCCTCCCGTAGGAGT
12	GCCTTGCCAGCCCGCTCAG	AGTCTC	GCTGCCTCCCGTAGGAGT
13	GCCTTGCCAGCCCGCTCAG	AGTGTC	GCTGCCTCCCGTAGGAGT
14	GCCTTGCCAGCCCGCTCAG	ATACGT	GCTGCCTCCCGTAGGAGT
15	GCCTTGCCAGCCCGCTCAG	ATACTC	GCTGCCTCCCGTAGGAGT
16	GCCTTGCCAGCCCGCTCAG	ATAGTC	GCTGCCTCCCGTAGGAGT
17	GCCTTGCCAGCCCGCTCAG	ATATGT	GCTGCCTCCCGTAGGAGT
18	GCCTTGCCAGCCCGCTCAG	ATCAGT	GCTGCCTCCCGTAGGAGT
19	GCCTTGCCAGCCCGCTCAG	ATCATC	GCTGCCTCCCGTAGGAGT
20	GCCTTGCCAGCCCGCTCAG	ATCGTC	GCTGCCTCCCGTAGGAGT
21	GCCTTGCCAGCCCGCTCAG	ATCTGT	GCTGCCTCCCGTAGGAGT
22	GCCTTGCCAGCCCGCTCAG	ATGAGT	GCTGCCTCCCGTAGGAGT
23	GCCTTGCCAGCCCGCTCAG	ATGATC	GCTGCCTCCCGTAGGAGT
24	GCCTTGCCAGCCCGCTCAG	ATGCGT	GCTGCCTCCCGTAGGAGT
25	GCCTTGCCAGCCCGCTCAG	ATGCTC	GCTGCCTCCCGTAGGAGT
26	GCCTTGCCAGCCCGCTCAG	ATGTGT	GCTGCCTCCCGTAGGAGT
27	GCCTTGCCAGCCCGCTCAG	CACAGT	GCTGCCTCCCGTAGGAGT
28	GCCTTGCCAGCCCGCTCAG	CACATC	GCTGCCTCCCGTAGGAGT
29	GCCTTGCCAGCCCGCTCAG	CACGTC	GCTGCCTCCCGTAGGAGT
30	GCCTTGCCAGCCCGCTCAG	CACTGT	GCTGCCTCCCGTAGGAGT
31	GCCTTGCCAGCCCGCTCAG	CAGAGT	GCTGCCTCCCGTAGGAGT
32	GCCTTGCCAGCCCGCTCAG	CAGATC	GCTGCCTCCCGTAGGAGT
33	GCCTTGCCAGCCCGCTCAG	CAGCGT	GCTGCCTCCCGTAGGAGT
34	GCCTTGCCAGCCCGCTCAG	CAGCTC	GCTGCCTCCCGTAGGAGT
35	GCCTTGCCAGCCCGCTCAG	CAGTGT	GCTGCCTCCCGTAGGAGT
36	GCCTTGCCAGCCCGCTCAG	CATAGT	GCTGCCTCCCGTAGGAGT
37	GCCTTGCCAGCCCGCTCAG	CATATC	GCTGCCTCCCGTAGGAGT
38	GCCTTGCCAGCCCGCTCAG	CATCGT	GCTGCCTCCCGTAGGAGT
39	GCCTTGCCAGCCCGCTCAG	CATCTC	GCTGCCTCCCGTAGGAGT
40	GCCTTGCCAGCCCGCTCAG	CATGTC	GCTGCCTCCCGTAGGAGT
41	GCCTTGCCAGCCCGCTCAG	CGACGT	GCTGCCTCCCGTAGGAGT
42	GCCTTGCCAGCCCGCTCAG	CGACTC	GCTGCCTCCCGTAGGAGT
43	GCCTTGCCAGCCCGCTCAG	CGAGTC	GCTGCCTCCCGTAGGAGT
44	GCCTTGCCAGCCCGCTCAG	CGATGT	GCTGCCTCCCGTAGGAGT
45	GCCTTGCCAGCCCGCTCAG	CGCAGT	GCTGCCTCCCGTAGGAGT
46	GCCTTGCCAGCCCGCTCAG	CGCATC	GCTGCCTCCCGTAGGAGT
47	GCCTTGCCAGCCCGCTCAG	CGCGTC	GCTGCCTCCCGTAGGAGT
48	GCCTTGCCAGCCCGCTCAG	CGCTGT	GCTGCCTCCCGTAGGAGT
49	GCCTTGCCAGCCCGCTCAG	CGTAGT	GCTGCCTCCCGTAGGAGT
50	GCCTTGCCAGCCCGCTCAG	CGTATC	GCTGCCTCCCGTAGGAGT

FIGURE 7D

REVERSE TAGGED ADAPTER PRIMER

Primer Pair	ADAPTER B	TAG	Reverse 16S rRNAPrimer
51	GCCTTGCCAGCCCGCTCAG	CGTCGT	GCTGCCTCCCGTAGGAGT
52	GCCTTGCCAGCCCGCTCAG	CGTCTC	GCTGCCTCCCGTAGGAGT
53	GCCTTGCCAGCCCGCTCAG	CGTGCA	GCTGCCTCCCGTAGGAGT
54	GCCTTGCCAGCCCGCTCAG	CGTGTC	GCTGCCTCCCGTAGGAGT
55	GCCTTGCCAGCCCGCTCAG	TACAGT	GCTGCCTCCCGTAGGAGT
56	GCCTTGCCAGCCCGCTCAG	TACATC	GCTGCCTCCCGTAGGAGT
57	GCCTTGCCAGCCCGCTCAG	TACGTC	GCTGCCTCCCGTAGGAGT
58	GCCTTGCCAGCCCGCTCAG	TACTGT	GCTGCCTCCCGTAGGAGT
59	GCCTTGCCAGCCCGCTCAG	TAGAGT	GCTGCCTCCCGTAGGAGT
60	GCCTTGCCAGCCCGCTCAG	TAGATC	GCTGCCTCCCGTAGGAGT
61	GCCTTGCCAGCCCGCTCAG	TAGCGT	GCTGCCTCCCGTAGGAGT
62	GCCTTGCCAGCCCGCTCAG	TAGCTC	GCTGCCTCCCGTAGGAGT
63	GCCTTGCCAGCCCGCTCAG	TAGTGT	GCTGCCTCCCGTAGGAGT
64	GCCTTGCCAGCCCGCTCAG	TATAGT	GCTGCCTCCCGTAGGAGT
65	GCCTTGCCAGCCCGCTCAG	TATATC	GCTGCCTCCCGTAGGAGT
66	GCCTTGCCAGCCCGCTCAG	TATCGT	GCTGCCTCCCGTAGGAGT
67	GCCTTGCCAGCCCGCTCAG	TATCTC	GCTGCCTCCCGTAGGAGT
68	GCCTTGCCAGCCCGCTCAG	TATGTC	GCTGCCTCCCGTAGGAGT
69	GCCTTGCCAGCCCGCTCAG	TCACGT	GCTGCCTCCCGTAGGAGT
70	GCCTTGCCAGCCCGCTCAG	TCACTC	GCTGCCTCCCGTAGGAGT
71	GCCTTGCCAGCCCGCTCAG	TCAGTC	GCTGCCTCCCGTAGGAGT
72	GCCTTGCCAGCCCGCTCAG	TCATGT	GCTGCCTCCCGTAGGAGT
73	GCCTTGCCAGCCCGCTCAG	TCGAGT	GCTGCCTCCCGTAGGAGT
74	GCCTTGCCAGCCCGCTCAG	TCGATC	GCTGCCTCCCGTAGGAGT
75	GCCTTGCCAGCCCGCTCAG	TCGCGT	GCTGCCTCCCGTAGGAGT
76	GCCTTGCCAGCCCGCTCAG	TCGCTC	GCTGCCTCCCGTAGGAGT
77	GCCTTGCCAGCCCGCTCAG	TCGTGT	GCTGCCTCCCGTAGGAGT
78	GCCTTGCCAGCCCGCTCAG	TCTAGT	GCTGCCTCCCGTAGGAGT
79	GCCTTGCCAGCCCGCTCAG	TCTATC	GCTGCCTCCCGTAGGAGT
80	GCCTTGCCAGCCCGCTCAG	TCTCGT	GCTGCCTCCCGTAGGAGT
81	GCCTTGCCAGCCCGCTCAG	TCTCTC	GCTGCCTCCCGTAGGAGT
82	GCCTTGCCAGCCCGCTCAG	TCTGTC	GCTGCCTCCCGTAGGAGT
83	GCCTTGCCAGCCCGCTCAG	AGAGCA	GCTGCCTCCCGTAGGAGT
84	GCCTTGCCAGCCCGCTCAG	AGATCA	GCTGCCTCCCGTAGGAGT
85	GCCTTGCCAGCCCGCTCAG	AGCACA	GCTGCCTCCCGTAGGAGT
86	GCCTTGCCAGCCCGCTCAG	AGCGCA	GCTGCCTCCCGTAGGAGT
87	GCCTTGCCAGCCCGCTCAG	AGCTCA	GCTGCCTCCCGTAGGAGT
88	GCCTTGCCAGCCCGCTCAG	AGTACA	GCTGCCTCCCGTAGGAGT
89	GCCTTGCCAGCCCGCTCAG	AGTGCA	GCTGCCTCCCGTAGGAGT
90	GCCTTGCCAGCCCGCTCAG	ATAGCA	GCTGCCTCCCGTAGGAGT
91	GCCTTGCCAGCCCGCTCAG	ATATCA	GCTGCCTCCCGTAGGAGT
92	GCCTTGCCAGCCCGCTCAG	ATCACA	GCTGCCTCCCGTAGGAGT
93	GCCTTGCCAGCCCGCTCAG	ATCGCA	GCTGCCTCCCGTAGGAGT
94	GCCTTGCCAGCCCGCTCAG	ATCTCA	GCTGCCTCCCGTAGGAGT
95	GCCTTGCCAGCCCGCTCAG	ATGACA	GCTGCCTCCCGTAGGAGT
96	GCCTTGCCAGCCCGCTCAG	ATGTCA	GCTGCCTCCCGTAGGAGT

Transcriptome **sequencing** with the **Genome** Sequencer FLX system

T Jarvie, T Harkins - Nature Methods, 2008 - nature.com

... Information on how to prepare small RNAs for 454 **Sequencing** analysis is available (Jarvie, T. & Harkins ... The **Genome** Sequencer FLX supports a number of formats, allowing users to customize the number of ... Multiplexing of samples is also supported by **Multiplex Identifier** tags. ...

Cited by 25 Related articles All 5 versions Cite Save

De novo 454 **sequencing** of barcoded BAC pools for comprehensive gene survey and **genome** analysis in the complex **genome** of barley

B Steuernagel, S Taudien... - BMC ..., 2009 - bmcgenomics.biomedcentral.com

... Due to the lack of a reference **genome** this BAC pool **sequencing** approach is ... short sequence tags that can be introduced either before the 454 **sequencing** library preparation [27] or by ligation of individual adaptors ("MID" = **Multiplex Identifier**", Roche Diagnostics ...

Cited by 69 Related articles All 14 versions Cite Save More

Digital PCR provides sensitive and absolute calibration for high throughput **sequencing**

RA White, PC Blainey, HC Fan... - BMC ..., 2009 - bmcgenomics.biomedcentral.com

... These results indicate that better than one-fold coverage of the ~4.7 Mbp K12 **genome** can be obtained from less than 1 ng input DNA. Detailed information about these **sequencing** results is presented in Table 3. Table 3 454 FLX trace library sequence results. Sample ID. ...

Cited by 149 Related articles All 39 versions Cite Save More

High-resolution, high-throughput HLA genotyping by next-generation **sequencing**

G Bentley, R Higuchi, B Hoglund, D Goodridge... - Tissue ..., 2009 - Wiley Online Library

... which allows the 454-**genome** sequencer software to differentiate HLA amplicon derived sequences from internal control sequences. We added 4-base **multiplex identifier** (MID) sequences (18) immediately following the library key tag to allow for multiplexed **sequencing** of HLA ...

Cited by 136 Related articles All 12 versions Cite Save

Phylotyping and functional analysis of two ancient human microbiomes

RY Tito, S Macmil, G Wiley, F Najar, L Cleeland, C Qu... - PLoS ..., 2008 - journals.plos.org

... Our matches represent sequences that are random areas of the **genome**; they are unlikely to be as robust as matches derived from ... False matches during shotgun **sequencing** are a concern [26]. ... Library formation employed **Multiplex Identifier** Kits (Roche Applied Science). ...

Cited by 41 Related articles All 20 versions Cite Save More

Rapid high-throughput human leukocyte antigen typing by massively parallel pyrosequencing for high-resolution allele identification

C Gabriel, M Danzer, C Hackl, G Kopal, P Hufnagl... - Human immunology, 2009 - Elsevier

... the readout data per flow, which was then used by a **Genome** Sequencer-specific ... 2.0) of the GS-FLX amplicon variant analyzer (AVA) software supporting **multiplex identifier** sequence tags. In general, the AVA software maps the amplicon **sequencing** reads against a reference ...

Cited by 68 Related articles All 6 versions Cite Save

System and method for identification of individual samples from a multiplex mixture

MS Braverman, JF Simons, M Srinivasan ... - US Patent App. 12/ ..., 2008 - Google Patents

... a unique identifier (UID) element, which is sometimes also referred to as a **multiplex identifier** (MID), with ... refer to a nucleic acid molecule that is the subject of a **sequencing** reaction from ... refers to a collection of molecules derived from and/or representing an entire **genome** (ie all ...

Cited by 50 Related articles All 2 versions Cite Save

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00792

Supplemental Information

C Vilek, BJ Kronschnabel, A Jeannard, G Blanc... - 2002 - researchgate.net

... 22 Page 2. Page2 of69 Selected DNA fragments were ligated to a Rapid Library **Multiplex Identifier** (MID) 23 adaptor designed for GS FLX Titanium chemistry. ... **genome** from duplicate **sequencing** runs; however, some regions had approximately100X 62 ...

Related articles All 25 versions Cite Save More

Improving Alfalfa as a Biofuel Feedstock

MS Genes - dl.sciencesocieties.org

... Using Next-Generation **Sequencing** in Amaranth Plant **Genome** 260–270 Although mostly underutilized ... from several individuals, and does not require a priori **genome** sequence information. ... with each sample labeled inde- pendently using a unique **multiplex identifier** barcode. ...

Cite Save

A NEW STRATEGY TO IDENTIFY THE DISEASE CAUSING MUTATION FOR NEURONAL CEROID LIPOFUSCINOSIS IN SOUTH HAMSPHIRE SHEEP

IFM Ismail, JAL Cavanagh, NL Mitchell... - Proc. Assoc. Advmt. ..., 2009 - aaabg.org

... The resulting products will be individually **multiplex identifier** (MID) tagged and 454 sequenced. CONCLUSION Whole **genome sequencing** has not been completed for the sheep and the publically available sequence information for our region of interest had large gaps. ...

Related articles All 6 versions Cite Save More

SNP discovery via genomic reduction, barcoding, and 454-pyrosequencing in amaranth

PJ Maughan, SM Yourstone, EN Jellen ... - ... plant **genome**, 2009 - dl.sciencesocieties.org

... based on restriction site conservation (GR-RSC) with each sample labeled independently using a unique **multiplex identifier** (MID) barcode ... bead separation and size selection via gel electrophoresis, followed by >10-fold **sequencing** coverage of the remaining **genome** via 454 ...

Cited by 67 Related articles All 6 versions Cite Save

Major histocompatibility complex genotyping with massively parallel pyrosequencing

RW Wiseman, JA Karl, BN Bimber, CE O'leary... - Nature medicine, 2009 - nature.com

... Chinese-origin rhesus macaques in a single pilot run on a **Genome** Sequencer FLX ... products from 12 macaques that were distinguished by 10-bp **multiplex identifier** (MID) tags ... detected five sequences not previously observed by cloning and Sanger **sequencing** (transcript levels ...

Cited by 92 Related articles All 15 versions Cite Save

Shifting the genomic gold standard for the prokaryotic species definition

M Richter, R Rosselló-Móra - Proceedings of the National ..., 2009 - National Acad Sciences

... For this, each **genome** was tagged with a different **multiplex identifier** for 454 libraries (Roche Applied ... almost duplicated until the bias is solved by the company producing the **sequencing** kits ... strain K12 that accounted for ≈3,158 reads covering ≈0.7 Mb (≈15% of the **genome**). ...

Cited by 869 Related articles All 13 versions Cite Save

CO dehydrogenase genes found in metagenomic fosmid clones from the deep Mediterranean Sea

AB Martin-Cuadrado, R Ghai, A Gonzaga... - Applied and ..., 2009 - Am Soc Microbiol

... approximately 7.2 Mb of DNA sequence (ie, roughly two prokaryotic **genome** equivalents ... For the **sequencing** process, the concentration of each DNA fosmid was measured using ... GATC, Constance, Germany), tagging each one individually using a **multiplex identifier** adaptor that ...

Cited by 23 Related articles All 12 versions Cite Save

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00793

Identification of novel MHC class I sequences in pig-tailed macaques by amplicon pyrosequencing and full-length cDNA cloning and **sequencing**

CE O'Leary, RW Wiseman, JA Karl, BN Bimber ... - Immunogenetics, 2009 - Springer

... 3'), which binds a conserved region of exon 3. Forward and reverse PCR primer pairs contained one of 12 **multiplex identifier** (MID) tags ... steps were performed following the manufacturer's GS FLX protocols at the University of Illinois—Urbana Champaign **Sequencing** Center. ...

Cited by 28 Related articles All 13 versions Cite Save

Cost-effective sequence-based nonhuman primate MHC class I genotyping from RNA

JA Karl, RW Wiseman, DH O'Connor - Methods, 2009 - Elsevier

... based genotyping that amplifies full-length NHP class I cDNAs that are molecularly barcoded with **multiplex identifier** (MID) tags. ... Finally, this method can also be adapted for use on next-generation **sequencing** platforms such as the Roche/454 **Genome** Sequencer FLX ...

Cited by 10 Related articles All 9 versions Cite Save

System and method for improved processing of nucleic acids for production of sequencable libraries

GC Ferreri, JF Simons, MT Ronan , - US Patent App. 12/ ..., 2009 - Google Patents

... data for each amplified member that comprises a sequence composition for the **multiplex identifier** element; and ... from and/or representing an entire **genome** (ie all regions of a **genome**) of an ... term "keypass" or "keypass well" as used herein generally refers to the **sequencing** of a ...

Cited by 2 Related articles All 2 versions Cite Save

System and method for detection of HIV integrase variants

BB Simen, EPS John - US Patent App. 12/592,243, 2009 - Google Patents

... from and/or representing an entire **genome** (ie all regions of a **genome**) of an ... functions including, but not limited to, primer sequences for amplification and/or **sequencing** methods, quality control elements, unique identifiers (also referred to as a **multiplex identifier** or "MID ...

Related articles All 2 versions Cite Save

Mycobacteriophages BPs, Angel and Halo: comparative genomics reveals a novel class of ultra-small mobile genetic elements

T Sampson, GW Broussard, EJ Marinelli, ... - ..., 2009 - mic.microbiologyresearch.org

... and annealed to primer-coated beads; the 'A' adaptor contained a 10 bp **Multiplex Identifier** (MID) tag ... per read, providing a total of 1 636 726 bases of raw **sequencing** information. ... one large contig with consensus quality values of 60 or greater and average **genome** coverage of ...

Cited by 47 Related articles All 13 versions Cite Save

Best Abstracts

A Amir, L Dorscgna, M van Loenen, M Kester... - 2009 - Wiley Online Library

... Gene **sequencing** showed the presence of a new type of mutation in the TAP1 gene. ... Page 3. O6 Analysis of the KIR in the CEPH human **genome** diversity panel reveals evidence of different evolutionary histories for the centromeric and telomeric portions of the gene cluster Jill A ...

Cite Save

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00794

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L23	21	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (nucleic or dna or polynucleotide)same (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
L24	8	23 and sequencing.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
L25	1	24 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:34
L26	134	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:35
L27	3	26 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)same sequence same (identif$4 or detect$3 or determin$3 or distinguish$3) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:36
L28	29	"9410150"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:04
L29	8	"9410149".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:05

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L30	3	"8563274".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:06
L31	2	"8679756".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:08

1/ 23/ 2017 6:12:23 PM
C:\ Users\ nbhat1\ Documents\ EAST\ Workspaces\ 14792094-1-23-17.wsp

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	29794	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:50
S2	134	S1 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:51
S3	536	S1 and (dna or nucleic or polynucleotide) same (pcr or amplif$7)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:54
S4	18	S3 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:56
S5	4	S4 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:58
S6	139392	(single or individual or one) near3 cell same (dna or nucleic or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:01
S7	3492	S6 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:02
S8	872	S7 and sequencing same (mid or	US-PGPUB;	OR	ON	2017/01/23

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		"multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			11:02
S9	88	S8 and (single or individual or one) near3 cell near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S10	84	S8 and (single or individual or one) near3 cell near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S11	66	S10 and first near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:05
S12	47	S11 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:05
S13	43263	"first cell" and "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:15
S14	1761	S13 and (dna or nucleic or polynucleotide) same "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:16
S15	1549	S14 and (dna or nucleic or polynucleotide) same "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:17
S16	143	S15 and (adapt$3 or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2017/01/23 11:18

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			IBM_TDB			
S17	27	S16 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:19
S18	35	S16 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:20
S19	870	(single or individual or one) near3 cell near5 isolat$4 near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:29
S20	315	S19 and (single or individual or one) near3 cell near5 isolat$4 near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:30
S21	120	S20 and (dna or nucleic or polynucleotide) near5 cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:31
S22	25	S21 and (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:31
S23	12	S21 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:34
S24	540	sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same cell near5 (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:36
S25	381	S24 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2017/01/23 11:37

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		sequence same (identif$4 or detect$3 or dtermin$3) same cell near5 (type or individual$3 or single)	JPO; DERWENT; IBM_TDB			
S26	67	S25 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or dtermin$3) same cell adj (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:38
S27	2799	"first cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:47
S28	1549	S27 and "second cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:48
S29	1	S28 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:49
S30	8	S27 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:51
S36	2321	(mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near5 sequence near5 (identif$4 or detect$3 or determin$3 or distinguish$3) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:59
S37	46	S36 and (append$3 or ligat$3) near5 (adpator or adapter or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) same (dna or nucleic or polynucleotide) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 12:02

1/23/2017 4:58:45 PM
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.		

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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UNITED STATES PATENT AND TRADEMARK OFFICE

USPTO Automated Interview Request (AIR)

Feb 27 2017

This paper requesting to schedule and/or conduct an interview is appropriate because:

This submission is requested to be accepted as an authorization for this interview to communicate via the internet. Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned concerning scheduling of the interview via video conference, instant messaging, or electronic mail, and to conduct the interview in accordance with office practice including video conferencing.

Name(s):
Gargi Talukder

S-signature:
/Gargi Talukder/

Registration Number:
61368

U.S. Application Number:
14792094

Confirmation Number:
9697

E-mail Address:
gargi.talukder@morganlewis.com

Phone Number:
4154421266

Proposed Time of Interview:
3-16-2017 3:00 PM ET

Prefered Interview Type:
Telephonic

I am the applicant or applicant's representative for this application.

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PALM-SILVER

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 03/21/2017
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
03/21/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

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Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	14/792,094	BRENNER ET AL.	
	Examiner	Art Unit	
	NARAYAN BHAT	1634	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Examiner: Bhat*. (3)_____.

(2) *Representative: Ms. Talukder*. (4)_____.

Date of Interview: *16 March 2017*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☒112 ☒102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *40*.

Identification of prior art discussed: *Braverman*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

In view of Automated Interview Request filed on 2/27/17, the interview was scheduled on 3/16/2017. The representative discussed that claim 40 languages are similar to the allowed claims of US 8,679,756 and 8,563,274 and further specifies the nucleic acid is from first and second isolated single cells and thus would be non-obvious over Braverman. The examiner informed that the allowed claims require "different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules" which is different from what is claimed in claim 40 and there are no support for the phrase "isolated single cell" in the instant specification and the recitation of "first and second isolated cell" could be the same. The representative asked if the support for said phrase is identified in the instant specification, will the pending claims be allowable. The examiner informed that instant claim 40 would be obvious over the arts of the record. No specific claim languages were agreed upon during the interview .

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☒ Attachment

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

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Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted.
2) an identification of the claims discussed.
3) an identification of the specific prior art discussed.
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner.
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Attachment: IMIS (2)

From:	PTO Automated Interview Request
To:	Bhat, Narayan
Cc:	PTO Automated Interview Request
Subject:	USPTO Automated Interview Request (AIR): Action Required - Received interview for US Application Number 14792094
Date:	Monday, February 27, 2017 2:37:33 PM
Importance:	High

NARAYAN BHAT,

This is an Automated Interview Request (AIR) made by Gargi Talukder for application number 14792094 for 3-16-2017 3:00 PM ET. The preferred interview type is: **Telephonic**. Please respond to this interview request within **1 business day**.

Please contact Gargi Talukder by calling 4154421266 or emailing gargi.talukder@morganlewis.com to set up an interview or deny if appropriate.

By receiving this AIR submission, the applicant has certified that:
This submission is requested to be accepted as an authorization for this interview to communicate via the internet. Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned concerning scheduling of the interview via video conference, instant messaging, or electronic mail, and to conduct the interview in accordance with office practice including video conferencing.

If you're going to deny the interview, please contact your SPE to clear the tracking off of your dashboard.

If you need assistance please contact your SPE, an Interview Specialist from your TC or see Quick Reference Guide. Thank you.

Please do not reply to this message; it was sent from an unmonitored e-mail address.

From:	Talukder, Gargi
To:	Bhat, Narayan
Subject:	RE: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05) 3.30 EST
Date:	Wednesday, March 15, 2017 4:48:03 PM

Examiner Bhat,

Yes, I will give you a call at 3:30 pm EST tomorrow.

Thanks,
Gargi

Gargi Talukder, J.D., Ph.D.
Morgan, Lewis & Bockius LLP
One Market, Spear Street Tower | San Francisco, CA 94105
Direct: +1.415.442.1266 | Main: +1.415.442.1000 | Fax: +1.415.442.1001
gargi.talukder@morganlewis.com | www.morganlewis.com
Assistant: Ronn R. Uchihara | +1.415.442.1715 | ronn.uchihara@morganlewis.com

From: Bhat, Narayan [mailto:Narayan.Bhat@USPTO.GOV]
Sent: Wednesday, March 15, 2017 1:36 PM
To: Talukder, Gargi
Subject: RE: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05) 3.30 EST

Ms. Talukder,
Sorry for the mix up. I have an interview tomorrow at 2 pm. Can we have the interview tomorrow @ 3.30 EST instead at 3 pm ? Thank you very much for your cooperation.

Narayan K Bhat
Primary Examiner, AU 1634,
Remsen 3C09, TC 1600, USPTO, DOC, Alexandria, VA-22313
T: 571-272-5540
Fax: 571-273-5540

From: Talukder, Gargi [mailto:gargi.talukder@morganlewis.com]
Sent: Wednesday, March 15, 2017 4:29 PM
To: Bhat, Narayan <Narayan.Bhat@USPTO.GOV>
Subject: FW: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05)

Dear Examiner Bhat,

Further to my voicemail, please see the forwarded e-mail below confirming that our interview is scheduled for tomorrow. Please let me know if tomorrow at 3 pm ET still works for you.

Thank you,
Gargi Talukder

Gargi Talukder, J.D., Ph.D.
Morgan, Lewis & Bockius LLP
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From: Talukder, Gargi
Sent: Monday, February 27, 2017 5:46 PM
To: narayan.bhat@uspto.gov
Cc: Gloss, Sheila R.
Subject: In preparation for Interview on March 16, 2017 for USSN 14/792,094 (MLB Ref: 104371-5016-US05)

Dear Examiner Bhat,

Thank you for taking the time to speak with me today, and I look forward to our further discussion in a couple of weeks.

Per our discussion today, please refer to US Patent No. 8,679,756 (USSN 14/021,790) and US Patent No. 8,563,274 (USSN 13/859,450). These are the patents on which we modeled the claims for the current case.

Best regards,
Gargi Talukder

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	RESPONSE TO OFFICE ACTION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Office Action mailed February 1, 2017, please enter the following amendments and remarks:

Amendments to the Claims are reflected in the listing of claims which begins on page **2** of this paper.

Remarks/Arguments begin on page **5** of this paper.

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Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-39 (Cancelled)

40. (Currently amended) A method of analyzing nucleic acids, the method comprising:

(a) providing a first plurality of segments of nucleic acid molecules from a contiguous region of a genome from a first single isolated cell, wherein each of the first plurality of segments is appended to a first multiplex identifier (MID) sequence prior to or during an amplification reaction to form first appended segments;

(b) providing a second plurality of segments of nucleic acid molecules from a contiguous region of a genome from a second single isolated cell, wherein each of the second plurality of segments is appended to a second MID sequence prior to or during an amplification reaction to produce second appended segments, wherein the first single isolated cell is a different cell than the second single isolated cell, and wherein the first MID sequence and the second MID sequence are different from each other;

(c) sequencing the first appended segments and the second appended segments to provide a plurality of sequences,

wherein each sequence comprises (i) nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, and wherein the MID sequences uniquely identify the segments with a single isolated cell.

41. (Previously presented) The method of claim 40, wherein the method further comprises amplifying the first appended segments and the second appended segments before the sequencing step (c).

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42. (Previously presented) The method of claim 40, wherein the nucleic acid molecules are selected from DNA and RNA.

43. (Previously presented) The method of claim 42, wherein the nucleic acid molecules comprise mRNA.

44. (Previously presented) The method of claim 40, wherein the MID sequences are appended to the segments through ligation.

45. (Previously presented) The method of claim 40, wherein the MID sequences are appended to the segments by performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

46. (Previously presented) The method of claim 40, wherein the sequences of the segments are attributed to different isolated cells based on the associated MID sequence.

47. (Previously presented) The method of claim 40, wherein the MID sequences further comprise a sequencing primer sequence.

48. (Previously presented) The method of claim 40, wherein the MID sequences are from 2 to 100 bases in length.

49. (Previously presented) The method of claim 40, wherein the segments of (a) and (b) are in the range of 100 to 5,000 bp in length.

50. (Previously presented) The method of claim 40, wherein the segments of (a) and (b) are 400 bp or less in length.

51. (Previously presented) The method of claim 40, wherein the method further comprises providing a third plurality of segments of nucleic acid molecules from a contiguous region of a genome from a third single isolated cell, wherein each of the third plurality of segments is appended to a third MID sequence to produce third

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appended segments, and the third MID sequence is different from the first MID sequence and the second MID sequence.

52. (Previously presented) The method of claim 40, wherein the contiguous region is greater than 2 kb in length.

53. (Currently amended) A method of analyzing a plurality of contiguous genomic sequences, comprising:

(a) providing a sample comprising the plurality of contiguous genomic sequences, wherein the plurality of contiguous genomic sequences are from a plurality of single isolated cells;

(b) tagging a plurality of fragments of the contiguous genomic sequences with a multiplex identifier (MID) sequence prior to or during an amplification reaction such that fragments of a first contiguous nucleic acid sequence share a ~~common~~ MID sequence that is different from ~~an~~ a MID sequence of fragments of a second contiguous nucleic acid sequence; and

(c) sequencing the plurality of fragments and MID sequences to identify fragments of the contiguous sequence.

54. (Previously presented) The method of claim 53, wherein the first contiguous nucleic acid sequence is from a first single isolated cell and the second contiguous nucleic acid sequence is from a second single isolated cell.

55. (Previously presented) The method of claim 53, wherein sequences of the fragments are attributed to different single isolated cells based on the associated MID sequence.

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Remarks/Arguments

Applicants have not dedicated or abandoned any unclaimed subject matter and have not acquiesced to any rejections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

Status of the Claims

Claims 40-55 are pending and under consideration in this application. Claims 40 and 53 have been amended, and these amendments are fully supported in the specification as filed.

For example, the amendment that the first and second MID sequences are appended "prior to or during an amplification reaction" is supported on pages 4-5 and 24-25 of the specification as filed.

The amendment clarifying that the first and second cells are different cells is supported for example on page 8, which states that "a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues *or cells*, or polynucleotides isolated at different times points)..." (emphasis added).

Applicants respectfully request entry of the claims as amended into the record.

Interview summary

Examiner Bhat and Applicants' representative, Gargi Talukder, (herein together referred to as "the Parties") participated in a telephonic interview on March 16, 2017. Applicants thank Examiner Bhat for his attention and time to this matter.

The Parties discussed the pending claims and the outstanding rejections in the present office action. No agreement was reached on the claims, and the Examiner suggested that a response to office action be filed with further amendments and arguments for consideration. The

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Examiner indicated he may need to conduct further searches depending on any new amendments to the claims.

Response to Office Action

Claim Objections

Claim 53 is objected to because of a typographical error to the recitation of "an MID sequence" in step 'b' lines 3 and 4. Claim 53 has been amended as suggested by the Examiner, thus overcoming this objection. Applicants respectfully request that this rejection be withdrawn.

Claim Rejections – 35 USC § 112-first paragraph – written description

Claims 40-55 are rejected under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AIA), first paragraph, as allegedly failing to comply with the written description requirement. Applicants respectfully disagree.

The Office asserts on page 7 of the current Office Action that the claim elements "first single isolated cell," "second single isolated cell," and "a common MID sequence" constitute new matter.

Applicants respectfully remind the Office that word-for-word support for every element of a claim is not the standard for fulfillment of the written description requirement. Rather, "If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met. See, e.g., *Vas-Cath,* 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson,* 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in *ipsis verbis* [i.e., "in the same words"] to be sufficient")." *MPEP 2163.* Applicants submit that the specification as filed provides an adequate description of all of the elements of the present claims, including the elements identified in the present Office Action.

Taking the "first single isolated cell" and "second single isolated cell" elements first, Applicants note that the specification explicitly states that samples can include polynucleotides

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from different sources, including "different tissues or cells." *See page 8, line 5 of the present specification.* In addition, the specification further provides that the nucleic acid sample can be from a single source, such as a cell, or from of a plurality of cells where by plurality is meant "two or more." *See page 19, first full paragraph of the specification as filed.* As such, the skilled artisan would readily understand that the specification as filed supports methods in which the polynucleotides to be analyzed can be sourced from two different cells, because the specification states that two or more cells could be the source of the nucleic acid sample. As will be appreciated, an adequate written description of the invention "may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention." *MPEP §2163.* Applicants submit that the above excerpts from the present specification provides sufficient identifying characteristics of the claimed invention to fulfill the written description requirement.

For the element of a "common MID sequence" Applicants have, solely to further prosecution of this application and without conceding to the propriety of this rejection, amended claim 53 to remove the term "common," thus clarifying that the claimed invention involves polynucleotides where fragments of a first contiguous nucleic acid sequence share an MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence. As such, the written description rejection as it pertains to this element of the claims is rendered moot.

For at least the foregoing reasons, Applicants respectfully request that all rejections under § 112 for lack of written description be withdrawn.

Claim Rejections – 35 USC § 112-second paragraph

Claims 53-55 are rejected under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention. Applicants respectfully disagree.

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The Office Action states on page 9 that claim 53 is indefinite because "in step 'b' it is unclear "how sharing sequences is different." Applicants note that the claim recites that fragments of a first contiguous nucleic acid sequence share a MID sequence that is different from *a MID sequence of fragments of a second contiguous nucleic acid sequence*. In other words, the fragments from the first contiguous nucleic acid sequence are tagged with the same MID sequence, and that MID sequence is different than the MID sequence that tags the fragments of the second nucleic acid sequence. Applicants respectfully submit that the claim is clear as currently pending and request that this rejection be withdrawn.

Claim Rejections – 35 USC § 103

Claims 40-55 are rejected under 35 U.S.C. § 103 as being allegedly anticipated by Braverman et al., US 2009/0105959 ("Braverman") in view of Drmanac, US 2007/0072208 ("Drmanac"). Applicants respectfully traverse these rejections.

Applicants submit that the skilled artisan would not have found it obvious to combine the teachings of Braverman and Drmanac to practice the claimed invention because (i) Braverman teaches away from elements of the claimed invention, (ii) the skilled artisan would have no reasonable expectation of success in practicing the claimed invention by combining the teachings of Braverman and Drmanac, and (iii) the combined teachings of Braverman and Drmanac fail to disclose all elements of the claimed invention.

As discussed in *KSR v. Teleflex*, "when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." *KSR Int'l Co. v. Teleflex, Inc., 550 U.S. 398 (2007) citing United States v. Adams, 383 U.S. 39, 51-52 (1966).* In addition, as described in MPEP § 2141.02, a prior art reference must be considered "in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 851 (1984).

When considered as a whole, Braverman is directed to associating nucleic acids with a "UID element" that is "resistant to introduced error in sequence data." *Braverman, paragraph*

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[0028]. Toward its purpose of producing nucleic acids tagged with UID's that are resistant to introduced error, Braverman teaches that methods of attaching the UID molecules after amplification provide distinct advantages: "By coupling the UID elements to the adapted template molecules post-amplification, errors introduced by the amplification method are avoided." *Braverman, paragraph [0084]*. The present claims are directed to methods in which an MID are appended prior to or during an amplification reaction. According to Braverman, any such tags would be prone to errors, and as such, Braverman teaches away from the claimed invention. The skilled artisan would not find it obvious to combine these teachings in Braverman with those in Drmanac to practice the claimed invention.

Furthermore, the skilled artisan would have no reasonable expectation of success in practicing the claimed invention by combining the teachings of Braverman with those of Drmanac. Drmanac describes a method that relies upon a dilution process that results in forming aliquots containing mixtures of non-overlapping fragments. In fact, the title of Drmanac is "Nucleic acid analysis by random mixtures of non-overlapping fragments." The methods in Drmanac rely on the dilution process to order sequence information. For example, Drmanac states that "When the fragments in the final tier are sequenced, overlapping sequence regions of fragments in different aliquots are used to register the different fragments so that non-overlapping regions are ordered." *Drmanac, paragraph [0015]*. As further explained in Drmanac, an overriding feature of the process in Drmanac is that different aliquots at most contain one molecule of a particular sequence of interest: "A common feature in all applications of this invention (genome sequencing, SNP or other marker mapping or cDNA analysis) is aliquoting nucleic acid sample such that sequences of predetermined type of relatedness (overlapped fragment, fragments with high similarity, homologous chromosomes, messengers transcribed from the same gene) occur mostly once e.g. as a single molecule per aliquot." *Drmanac, paragraph [0017]*. The skilled artisan would have no reason to use the teachings of Drmanac in combination with those of Braverman to practice the claimed invention, because the purpose of Drmanac is to divide out the nucleic acids from a source to sub-haploid levels, whereas the presently claimed invention would result in nucleic acids from a first cell to be tagged with an MID that is different from the MID of nucleic acids form a second cell. In other words, the sequencing step of the presently claimed methods would necessarily involve

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sequencing overlapping segments from the original isolated cell. As such, the skilled artisan would have no expectation of success in practicing the claimed invention based on the combined teachings of Drmanac and Braverman, because Drmanac is directed to a method that is not in keeping with the presently claimed invention.

Applicants note that the Office has cited to Drmanac to provide the teaching that the samples can be from two isolated cells. However, as discussed above, in supporting a rejection for obviousness, a prior art reference must be considered "in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 851 (1984). *MPEP § 2141.02.* When taken as a whole, Drmanac does not support a rejection for obviousness.

Applicants further submit that the combined teachings of Braverman and Drmanac fail to disclose or suggest elements of the claims, including a method in which each of a first plurality of segments from a first isolated cell is appended to a first multiplex identifier (MID) and each of a second plurality of segments from a second isolated cell are appended to a second MID, as is recited in independent claim 40. In addition, the combined teachings of Braverman and Drmanac fail to provide a method in which a plurality of fragments of contiguous genomic sequences are tagged with a MID sequence such that fragments of a first contiguous nucleic acid sequence share a common MID sequence that is different from an MID sequence of fragments of a second contiguous nucleic acid sequence, as is recited in independent claim 53. Furthermore, the combined teachings of Braverman and Drmanac fails to disclose methods in which segments appended to or tagged with MIDs can be attributed to their single cell of origin.

For at least the foregoing reasons, Applicants respectfully request that all rejections under § 103 be withdrawn.

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Claim Rejections – Double Patenting

Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Drmanac (US 2007/0072208).

For the reasons recited above, Applicants respectfully submit the presently claimed invention does not support a rejection on the ground of nonstatutory double patenting, as the present claims are not obvious in light of U.S. Patent No. 8,679,756 in view of Drmanac. Applicants respectfully request that this rejection be withdrawn.

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CONCLUSION

All the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action, and as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided below.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any necessary fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17, which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310 (Order No.: 104371-5016-US05).

Prompt and favorable consideration of this Amendment and Response is respectfully requested.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: May 1, 2017 By: Gargi Talukder
Gargi Talukder, Reg. No. 61,368

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10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00821

Electronic Acknowledgement Receipt

EFS ID:	29077048
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	01-MAY-2017
Filing Date:	06-JUL-2015
Time Stamp:	14:20:00
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		104371_5016_US05_RSP.pdf	578756 2f64ec68f47177fdc921d9212ca436981866b7ca1	yes	12

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	4
	Applicant Arguments/Remarks Made in an Amendment	5	12
Warnings:			
Information:			
	Total Files Size (in bytes):	578756	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 = *		X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 = *		X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 05/01/2017	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 16	Minus	** 20	= 0	X $40 =	0
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
ROBERT TALBERT

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 05/31/2017
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One Market, Spear Street Tower, Suite 2800
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EXAMINER
BHAT, NARAYAN KAMESHWAR

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
05/31/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

Office Action Summary	Application No. 14/792,094	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on 5/01/2017.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) 40-55 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) 40-55 is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on 7/6/2015 is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.

4) ☐ Other: _____.

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00826

Final action

1. The present application is being examined under the pre-AIA first to invent provisions. This office action is in response to the papers filed on 5/1/2017. The amendments to independent claims 40 and 53 changing the scope of previously rejected said claims necessitated the new grounds of rejections as set forth in this office action. ***Accordingly this office action has been made final.***

Track1 Request

2. Track1 request filed by the applicant on December 22, 2016 has been granted by the office on January 10, 2017. Therefore instant application has been examined on a priority basis. To maintain its status applicant is required to follow the requirements listed in the Track1 grant document.

3. The present application is being examined under the pre-AIA first to invent provisions. The instant application is a continuation of 14/172,694, now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450, now U.S. Patent 8,563,274, which is a continuation of 13/622,872, now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, claiming priority from provisional application 61288792, filed on 12/21/2009 and from provisional application 61235595, filed on 08/20/2009.

Claim Status

4. In the claim listing of 5/1/2017 claims 40-55 are pending in this application and are under prosecution. Claims 40 and 53 are amended. Claims 1-39 are canceled. Claims have been reviewed and entered. The claim amendments have been reviewed and entered. No new matter has been introduced by the claim amendments as applicant has identified the support in the instant specification (Remarks, pg. 5).

5. The interview summary filed by the applicant has been acknowledged by the examiner (Remarks, pg. 5)

Withdrawn Objection, Rejections and Response to the Remarks

6. The previous objection of claim 53 has been withdrawn in view of fixing the minor informality in said claim (Remarks, pg. 6).

7. The previous new matter rejection of claims 40-55 under 35 USC pre-AIA first paragraph has been withdrawn in view of claim amendments and clarifying on the record that the instant specification at the time of filing has the support for the first and the second isolated single cell (Remarks, pgs. 6 and 7).

8. The previous rejection of claims 53-55 under 35 USC pre-AIA second paragraph has been withdrawn in view of claim amendments (Remarks, pgs. 7 and 8).

9. The previous rejection of claims 40-55 under 35 USC 103(a) as being unpatentable over Braverman in view of Drmanac has been withdrawn in view of the claim amendments, persuasive arguments made by the applicant that Braverman does

not teach the step of appending MID sequences prior to or during an amplification reaction and solely to expedite the prosecution (Remarks, pgs. 8-10).

10. The previous ODP rejections on the record have been withdrawn necessitated by the claim amendments. The arguments regarding ODP rejection of instant claims 40-55 over claims 1-12 of '756 patent in view of Drmanac have been fully considered (Remarks, pg. 11). They are moot in view of withdrawn ODP rejections necessitated by the claim amendments.

However, upon further consideration new grounds of rejections are set forth in this office action necessitated by the claim amendments.

Claim Rejections - 35 USC § 103

11. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

12. Claims 40-55 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Gillevet (WO 2008/061193, cited in the previous office action) in view Wangh et al (US 2003/0022231 published Jan 30, 2003).

Gillevet and Wangh teach a method for analyzing nucleic acid from plurality of samples and therefore are analogous arts. Gillevet teaches the steps of claims 40 and 53 including analyzing the nucleic acid from a plurality of nucleic acid template from multiple samples and sequencing the segments of the nucleic acid template (i.e., contiguous region of the genome) from each sample and identifying the nucleic acid segments from each sample by a tag sequence (i.e., a multiplex identifier) specific to

each of sample. Gillevet does not specifically teach that the nucleic acid segments from single isolated cell from each sample. However, the concept of using the nucleic acid from single isolated cell from each sample was known in the art at the time the claimed invention was made as taught by Wangh, which is very useful for a variety of uses, including research, forensic science and diagnostic applications least two isolated single cells.

Regarding claim 40, Gillevet teaches a multiplex method for the quantitative determination of polynucleotides in two or more samples, comprising: hybridizing a first primer to polynucleotides in a first sample, said first primer comprising a first tag sequence (i.e., a first multiplex identifier (MID)) and a first probe sequence specific for a first target sequence, wherein said first target sequence is 3' to a variable genetic region; elongating primer templates formed thereby to form a first population of tagged polynucleotides comprising: said first primer including said first tag sequence (i.e., first MID sequence); and sequences of said variable genetic region (encompasses the step of appending first MID); hybridizing a second primer to polynucleotides in a second sample, said second primer comprising a second tag sequence (i.e., a second MID) and a second probe sequence specific for a second target sequence, wherein said second target sequence is 3' to the same variable genetic region as said first target sequence, wherein further said second probe sequence may be the same as or different from said first probe sequence; elongating primer templates formed thereby to form a second population of tagged polynucleotides comprising: said second primer including said second tag sequence (encompasses the step of appending second MID); and

sequences of said variable genetic region; mixing said first and second populations together; determining sequences of polynucleotides comprising tag sequences and the sequences of the variable genetic element in said mixture; from the tag sequences comprised in the polynucleotide sequences thus determined identifying the sample in which polynucleotide sequences occurred; from the sequences of the variable genetic region comprised in the polynucleotide sequences thus determined identifying particular variants of said variable genetic element; from this information determining the number of time one or more given variants occur in each sample, and from the number for each variant in the polynucleotides thus determined, quantifying said polynucleotides in said samples (Fig. 1 and its legend in pgs. 13 and 14 and Gillevet claim 1). Gillevet also teaches that the tags are comprised in primers for PCR amplification (pg. 18, lines 14 and 15), which meets the limitation of first and second MID sequences are appended during an amplification reaction to form the first and second appended segments.

Gillevet does not specifically teach the nucleic acid genome is from single isolated cells. However, nucleic acid genome isolated from single isolated cell was known in the art at the time the claimed invention was made as taught by Wangh, who teaches a method, wherein nucleic acid molecules are isolated from single cell from a sample (Wangh claims 25 and 45) and further teaches that the nucleic acid content from the single cell are useful for a variety of uses, including research, forensic science and diagnostic applications (paragraphs 0187-0202), thus providing motivation to include to use of isolated single cell from the sample(s) of Wangh in the method of Gillevet.

One having ordinary skill in the art would also recognize that while combining the step of Wangh in the method of Gillevet some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step of use of isolated single cell comes from Wangh and not gleaned from the instant invention. One having ordinary skill in the art would be motivated to analyze the nucleic acid sequences from two different single cells rather than from two different samples because that would allow the monitor nucleic acid differences from individual genome of the cell.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid molecules from individual single cell of Wangh in the method of Gillevet with a reasonable expectation of success with the expected benefit of analyzing the nucleic acid sequences from individual isolated cells having a variety of uses, including research, forensic science and diagnostic applications as taught by Wangh. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art as exemplified by Wangh.

The teachings of Gillevet in view of Wangh regarding dependent claims 41-55 are discussed below.

Regarding claim 41, Gillevet teaches an amplification of plurality of nucleic acid attached to the MID sequence before determining the nucleic acid sequence (pg. 18, lines 14-18).

Regarding claims 42 and 43, Gillevet teaches that the nucleic acids are DNA (pg. 18, line 25) and RNA (pg. 9, lines 24 and 25). Wangh teaches nucleic acid molecule is mRNA (paragraph 0046).

Regarding claim 44, Gillevet teaches that the tagging step comprises ligation with nucleic acid (pg.8, lines 10-12).

Regarding claims 45 and 47, as discussed above while rejecting claim 40, Gillevet teaches that the tagging step comprises performing PCR of the plurality of nucleic acids and the MID sequence is in the primer (pg. 18, lines 12-18).

Regarding claim 46, as discussed above while rejecting claim 40, Gillevet in view of Wangh teaches providing a plurality of nucleic acids from each of a plurality of single cell sources; the attaching step comprises attaching different MID sequences to the nucleic acids from each of the plurality of single cell sources; and wherein nucleic acid sequences are attributed to different individual cell sources based upon the MID sequences attached in the attaching step and a sequencing primer sequence (Gillevet Fig. 1 and its legend in pgs. 13 and 14 and Gillevet claim 1 and Wangh claims 25 and 45).

Regarding claim 48, Gillevet teaches that the MID sequences (i.e., the tag sequences) are 2, 3, 4, 5, 6, 8, 10, 12 bases long, which is within the range of 2 to 100 bases in length (pg. 13, lines 16-18).

Regarding claims 49 and 50, Gillevet teaches that the nucleic acid segments are in the range of about 300 to 1700 bp (Fig. 4), which is in the claimed range of 100 to 5000 bp.

Regarding claim 51, Gillevet teaches a plurality of samples in the range of 5 to 10,000 samples, wherein each sample comprises a specific sequence tag (pg. 8, lines 3-9). One having ordinary skill in the art would recognize that the steps of claim 51 are obvious over Gillevet in view of Wangh because as discussed above while rejecting claim 40, Wangh provide motivation to include isolated single cell from each sample of Gillevet.

Regarding claim 52, Gillevet teaches samples are fragmented genomic DNAs (pg. 18, line 25) and further teaches amplified fragment is about 1700 bp (Fig. 4). Also the genomic fragment size in the range of 10-30 kb is routine, well established and conventional in the art as further evidenced by Drmanac (cited in the previous office action, paragraph 0023) and therefore obvious over Gillevet in view of Wangh.

Regarding claims 53-55, it is noted that claims 54 and 55 further specifies plurality of single isolated cells comprise first and second single isolated cells, wherein each cell type comprise specific MID sequence. As discussed above while rejecting claim 40, the subject matter of analyzing the first contiguous nucleic acid sequence from first single isolated cell and second contiguous nucleic acid sequence from second single isolated cell using MID sequence for each single isolated cell is obvious over Gillevet in view of Wangh for the reasons discussed above. Therefore the subject matter of claims 53-55 are obvious improvements over Gillevet in view of Wangh and therefore not novel.

Double Patenting

13. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

14. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

Regarding instant independent claims 40 and 53, claims 1, 2 and 11 of '756 patent discloses a method comprising: a) obtaining a composition comprising a mixture

of: i. a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and ii. a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprises a unique MID sequence that uniquely identifies each of the nucleic acid molecules; and b) sequencing said first plurality of segments and said a second plurality of segments to provide a plurality of sequences; wherein each sequence comprises: (i) the nucleotide sequence of at least a portion of a segment and (ii) an associated MID sequence, wherein said genomic DNA is genomic DNA from a virus or a bacterium, wherein defining a linkage relationship between said sequences using the MID sequence that is associated with each of the sequences, wherein sequences that comprise the first MID sequence are linked and sequences that comprise the second MID sequence are linked (common steps between instant claims 40 and 53 are underlined by the examiner). One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell.

However, claims of '756 patent do not disclose nucleic acid segments are from first and second isolated single cell. However, providing the genomic DNA from two

different cell types was known in the art at the time the claimed invention was made as taught by Gillevet, who teaches multiplex method for the quantitative determination of polynucleotides in two or more samples, wherein plurality of nucleic acids from first and second sample are appended with tag sequence during amplification, wherein the first tag sequence is different from the second tag sequence and determining the sequences of the polynucleotides including the tag sequences and identifying the variable genetic element in each sample based on tag sequence that identifies the sample type (Gillevet, claims 2 and 6), which are useful for detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like (pg. 18, lines 31 and 32).

Claims of '756 patent in view of Gillevet does not specifically teach that the nucleic acids are from a genome from the single isolated cell, which is taught by Wangh, who teaches method, wherein nucleic acid molecules are isolated from single cell (Wangh claim 2) and further teaches that the nucleic acid content from the single cell are useful for a variety of uses, including research, forensic science and diagnostic applications (paragraphs 0187-0202).One having ordinary skill in the art would also recognize that while combining the steps of '756 patent with the step of Gillevet and Wangh some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Gillevet and Wangh. One having ordinary skill in the art would be motivated to analyze the nucleic acid sequences from two different single cells rather than from plurality of different samples because that would allow the monitor nucleic acid differences from individual genome.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid molecules from a plurality of samples using tag sequence specific to each sample type of Gillevet and nucleic acid from individual single cell of Wangh in the method of '756 patent with a reasonable expectation of success with the expected benefit of analyzing the nucleic acid sequences from individual isolated cells having a variety of uses, including research, forensic science and diagnostic applications as taught by Gillevet and Wangh. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '756 patent in view of Gillevet and Wangh.

With regard to instant dependent claims 41-52, 54 and 55, claims of '756 patent in view of Gillevet and Wangh disclose the steps and therefore are not patentably distinct.

15. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

Regarding instant claims 40 and 53, claims 1 and 10 of '274 patent is drawn to a composition comprising a mixture of: a) a first plurality of different segments of a first nucleic acid molecule of a contiguous region in genomic DNA, wherein said contiguous

region in genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of segments is appended to a first multiplex identifier (MID) sequence; and b) a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different, wherein, in said composition, all of the segments of each of the nucleic acid molecules of the contiguous region comprise a unique MID sequence that uniquely identifies each of the nucleic acid molecules, wherein said genomic DNA is genomic DNA from a virus or a bacterium.

One having ordinary skill in the art would recognize that genomic DNA from a bacterium amounts of a genome from a single isolated cell. One having ordinary skill in the art would also recognize that providing a genome from a second isolated cell would be a duplication of the steps of '274 patent because instant claims as recited merely require analysis of the first and second appended segments and not linking the sequence to the first and the second isolated single cell.

However, claims of '274 patent do not disclose nucleic acid segments are from first and second isolated single cell. However, providing the genomic DNA from two different cell types was known in the art at the time the claimed invention was made as taught by Gillevet, who teaches multiplex method for the quantitative determination of polynucleotides in two or more samples, wherein plurality of nucleic acids from first and second sample are appended with tag sequence during amplification, wherein the first tag sequence is different from the second tag sequence and determining the sequences

of the polynucleotides including the tag sequences and identifying the variable genetic element in each sample based on tag sequence that identifies the sample type (Gillevet, claims 2 and 6), which are useful for detecting, monitoring, profiling, prognosticating, and/or diagnosing a disorder, disease, or the like (pg. 18, lines 31 and 32).

Claims of '274 patent in view of Gillevet does not specifically teach that the nucleic acids are from a genome from the single isolated cell, which is taught by Wangh, who teaches method, wherein nucleic acid molecules are isolated from single cell (Wangh claim 2) and further teaches the nucleic acid content from the single cell are useful for a variety of uses, including research, forensic science and diagnostic applications (paragraphs 0187-0202).One having ordinary skill in the art would also recognize that while combining the steps of '756 patent with the step of Gillevet and Wangh some routine optimization is necessary which is within the skills of one having ordinary skill in the art and motivation to include the missing step comes from Gillevet and Wangh. One having ordinary skill in the art would be motivated to analyze the nucleic acid sequences from two different single cells rather than from plurality of different samples because that would allow the monitor nucleic acid differences from individual genome.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of analyzing nucleic acid molecules from a plurality of samples using tag sequence specific to each sample type of Gillevet and nucleic acid from individual single cell of Wangh in the method of '274 patent with a reasonable expectation of success with the expected benefit of analyzing

the nucleic acid sequences from individual isolated cells having a variety of uses, including research, forensic science and diagnostic applications as taught by Gillevet and Wangh. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the two single cell analysis which is routinely done in the art and therefore the steps of instant claims 40 and 53 are not patentably distinct from claims of '274 patent in view of Gillevet and Wangh.

With regard to instant dependent claims 41-52, 54 and 55, claims of '274 patent in view of Gillevet and Wangh disclose the steps and therefore are not patentably distinct.

16. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 9,410,149 in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

The subject matter of claims 1-13 of '149 patent is similar to claims 1-11 of '756 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-13 of '149 patent in view of Gillevet and Wangh.

17. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of in view of Gillevet (WO 2008/061193, cited in the previous office action) and Wangh et al (US 2003/0022231 published Jan 30, 2003) for the following reasons.

The subject matter of claims 1-19 of '150 patent is similar to claims 1-11 of '274 patent. Therefore it is maintained that steps of instant claims 40-55 are not significantly different from claims 1-19 of '150 patent in view of Gillevet and Wangh.

Conclusion

18. No claims are allowed.

19. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 7 pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

Notice of References Cited	Application/Control No. 14/792,094	Applicant(s)/Patent Under Reexamination BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-2003/0022231 A1	01-2003	Wangh, Lawrence	C12Q1/6806	435/6.11
	B	US-				
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	4	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and isolat$5 near5 (single or individual or one) near5 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 09:47
L2	20	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and isolat$5 same (single or individual or one) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 09:59
L3	0	2 and genomic same (dna or nucleic or polynucleotide) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:02
L4	1	2 and genom$3 same (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:03
L5	2389	genomic same (dna or nucleic or polynucleotide) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:04
L6	107	5 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or tag or addressable) same (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:06
L7	26	6 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or tag or addressable) same (different or distinct or distiguish$4) same (origin or cell or sample or library).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:08
L8	298	("multiplex identifier" or barcode or	US-PGPUB;	OR	ON	2017/05/25

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		"unique tag" or "unique identifier" "bar code" or tag or addressable) near5(different or distinct or distiguish$4) near5 (origin or cell or sample or library).clm.	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			10:11
L9	103	8 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or tag or addressable) near5 (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:12
L10	7298	(dna or nucleic) near5 (single or individual or one) near5 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:22
L11	36	10 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier" "bar code" or addressable) near5 (dna or nucleic or polynucleotide).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:23
L12	911	(isolat$3 or extract$3 or purif$5)near5 (dna or nucleic) near5 ("single cell" or "individual cell" or "one cell")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:58
L13	66	12 and(isolat$3 or extract$3 or purif$5)near5 (dna or nucleic) near5 ("single cell" or "individual cell" or "one cell").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/05/25 10:58
S1	29794	brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:50
S2	134	S1 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:51
S3	536	S1 and (dna or nucleic or polynucleotide) same (pcr or amplif$7)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2017/01/23 10:54

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			IBM_TDB			
S4	18	S3 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:56
S5	4	S4 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 10:58
S6	139392	(single or individual or one) near3 cell same (dna or nucleic or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:01
S7	3492	S6 and (adapt$3 or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:02
S8	872	S7 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:02
S9	88	S8 and (single or individual or one) near3 cell near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S10	84	S8 and (single or individual or one) near3 cell near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:03
S11	66	S10 and first near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:05
S12	47	S11 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2017/01/23 11:05

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			JPO; DERWENT; IBM_TDB			
S13	43263	"first cell" and "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:15
S14	1761	S13 and (dna or nucleic or polynucleotide) same "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:16
S15	1549	S14 and (dna or nucleic or polynucleotide) same "second cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:17
S16	143	S15 and (adapt$3 or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:18
S17	27	S16 and second near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:19
S18	35	S16 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:20
S19	870	(single or individual or one) near3 cell near5 isolat$4 near5 first	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:29
S20	315	S19 and (single or individual or one) near3 cell near5 isolat$4 near5 second	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:30
S21	120	S20 and (dna or nucleic or polynucleotide) near5 cell	US-PGPUB; USPAT;	OR	ON	2017/01/23 11:31

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			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S22	25	S21 and (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:31
S23	12	S21 and sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:34
S24	540	sequencing same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same cell near5 (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:36
S25	381	S24 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same (identif$4 or detect$3 or dtermin$3) same cell near5 (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:37
S26	67	S25 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or dtermin$3) same cell adj (type or individual$3 or single)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:38
S27	2799	"first cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:47
S28	1549	S27 and "second cell" same (nucleic or dna or polynucleotide)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:48
S29	1	S28 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence near5 (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:49

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S30	8	S27 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near3 sequence same (identif$4 or detect$3 or determin$3) near5 "first cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:51
S36	2321	(mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)near5 sequence near5 (identif$4 or detect$3 or determin$3 or distinguish$3) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 11:59
S37	46	S36 and (append$3 or ligat$3) near5 (adpator or adapter or linker) near5 (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier) same (dna or nucleic or polynucleotide) near5 (cell or sample)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 12:02
S38	21	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (nucleic or dna or polynucleotide)same (adaptor or adapter or linker) same (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
S39	8	S38 and sequencing.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:32
S40	1	S39 and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:34
S41	134	(brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "10X GENOMICS".as. or "COMPASS GENETICS".as. or "population genetics".as.) and (single or individual or one) near3 cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:35
S42	3	S41 and (mid or "multiplex identifier" or barcode or tag or zipcode or token or "bar code" or "bar-code" addressable or identifier)same sequence same (identif$4 or detect$3 or determin$3 or distinguish$3) same cell.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 17:36
S43	29	"9410150"	US-PGPUB; USPAT;	OR	ON	2017/01/23 18:04

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			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S44	8	"9410149".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:05
S45	3	"8563274".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:06
S46	2	"8679756".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/23 18:08
S47	239	"multiplex identifier"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:33
S48	16	S47 and ("single cell" or "Individual cell") near5 (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:34
S49	12	"multiplex identifier" near5 (adaptor or linker or adapter)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:35
S50	56	"multiplex identifier" same (adaptor or linker or adapter)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:40
S51	5080	("multiplex identifier" or barcode or "unique tag" or "unique identifier")same (adaptor or linker or adapter)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:49

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S52	233	S51 and ("single cell" or "Individual cell") near5 (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:50
S53	226	S52 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")same (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:50
S54	166	S53 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 sequence same(identif$4 or detect$3 or determin$3 or distinguish$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:52
S55	11	S54 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 contiguous	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:53
S56	56	S51 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 contiguous	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:53
S57	3	S51 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")same contiguous near5 genomic near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:57
S58	13	("multiplex identifier" or barcode or "unique tag" or "unique identifier")same contiguous near5 genomic near5 sequence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:58
S59	2	("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 contiguous near5 genomic	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 14:59
S60	179	("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 genomic near5 (dna or nucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2017/01/24 15:00

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			DERWENT; IBM_TDB			
S61	91	S60 and ("multiplex identifier" or barcode or "unique tag" or "unique identifier")near5 sequence same(identif$4 or detect$3 or determin$3 or distinguish$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/01/24 15:00

5/ 25/ 2017 11:02:58 AM
C:\ Users\ nbhat1\ Documents\ EAST\ Workspaces\ 14792094-1-23-17.wsp

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14792094	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.		
Updated the search for the topics in EAST. Search history is enclosed.	5/25/17	Bhat N.

INTERFERENCE SEARCH

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/NARAYAN BHAT/ Primary Examiner.Art Unit 1634

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Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (07-14)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	14/792,094	Filing Date	2015-07-06	Docket Number (if applicable)	104371-5016-US05	Art Unit	1634
First Named Inventor	Sydney Brenner			Examiner Name	Narayan Kameshwar Bhat		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not comply with the requirements of 35 U.S.C. 371, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV.

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 500310

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (07-14)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Signature of Registered U.S. Patent Practitioner			
Signature	/Gargi Talukder/	Date (YYYY-MM-DD)	2017-08-30
Name	Gargi Talukder	Registration Number	61368

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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ftIN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9697
Sidney Brenner et al.	Examiner: Narayan Kameshwar Bhat
Application No.: 14/792,094	Art Unit: 1634
Filed: July 6, 2015	
For: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID ARRANGEMENT	RESPONSE TO FINAL OFFICE ACTION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Final Office Action mailed May 31, 2017, please enter the following amendments and remarks:

Amendments to the Claims are reflected in the listing of claims which begins on page **2** of this paper.

Remarks/Arguments begin on page **4** of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-55. (Cancelled)

56. (New) A method of analyzing nucleic acids, the method comprising:

(a) providing a plurality of nucleic acid samples, each made from single isolated cells;

(b) tagging a plurality of segments of the nucleic acid molecules in the samples of (a) with first and second multiplex identifier (MID) sequences, wherein:

(i) the first MID sequences associated with a segment of a nucleic acid molecule of (a) uniquely identifies the single isolated cell from which the nucleic acid molecule is provided; and

(ii) the second MID sequences are all different from each other;

(c) sequencing the tagged segments of (b) to obtain the nucleotide sequences of the plurality of nucleic acids and the attached first and second MID sequences.

57. (New) The method of claim 56, wherein the nucleic acids are mRNA.

58. (New) The method of claim 56, wherein the method further comprises amplifying the segments and the appended MID sequences before the sequencing step (c).

59. (New) The method of claim 56, wherein the MID sequences are appended to the segments through ligation.

60. (New) The method of claim 56, wherein the MID sequences are appended to the segments by performing PCR or linear amplification of the plurality of nucleic acids using an adapter sequence comprising the MID sequence and an amplification primer.

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61. (New) The method of claim 56, wherein the MID sequences further comprise a sequencing primer sequence.

62. (New) The method of claim 56, wherein the MID sequences are from 2 to 100 bases in length.

63. (New) The method of claim 56, wherein the segments of (a) and (b) are in the range of 100 to 5,000 bp in length.

64. (New) The method of claim 40, wherein the segments are 400 bp or less in length.

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Remarks/Arguments

Applicants have not dedicated or abandoned any unclaimed subject matter and have not acquiesced to any rejections made by the Patent Office. Applicants reserve the right to pursue prosecution of any presently excluded claim embodiments in future continuation and/or divisional applications.

Status of the Claims

Claims 56-64 are newly added and are fully supported by the specification and claims as originally filed.

Applicants respectfully request entry of the claims as amended into the record.

Response to Office Action

Claim Rejections – 35 USC § 103

Claims 40-55 are rejected under 35 U.S.C. § 103 as being allegedly anticipated by Gillevet, WO 2008/061193 ("Gillevet") in view of Wangh et al., US 2003/0022231 ("Wangh"). Applicants respectfully traverse these rejections.

Applicants submit that the combined teachings of Gillevet and Wangh fail to teach all elements of the claimed invention. In particular, the combined teachings of these references fail to teach methods of analyzing nucleic acids from single isolated cells, where segments of the nucleic acids are appended to two MID sequences and where the first MID sequence identifies the source of the nucleic acid segments and the second MID sequence is unique from the other second MID sequences and can be used for further deep analysis of the sample sequences, including quantification. Neither Gillevet or Wangh alone or in combination teaches this element of the claimed invention.

Applicants further note that Gillevet teaches a method for sample indexing – such a method would have no need for a second MID sequence as recited in the present claims. In

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addition, Wangh is unrelated to a sequencing method – the methods taught in Wangh are real-time PCR assay methods that answer a "yes or no" question, i.e., whether a particular sequence is present in a sample. There is no analysis of segments appended to two MID sequences as recited in the present claims. As such, the skilled artisan would have no reasonable expectation of success in practicing the claimed invention based on the combined teachings of Gillevet and Wangh, because not only do both references lack elements of the claimed invention, but also the teachings in the two references are incompatible with each other.

For at least the foregoing reasons, Applicants respectfully submit that an obviousness rejection cannot be maintained based on the teachings of Gillevet and Wangh and request that all rejections under § 103 be withdrawn.

Claim Rejections – Double Patenting

Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Gillevet, WO 2008/061193 ("Gillevet") and Wangh et al., US 2003/0022231. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Gillevet, WO 2008/061193 ("Gillevet") and Wangh et al., US 2003/0022231. Claims 40-55 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of Gillevet, WO 2008/061193 ("Gillevet") and Wangh et al., US 2003/0022231.

For the reasons recited above, Applicants respectfully submit the presently claimed invention does not support a rejection on the ground of nonstatutory double patenting over any of the cited patents, as the present claims are not obvious in light of U.S. Patent No. 8,679,756 in view of Gillevet and Wangh. Applicants respectfully request that all double patenting rejections be withdrawn.

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CONCLUSION

All the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action, and as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided below.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any necessary fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17, which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310 (Order No.: 104371-5016-US05).

Prompt and favorable consideration of this Amendment and Response is respectfully requested.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: August 30, 2017 By: /Gargi Talukder/
Gargi Talukder, Reg. No. 61,368

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: RARE CELL ANALYSIS USING SAMPLE SPLITTING AND DNA TAGS

(57) Abstract: The present invention provides systems, apparatuses, and methods to detect the presence of fetal cells when mixed with a population of maternal cells in a sample and to test fetal abnormalities, e.g. aneuploidy. The present invention involves labeling regions of genomic DNA in each cell in said mixed sample with different labels wherein each label is specific to each cell and quantifying the labeled regions of genomic DNA from each cell in the mixed sample. More particularly the invention involves quantifying labeled DNA polymorphisms from each cell in the mixed sample.

WO 2007/147079 A2

RARE CELL ANALYSIS USING SAMPLE SPLITTING AND DNA TAGS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 60/804,819, filed June 14, 2006, which application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Analysis of specific cells can give insight into a variety of diseases. These analyses can provide non-invasive tests for detection, diagnosis and prognosis of diseases such as cancer or fetal disorders, thereby eliminating the risk of invasive diagnosis. Regarding fetal disorders, current prenatal diagnosis, such as amniocentesis and chorionic villus sampling (CVS), are potentially harmful to the mother and to the fetus. The rate of miscarriage for pregnant women undergoing amniocentesis is increased by 0.5-1%, and that figure is slightly higher for CVS. Because of the inherent risks posed by amniocentesis and CVS, these procedures are offered primarily to older women, e.g., those over 35 years of age, who have a statistically greater probability of bearing children with congenital defects. As a result, a pregnant woman at the age of 35 has to balance an average risk of 0.5-1% to induce an abortion by amniocentesis against an age related probability for trisomy 21 of less than 0.3%.

[0003] Regarding prenatal diagnostics, some non-invasive methods have already been developed to screen for fetuses at higher risk of having specific congenital defects. For example, maternal serum alpha-fetoprotein, and levels of unconjugated estriol and human chorionic gonadotropin can be used to identify a proportion of fetuses with Down's syndrome. However, these tests suffer from many false positive. Similarly, ultrasonography is used to determine congenital defects involving neural tube defects and limb abnormalities, but such methods are limited to time periods after fifteen weeks of gestation and are present unreliable results.

[0004] The presence of fetal cells within the blood of pregnant women offers the opportunity to develop a prenatal diagnostic that replaces amniocentesis and thereby eliminates the risk of today's invasive diagnosis. However, fetal cells represent a small number of cells against the background of a large number of maternal cells in the blood which make the analysis time consuming and prone to error.

[0005] With respect to cancer diagnosis, early detection is of paramount importance. Cancer is a disease marked by the uncontrolled proliferation of abnormal cells. In normal tissue, cells divide and organize within the tissue in response to signals from surrounding cells. Cancer cells do not respond in the same way to these signals, causing them to proliferate and, in many organs, form a tumor. As the growth of a tumor continues, genetic alterations may accumulate, manifesting as a more aggressive growth phenotype of the cancer cells. If left untreated, metastasis, the spread of cancer cells to distant areas of the body by way of the lymph system or bloodstream, may ensue. Metastasis results in the formation of secondary tumors at multiple sites, damaging healthy tissue. Most cancer death is caused by such secondary tumors. Despite decades of advances in cancer diagnosis and therapy, many cancers continue to go undetected until late in their development. As one example, most early-stage lung cancers are asymptomatic and are not detected in time for curative treatment, resulting in an overall five-year survival rate for patients with lung cancer of less than 15%. However, in those instances in which lung cancer is detected and treated at an early stage, the prognosis is much more favorable.

[0006] The methods of the present invention allow for the detection of fetal cells and fetal abnormalities when fetal cells are mixed with a population of maternal cells, even when the maternal cells dominate the mixture. In addition, the methods of the present invention can also be utilized to detect or diagnose cancer.

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SUMMARY OF THE INVENTION

[0007] The present invention relates to methods for the detection of fetal cells or cancer cells in a mixed sample. In one embodiment, the present invention provides methods for determining fetal abnormalities in a sample comprising fetal cells that are mixed with a population of maternal cells. In some embodiments, determining the presence of fetal cells and fetal abnormalities comprises labeling one or more regions of genomic DNA in each cell from a mixed sample comprising at least one fetal cell with different labels wherein each label is specific to each cell. In some embodiments, the genomic DNA to be labeled comprises one or more polymorphisms, particularly STRs or SNPs

[0008] In some embodiments, the methods of the invention allow for simultaneously detecting the presence of fetal cells and fetal abnormalities when fetal cells are mixed with a population of maternal cells, even when the maternal cells dominate the mixture. In some embodiments, the sample is enriched to contain at least one fetal and one non fetal cell, and in other embodiments, the cells of the enriched population can be divided between two or more discrete locations that can be used as addressable locations. Examples of addressable locations include wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles or in-situ within a cell or nuclear membrane.

[0009] In some embodiments, the methods comprise labeling one or more regions of genomic DNA in each cell in the enriched sample with different labels, wherein each label is specific to each cell, and quantifying the labeled DNA regions. The labeling methods can comprise adding a unique tag sequence for each cell in the mixed sample. In some embodiments, the unique tag sequence identifies the presence or absence of a DNA polymorphism in each cell from the mixed sample. Labels are added to the cells/DNA using an amplification reaction, which can be performed by PCR methods. For example, amplification can be achieved by multiplex PCR. In some embodiments, a further PCR amplification is performed using nested primers for the genomic DNA region(s).

[0010] In some embodiments, the DNA regions can be amplified prior to being quantified. The labeled DNA can be quantified using sequencing methods, which, in some embodiments, can precede amplifying the DNA regions. The amplified DNA region(s) can be analyzed by sequencing methods. For example, ultra deep sequencing can be used to provide an accurate and quantitative measurement of the allele abundances for each STR or SNP. In other embodiments, quantitative genotyping can be used to declare the presence of fetal cells and to determine the copy numbers of the fetal chromosomes. Preferably, quantitative genotyping is performed using molecular inversion probes.

[0011] The invention also relates to methods of identifying cells from a mixed sample with non-maternal genomic DNA and identifying said cells with non-maternal genomic DNA as fetal cells. In some embodiments, the ratio of maternal to paternal alleles is compared on the identified fetal cells in the mixed sample.

[0012] .In one embodiment, the invention provides for a method for determining a fetal abnormality in a maternal sample that comprises at least one fetal and one non fetal cell. The sample can be enriched to contain at least one fetal cell, and the enriched maternal sample can be arrayed into a plurality of discrete sites. In some embodiments, each discrete site comprises no more than one cell.

[0013] In some embodiments, the invention comprises labeling one or more regions of genomic DNA from the arrayed samples using primers that are specific to each DNA region or location, amplifying the DNA region(s), and quantifying the labeled DNA region. The labeling of the DNA region(s) can comprise labeling each region with a unique tag sequence, which can be used to identify the presence or absence of a DNA polymorphism on arrayed cells and the distinct location of the cells.

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[0014] The step of determining can comprise identifying non-maternal alleles at the distinct locations, which can result from comparing the ratio of maternal to paternal alleles at the location. In some embodiments, the method of identifying a fetal abnormality in an arrayed sample can further comprise amplifying the genomic DNA regions. The genomic DNA regions can comprise one or more polymorphisms e.g. STRs and SNPs, which can be amplified using PCR methods including multiplex PCR. An additional amplification step can be performed using nested primers.

[0015] The amplified DNA region(s) can be analyzed by sequencing methods. For example, ultra deep sequencing can be used to provide an accurate and quantitative measurement of the allele abundances for each STR or SNP. In other embodiments, quantitative genotyping can be sued to declare the presence of fetal cells and to determine the copy numbers of the fetal chromosomes. Preferably, quantitative genotyping is performed using molecular inversion probes.

[0016] In one embodiment, the invention provides methods for diagnosing a cancer and giving a prognosis by obtaining and enriching a blood sample from a patient for epithelial cells, splitting the enriched sample into discrete locations, and performing one or more molecular and/or morphological analyses on the enriched and split sample. The molecular analyses can include detecting the level of expression or a mutation of gene disclosed in Figure 10. Preferably, the method comprises performing molecular analyses on EGFR, EpCAM, GA733-2, MUC-1, HER-2, or Claudin-7 in each arrayed cell. The morphological analyses can include identifying, quantifying and /or characterizing mitochondrial DNA, telomerase, or nuclear matrix proteins.

[0017] In some embodiments, the sample can be enriched for epithelial cells by at least 10,000 fold, and the diagnosis and prognosis can be provided prior to treating the patient for the cancer. Preferably, the blood samples are obtained from a patient at regular intervals such as daily, or every 2, 3 or 4 days, weekly, bimonthly, monthly, bi-yearly or yearly.

[0018] In some embodiments, the step of enriching a patient's blood sample for epithelial cells involves flowing the sample through a first array of obstacles that selectively directs cells that are larger than a predetermined size to a first outlet and cells that are smaller than a predetermined size to a second outlet. Optionally, the sample can be subjected to further enrichment by flowing the sample through a second array of obstacles, which can be coated with antibodies that selectively bind to white blood cells or epithelial cells. For example, the obstacles of the second array can be coated with anti-EpCAM antibodies.

[0019] Splitting the sample of cells of the enriched population can comprises splitting the enriched sample to locate individual cells at discrete sites that can be addressable sites. Examples of addressable locations include wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles or in-situ within a cell or nuclear membrane.

[0020] In some embodiments there are provided kits comprising devices for enriching the sample and the devices and reagents needed to perform the genetic analysis. The kits may contain the arrays for size-based separation, reagents for uniquely labeling the cells, devices for splitting the cells into individual addressable locations and reagents for the genetic analysis.

SUMMARY OF THE DRAWINGS

[0021] **Figures 1A-1D** illustrate various embodiments of a size-based separation module.

[0022] **Figures 2A-2C** illustrate one embodiment of an affinity separation module.

[0023] **Figure 3** illustrate one embodiment of a magnetic separation module.

[0024] **Figure 4** illustrates an overview for diagnosing, prognosing, or monitoring a prenatal condition in a fetus.

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[0025] **Figure 5** illustrates an overview for diagnosing, prognosing, or monitoring a prenatal condition in a fetus.

[0026] **Figure 6** illustrates an overview for diagnosing, prognosing or monitoring cancer in a patient.

[0027] **Figures 7A-7B** illustrate an assay using molecular inversion probes. Figure 7 C illustrates an overview of the use of nucleic acid tags.

[0028] **Figures 8A-8C** illustrate one example of a sample splitting apparatus.

[0029] **Figure 9** illustrates the probability of having 2 or more CTC's loaded into a single sample well.

[0030] **Figure 10** illustrates genes whose expression or mutations can be associated with cancer or another condition diagnosed herein.

[0031] **Figure 11** illustrates primers useful in the methods herein.

[0032] **Figure 12A-B** illustrate cell smears of the product and waste fractions.

[0033] **Figure 13A-F** illustrate isolated fetal cells confirmed by the reliable presence of male cells.

[0034] **Figure 14** illustrates cells with abnormal trisomy 21 pathology.

[0035] **Figure 15** illustrates performance of a size-based separation module.

[0036] **Figure 16** illustrates histograms of these cell fractions resulting from a size-based separation module.

[0037] **Figure 17** illustrates a first output and a second output of a size-based separation module.

[0038] **Figure 18** illustrates epithelial cells bound to a capture module of an array of obstacles coated with anti-EpCAM.

[0039] **Figures 19A-C** illustrate one embodiment of a flow-through size-based separation module adapted to separate epithelial cells from blood and alternative parameters that can be used with such device.

[0040] **Figure 20A-D** illustrate various targeted subpopulations of cells that can be isolated using size-based separation and various cut-off sizes that can be used to separate such targeted subpopulations.

[0041] **Figure 21** illustrates a device of the invention with counting means to determine the number of cells in the enriched sample.

[0042] **Figure 22** illustrates an overview of one aspect of the invention for diagnosing, prognosing, or monitoring cancer in a patient.

[0043] **Figure 23** illustrates the use of EGFR mRNA for generating sequencing templates.

[0044] **Figure 24** illustrates performing real-time quantitative allele-specific PCR reactions to confirm the sequence of mutations in EGFR mRNA.

[0045] **Figure 25** illustrates confirmation of the presence of a mutation is when the signal from a mutant allele probe rises above the background level of fluorescence.

[0046] **Figure 26A-B** illustrate the presence of EGFR mRNA in epithelial cells but not leukocytes.

[0047] **Figure 27** illustrate results of the first and second EGFR PCR reactions.

[0048] **Figure 28A-B** results of the first and second EGFR PCR reactions.

[0049] **Figure 29** illustrates that EGFR wild type and mutant amplified fragments are readily detected, despite the high leukocyte background.

[0050] **Figure 30** illustrates the detection of single copies of a fetal cell genome by qPCR.

[0051] **Figure 31** illustrates detection of single fetal cells in binned samples by SNP analysis.

[0052] **Figure 32** illustrates a method of trisomy testing. The trisomy 21 screen is based on scoring of target cells obtained from maternal blood. Blood is processed using a cell separation module for hemoglobin enrichment (CSM-HE). Enriched cells are transferred to slides that are first stained and subsequently probed by FISH. Images are acquired, such as from bright field or fluorescent microscopy, and scored. The proportion of trisomic cells of certain classes serves as a classifier for risk of fetal trisomy 21. Fetal genome identification can performed using

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assays such as: (1) STR markers; (2) qPCR using primers and probes directed to loci, such as the multi-repeat DYZ locus on the Y-chromosome; (3) SNP detection; and (4) CGH (comparative genome hybridization) array detection.

[0053] **Figure 33** illustrates assays that can produce information on the presence of aneuploidy and other genetic disorders in target cells. Information on aneuploidy and other genetic disorders in target cells may be acquired using technologies such as: (1) a CGH array established for chromosome counting, which can be used for aneuploidy determination and/or detection of intra-chromosomal deletions; (2) SNP/taqman assays, which can be used for detection of single nucleotide polymorphisms; and (3) ultra-deep sequencing, which can be used to produce partial or complete genome sequences for analysis.

[0054] **Figure 34** illustrates methods of fetal diagnostic assays. Fetal cells are isolated by CSM-HE enrichment of target cells from blood. The designation of the fetal cells may be confirmed using techniques comprising FISH staining (using slides or membranes and optionally an automated detector), FACS, and/or binning. Binning may comprise distribution of enriched cells across wells in a plate (such as a 96 or 384 well plate), microencapsulation of cells in droplets that are separated in an emulsion, or by introduction of cells into microarrays of nanofluidic bins. Fetal cells are then identified using methods that may comprise the use of biomarkers (such as fetal (gamma) hemoglobin), allele-specific SNP panels that could detect fetal genome DNA, detection of differentially expressed maternal and fetal transcripts (such as Affymetrix chips), or primers and probes directed to fetal specific loci (such as the multi-repeat DYZ locus on the Y-chromosome). Binning sites that contain fetal cells are then be analyzed for aneuploidy and/or other genetic defects using a technique such as CGH array detection, ultra deep sequencing (such as Solexa, 454, or mass spectrometry), STR analysis, or SNP detection.

Figure 35 illustrates methods of fetal diagnostic assays, further comprising the step of whole genome amplification prior to analysis of aneuploidy and/or other genetic defects.

INCORPORATION BY REFERENCE

[0055] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

DETAILED DESCRIPTION OF THE INVENTION

[0056] The present invention provides systems, apparatus, and methods to detect the presence of or abnormalities of rare analytes or cells, such as hematapoeitic bone marrow progenitor cells, endothelial cells, fetal cells, epithelial cells, or circulating tumor cells in a sample of a mixed analyte or cell population (e.g., maternal peripheral blood samples).

I. Sample Collection/Preparation

[0057] Samples containing rare cells can be obtained from any animal in need of a diagnosis or prognosis or from an animal pregnant with a fetus in need of a diagnosis or prognosis. In one example, a sample can be obtained from animal suspected of being pregnant, pregnant, or that has been pregnant to detect the presence of a fetus or fetal abnormality. In another example, a sample is obtained from an animal suspected of having, having, or an animal that had a disease or condition (e.g. cancer). Such condition can be diagnosed, prognosed, monitored and therapy can be determined based on the methods and systems herein. Animal of the present invention can be a human or a domesticated animals such as a cow, chicken, pig, horse, rabbit, dogs, cat, or goat. Samples derived from an animal or human can include, e.g., whole blood, sweat, tears, ear flow, sputum, lymph, bone marrow suspension, lymph, urine, saliva, semen, vaginal flow, cerebrospinal fluid, brain fluid, ascites, milk, secretions of the respiratory, intestinal or genitourinary tracts fluid.

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[0058] To obtain a blood sample, any technique known in the art may be used, e.g. a syringe or other vacuum suction device. A blood sample can be optionally pre-treated or processed prior to enrichment. Examples of pre-treatment steps include the addition of a reagent such as a stabilizer, a preservative, a fixant, a lysing reagent, a diluent, an anti-apoptotic reagent, an anti-coagulation reagent, an anti-thrombotic reagent, magnetic property regulating reagent, a buffering reagent, an osmolality regulating reagent, a pH regulating reagent, and/or a cross-linking reagent.

[0059] When a blood sample is obtained, a preservative such an anti-coagulation agent and/or a stabilizer is often added to the sample prior to enrichment. This allows for extended time for analysis/detection. Thus, a sample, such as a blood sample, can be enriched and/or analyzed under any of the methods and systems herein within 1 week, 6 days, 5 days, 4 days, 3 days, 2 days, 1 day, 12 hrs, 6 hrs, 3 hrs, 2 hrs, or 1 hr from the time the sample is obtained.

[0060] In some embodiments, a blood sample can be combined with an agent that selectively lyses one or more cells or components in a blood sample. For example, fetal cells can be selectively lysed releasing their nuclei when a blood sample including fetal cells is combined with deionized water. Such selective lysis allows for the subsequent enrichment of fetal nuclei using, e.g., size or affinity based separation. In another example platelets and/or enucleated red blood cells are selectively lysed to generate a sample enriched in nucleated cells, such as fetal nucleated red blood cells (fnRBC's), maternal nucleated blood cells (mnBC), epithelial cells and circulating tumor cells. fnRBC's can be subsequently separated from mnBC's using, e.g., antigen-i affinity or differences in hemoglobin

[0061] When obtaining a sample from an animal (e.g., blood sample), the amount can vary depending upon animal size, its gestation period, and the condition being screened. In some embodiments, up to 50, 40, 30, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 mL of a sample is obtained. In some embodiments, 1-50, 2-40, 3-30, or 4-20 mL of sample is obtained. In some embodiments, more than 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mL of a sample is obtained.

[0062] To detect fetal abnormality, a blood sample can be obtained from a pregnant animal or human within 36, 24, 22, 20, 18, 16, 14, 12, 10, 8, 6 or 4 weeks of gestation.

II. Enrichment

[0063] A sample (e.g. blood sample) can be enriched for rare analytes or rare cells (e.g. fetal cells, epithelial cells or circulating tumor cells) using one or more any methods known in the art (e.g. Guetta, EM et al. Stem Cells Dev, 13(1):93-9 (2004)) or described herein. The enrichment increases the concentration of rare cells or ratio of rare cells to non-rare cells in the sample. For example, enrichment can increase concentration of an analyte of interest such as a fetal cell or epithelial cell or CTC by a factor of at least 2, 4, 6, 8, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 2,000,000, 5,000,000, 10,000,000, 20,000,000, 50,000,000, 100,000,000, 200,000,000, 500,000,000, 1,000,000,000, 2,000,000,000, or 5,000,000,000 fold over its concentration in the original sample. In particular, when enriching fetal cells from a maternal peripheral venous blood sample, the initial concentration of the fetal cells may be about 1:50,000,000 and it may be increased to at least 1:5,000 or 1:500. Enrichment can also increase concentration of rare cells in volume of rare cells / total volume of sample (removal of fluid). A fluid sample (e.g., a blood sample) of greater than 10, 15, 20, 50, or 100 mL total volume comprising rare components of interest, and it can be concentrated such that the rare component of interest into a concentrated solution of less than 0.5, 1, 2, 3, 5, or 10 mL total volume.

[0064] Enrichment can occur using one or more types of separation modules. Several different modules are described herein, all of which can be fluidly coupled with one another in the series for enhanced performance.

[0065] In some embodiments, enrichment occurs by selective lysis as described above.

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[0066] In one embodiment, enrichment of rare cells occurs using one or more size-based separation modules. Examples of size-based separation modules include filtration modules, sieves, matrixes, etc. Examples of size-based separation modules contemplated by the present invention include those disclosed in International Publication No. WO 2004/113877. Other size based separation modules are disclosed in International Publication No. WO 2004/0144651.

[0067] In some embodiments, a size-based separation module comprises one or more arrays of obstacles forming a network of gaps. The obstacles are configured to direct particles as they flow through the array/network of gaps into different directions or outlets based on the particle's hydrodynamic size. For example, as a blood sample flows through an array of obstacles, nucleated cells or cells having a hydrodynamic size larger than a predeterminedcertain size such as a cuttoff or predetermined size, e.g., 8 microns, are directed to a first outlet located on the opposite side of the array of obstacles from the fluid flow inlet, while the enucleated cells or cells having a hydrodynamic size smaller than a predetermined size, e.g., 8 microns, are directed to a second outlet also located on the opposite side of the array of obstacles from the fluid flow inlet.

[0068] An array can be configured to separate cells smaller or larger than a predetermined size by adjusting the size of the gaps, obstacles, and offset in the period between each successive row of obstacles. For example, in some embodiments, obstacles or gaps between obstacles can be up to 10, 20, 50, 70, 100, 120, 150, 170, or 200 microns in length or about 2, 4, 6, 8 or 10 microns in length. In some embodiments, an array for size-based separation includes more than 100, 500, 1,000, 5,000, 10,000, 50,000 or 100,000 obstacles that are arranged into more than 10, 20, 50, 100, 200, 500, or 1000 rows. Preferably, obstacles in a first row of obstacles are offset from a previous (upstream) row of obstacles by up to 50% the period of the previous row of obstacles. In some embodiments, obstacles in a first row of obstacles are offset from a previous row of obstacles by up to 45, 40, 35, 30, 25, 20, 15 or 10% the period of the previous row of obstacles. Furthermore, the distance between a first row of obstacles and a second row of obstacles can be up to 10, 20, 50, 70, 100, 120, 150, 170 or 200 microns. A particular offset can be continuous (repeating for multiple rows) or non-continuous. In some embodiments, a separation module includes multiple discrete arrays of obstacles fluidly coupled such that they are in series with one another. Each array of obstacles has a continuous offset. But each subsequent (downstream) array of obstacles has an offset that is different from the previous (upstream) offset. Preferably, each subsequent array of obstacles has a smaller offset that the previous array of obstacles. This allows for a refinement in the separation process as cells migrate through the array of obstacles. Thus, a plurality of arrays can be fluidly coupled in series or in parallel, (e.g., more than 2, 4, 6, 8, 10, 20, 30, 40, 50). Fluidly coupling separation modules (e.g., arrays) in parallel allows for high-throughput analysis of the sample, such that at least 1, 2, 5, 10, 20, 50, 100, 200, or 500 mL per hour flows through the enrichment modules or at least 1, 5, 10, or 50 million cells per hour are sorted or flow through the device.

[0069] **Figure 1A** illustrates an example of a size-based separation module. Obstacles (which may be of any shape) are coupled to a flat substrate to form an array of gaps. A transparent cover or lid may be used to cover the array. The obstacles form a two-dimensional array with each successive row shifted horizontally with respect to the previous row of obstacles, where the array of obstacles directs component having a hydrodynamic size smaller than a predetermined size in a first direction and component having a hydrodynamic size larger that a predetermined size in a second direction. For enriching epithelial or circulating tumor cells from enucleated, the predetermined size of an array of obstacles can be get at 6-12 μm or 6-8 μm. For enriching fetal cells from a mixed sample (e.g. maternal blood sample) the predetermined size of an array of obstacles can be between 4-10 μm or 6-8 μm. The flow of sample into the array of obstacles can be aligned at a small angle (flow angle) with respect to a line-of-sight of the array. Optionally, the array is coupled to an infusion pump to perfuse the sample through the obstacles. The flow

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conditions of the size-based separation module described herein are such that cells are sorted by the array with minimal damage. This allows for downstream analysis of intact cells and intact nuclei to be more efficient and reliable.

[0070] In some embodiments, a size-based separation module comprises an array of obstacles configured to direct cells larger than a predetermined size to migrate along a line-of-sight within the array (e.g. towards a first outlet or bypass channel leading to a first outlet), while directing cells and analytes smaller than a predetermined size to migrate through the array of obstacles in a different direction than the larger cells (e.g. towards a second outlet). Such embodiments are illustrated in part in **Figures 1B-1D**.

[0071] A variety of enrichment protocols may be utilized although gentle handling of the cells is needed to reduce any mechanical damage to the cells or their DNA. This gentle handling also preserves the small number of fetal or rare cells in the sample. Integrity of the nucleic acid being evaluated is an important feature to permit the distinction between the genomic material from the fetal or rare cells and other cells in the sample. In particular, the enrichment and separation of the fetal or rare cells using the arrays of obstacles produces gentle treatment which minimizes cellular damage and maximizes nucleic acid integrity permitting exceptional levels of separation and the ability to subsequently utilize various formats to very accurately analyze the genome of the cells which are present in the sample in extremely low numbers.

[0072] In some embodiments, enrichment of rare cells (e.g. fetal cells, epithelial cells or circulating tumor cells (CTCs)) occurs using one or more capture modules that selectively inhibit the mobility of one or more cells of interest. Preferable a capture module is fluidly coupled downstream to a size-based separation module. Capture modules can include a substrate having multiple obstacles that restrict the movement of cells or analytes greater than a predetermined size. Examples of capture modules that inhibit the migration of cells based on size are disclosed in U.S. Patent No. 5,837,115 and 6,692,952.

[0073] In some embodiments, a capture module includes a two dimensional array of obstacles that selectively filters or captures cells or analytes having a hydrodynamic size greater than a particular gap size (predetermined size), International Publication No. WO 2004/113877.

[0074] In some cases a capture module captures analytes (e.g., cells of interest or not of interest) based on their affinity. For example, an affinity-based separation module that can capture cells or analytes can include an array of obstacles adapted for permitting sample flow through, but for the fact that the obstacles are covered with binding moieties that selectively bind one or more analytes (e.g., cell populations) of interest (e.g., red blood cells, fetal cells, epithelial cells or nucleated cells) or analytes not-of-interest (e.g., white blood cells). Arrays of obstacles adapted for separation by capture can include obstacles having one or more shapes and can be arranged in a uniform or non-uniform order. In some embodiments, a two-dimensional array of obstacles is staggered such that each subsequent row of obstacles is offset from the previous row of obstacles to increase the number of interactions between the analytes being sorted (separated) and the obstacles.

[0075] Binding moieties coupled to the obstacles can include e.g., proteins (e.g., ligands/receptors), nucleic acids having complementary counterparts in retained analytes, antibodies, etc. In some embodiments, an affinity-based separation module comprises a two-dimensional array of obstacles covered with one or more antibodies selected from the group consisting of: anti-CD71, anti-CD235a, anti-CD36, anti-carbohydrates, anti-selectin, anti-CD45, anti-GPA, anti-antigen-i, anti-EpCAM, anti-E-cadherin, and anti-Muc-1.

[0076] **Figure 2A** illustrates a path of a first analyte through an array of posts wherein an analyte that does not specifically bind to a post continues to migrate through the array, while an analyte that does bind a post is captured by the array. **Figure 2B** is a picture of antibody coated posts. **Figure 2C** illustrates coupling of antibodies to a

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substrate (e.g., obstacles, side walls, etc.) as contemplated by the present invention. Examples of such affinity-based separation modules are described in International Publication No. WO 2004/029221.

[0077] In some embodiments, a capture module utilizes a magnetic field to separate and/or enrich one or more analytes (cells) based on a magnetic property or magnetic potential in such analyte of interest or an analyte not of interest. For example, red blood cells which are slightly diamagnetic (repelled by magnetic field) in physiological conditions can be made paramagnetic (attributed by magnetic field) by deoxygenation of the hemoglobin into methemoglobin. This magnetic property can be achieved through physical or chemical treatment of the red blood cells. Thus, a sample containing one or more red blood cells and one or more white blood cells can be enriched for the red blood cells by first inducing a magnetic property in the red blood cells and then separating the red blood cells from the white blood cells by flowing the sample through a magnetic field (uniform or non-uniform).

[0078] For example, a maternal blood sample can flow first through a size-based separation module to remove enucleated cells and cellular components (e.g., analytes having a hydrodynamic size less than 6 μms) based on size. Subsequently, the enriched nucleated cells (e.g., analytes having a hydrodynamic size greater than 6 μms) white blood cells and nucleated red blood cells are treated with a reagent, such as CO_2, N_2, or $NaNO_2$, that changes the magnetic property of the red blood cells' hemoglobin. The treated sample then flows through a magnetic field (e.g., a column coupled to an external magnet), such that the paramagnetic analytes (e.g., red blood cells) will be captured by the magnetic field while the white blood cells and any other non-red blood cells will flow through the device to result in a sample enriched in nucleated red blood cells (including fetal nucleated red blood cells or fnRBC's). Additional examples of magnetic separation modules are described in US Application Serial No. 11/323,971, filed December 29, 2005 entitled "Devices and Methods for Magnetic Enrichment of Cells and Other Particles" and US Application Serial No. 11/227,904, filed September 15, 2005, entitled "Devices and Methods for Enrichment and Alteration of Cells and Other Particles".

[0079] Subsequent enrichment steps can be used to separate the rare cells (e.g. fnRBC's) from the non-rare cells maternal nucleated red blood cells. In some embodiments, a sample enriched by size-based separation followed by affinity/magnetic separation is further enriched for rare cells using fluorescence activated cell sorting (FACS) or selective lysis of a subset of the cells.

[0080] In some embodiments, enrichment involves detection and/or isolation of rare cells or rare DNA (e.g. fetal cells or fetal DNA) by selectively initiating apoptosis in the rare cells. This can be accomplished, for example, by subjecting a sample that includes rare cells (e.g. a mixed sample) to hyperbaric pressure (increased levels of CO_2; e.g. 4% CO_2). This will selectively initiate apoptosis in the rare or fragile cells in the sample (e.g. fetal cells). Once the rare cells (e.g. fetal cells) begin apoptosis, their nuclei will condense and optionally be ejected from the rare cells. At that point, the rare cells or nuclei can be detected using any technique known in the art to detect condensed nuclei, including DNA gel electrophoresis, in situ labeling of DNA nick using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP in situ nick labeling (TUNEL) (Gavrieli, Y., et al. J. Cell Biol. 119:493-501 (1992)), and ligation of DNA strand breaks having one or two-base 3' overhangs (Taq polymerase-based in situ ligation). (Didenko V., et al. J. Cell Biol. 135:1369-76 (1996)).

[0081] In some embodiments ejected nuclei can further be detected using a size based separation module adapted to selectively enrich nuclei and other analytes smaller than a predetermined size (e.g. 6 microns) and isolate them from cells and analytes having a hydrodynamic diameter larger than 6 microns. Thus, in one embodiment, the present invention contemplated detecting fetal cells/fetal DNA and optionally using such fetal DNA to diagnose or prognose a condition in a fetus. Such detection and diagnosis can occur by obtaining a blood sample from the female pregnant with the fetus, enriching the sample for cells and analytes larger than 8 microns using, for example,

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an array of obstacles adapted for size-base separation where the predetermined size of the separation is 8 microns (e.g. the gap between obstacles is up to 8 microns). Then, the enriched product is further enriched for red blood cells (RBC's) by oxidizing the sample to make the hemoglobin puramagnetic and flowing the sample through one or more magnetic regions. This selectively captures the RBC's and removes other cells (e.g. white blood cells) from the sample. Subsequently, the fnRBC's can be enriched from mnRBC's in the second enriched product by subjecting the second enriched product to hyperbaric pressure or other stimulus that selectively causes the fetal cells to begin apoptosis and condense / eject their nuclei. Such condensed nuclei are then identified/isolated using e.g. laser capture microdissection or a size based separation module that separates components smaller than 3, 4, 5 or 6 microns from a sample. Such fetal nuclei can then by analyzed using any method known in the art or described herein.

[0082] In some embodiments, when the analyte desired to be separated (e.g., red blood cells or white blood cells) is not ferromagnetic or does not have a potential magnetic property, a magnetic particle (e.g., a bead) or compound (e.g., Fe^{3+}) can be coupled to the analyte to give it a magnetic property. In some embodiments, a bead coupled to an antibody that selectively binds to an analyte of interest can be decorated with an antibody elected from the group of anti CD71 or CD75. In some embodiments a magnetic compound, such as Fe^{3+}, can be couple to an antibody such as those described above. The magnetic particles or magnetic antibodies herein may be coupled to any one or more of the devices herein prior to contact with a sample or may be mixed with the sample prior to delivery of the sample to the device(s). Magnetic particles can also be used to decorate one or more analytes (cells of interest or not of interest) to increase the size prior to performing size-based separation.

[0083] Magnetic field used to separate analytes/cells in any of the embodiments herein can uniform or non-uniform as well as external or internal to the device(s) herein. An external magnetic field is one whose source is outside a device herein (e.g., container, channel, obstacles). An internal magnetic field is one whose source is within a device contemplated herein. An example of an internal magnetic field is one where magnetic particles may be attached to obstacles present in the device (or manipulated to create obstacles) to increase surface area for analytes to interact with to increase the likelihood of binding. Analytes captured by a magnetic field can be released by demagnetizing the magnetic regions retaining the magnetic particles. For selective release of analytes from regions, the demagnetization can be limited to selected obstacles or regions. For example, the magnetic field can be designed to be electromagnetic, enabling turn-on and turn-off off the magnetic fields for each individual region or obstacle at will.

[0084] **Figure 3** illustrates an embodiment of a device configured for capture and isolation of cells expressing the transferrin receptor from a complex mixture. Monoclonal antibodies to CD71 receptor are readily available off-the-shelf and can be covalently coupled to magnetic materials comprising any conventional ferroparticles, such as, but not limited to ferrous doped polystyrene and ferroparticles or ferro-colloids (e.g., from Miltenyi and Dynal). The anti CD71 bound to magnetic particles is flowed into the device. The antibody coated particles are drawn to the obstacles (e.g., posts), floor, and walls and are retained by the strength of the magnetic field interaction between the particles and the magnetic field. The particles between the obstacles and those loosely retained with the sphere of influence of the local magnetic fields away from the obstacles are removed by a rinse.

[0085] One or more of the enrichment modules herein (e.g., size-based separation module(s) and capture module(s)) may be fluidly coupled in series or in parallel with one another. For example a first outlet from a separation module can be fluidly coupled to a capture module. In some embodiments, the separation module and capture module are integrated such that a plurality of obstacles acts both to deflect certain analytes according to size

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and direct them in a path different than the direction of analyte(s) of interest, and also as a capture module to capture, retain, or bind certain analytes based on size, affinity, magnetism or other physical property.

[0086] In any of the embodiments herein, the enrichment steps performed have a specificity and/or sensitivity greater than 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 99.1, 99.2, 99.3, 99.4, 99.5, 99.6, 99.7, 99.8, 99.9 or 99.95% The retention rate of the enrichment module(s) herein is such that ≥50, 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.9 % of the analytes or cells of interest (e.g., nucleated cells or nucleated red blood cells or nucleated from red blood cells) are retained. Simultaneously, the enrichment modules are configured to remove ≥50, 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.9 % of all unwanted analytes (e.g., red blood-platelet enriched cells) from a sample.

[0087] Any of the enrichment methods herein may be further supplemented by splitting the enriched sample into aliquots or sub-samples. In some embodiments, an enriched sample is split into at least 2, 5, 10, 20, 50, 100, 200, 500, or 1000 sub-samples. Thus when an enriched sample comprises about 500 cells and is split into 500 or 1000 different sub-samples, each sub-sample will have 1 or 0 cells.

[0088] In some cases a sample is split or arranged such that each sub-sample is in a unique or distinct location (e.g. well). Such location may be addressable. Each site can further comprise a capture mechanism to capture cell(s) to the site of interest and/or release mechanism for selectively releasing cells from the cite of interest. In some cases, the well is configured to hold a single cell.

III. Sample Analysis

[0089] In some embodiments, the methods herein are used for detecting the presence or conditions of rare cells that are in a mixed sample (optionally even after enrichment) at a concentration of up to 90%, 80%, 70%, 60%, 50 %, 40%, 30%, 20%, 10%, 5% or 1% of all cells in the mixed sample, or at a concentration of less than 1:2, 1:4, 1:10, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10,000, 1:20,000, 1:50,000, 1:100,000, 1:200,000, 1:1,000,000, 1:2,000,000, 1:5,000,000, 1:10,000,000, 1:20,000,000, 1:50,000,000 or 1:100,000,000 of all cells in the sample, or at a concentration of less than 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, or 1×10^{-7} cells/μL of a fluid sample. In some embodiments, the mixed sample has a total of up to 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, or 100 rare cells (e.g. fetal cells or epithelial cells).

[0090] Enriched target cells (e.g., fnRBC) may be "binned" prior to further analysis of the enriched cells (**Figures 34 &35**). Binning is any process which results in the reduction of complexity and/or total cell number of the enriched cell output. Binning may be performed by any method known in the art or described herein. One method of binning is by serial dilution. Such dilution may be carried out using any appropriate platform (e.g., PCR wells, microtiter plates) and appropriate buffers. Other methods include nanofluidic systems which can separate samples into droplets (e.g., BioTrove, Raindance, Fluidigm). Such nanofluidic systems may result in the presence of a single cell present in a nanodroplet.

[0091] Binning may be preceded by positive selection for target cells including, but not limited to, affinity binding (e.g. using anti-CD71 antibodies). Alternately, negative selection of non-target cells may precede binning. For example, output from a size-based separation module may be passed through a magnetic hemoglobin enrichment module (MHEM) which selectively removes WBCs from the enriched sample by attracting magnetized hemoglobin-containing cells.

[0092] For example, the possible cellular content of output from enriched maternal blood which has been passed through a size-based separation module (with or without further enrichment by passing the enriched sample through a MHEM) may consist of: 1) approximately 20 fnRBC; 2) 1,500 mnRBC; 3) 4,000-40,000 WBC; 4) 15×10^6 RBC. If this sample is separated into 100 bins (PCR wells or other acceptable binning platform), each bin would be

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expected to contain: 1) 80 negative bins and 20 bins positive for one fnRBC; 2) 150 mnRBC; 3) 400-4,000 WBC; 4) $15x10^4$ RBC. If separated into 10,000 bins, each bin would be expected to contain: 1) 9,980 negative bins and 20 bins positive for one fnRBC; 2) 8,500 negative bins and 1,500 bins positive for one mnRBC; 3) <1-4 WBC; 4) $15x10^2$ RBC. One of skill in the art will recognize that the number of bins may be increased or decreased depending on experimental design and/or the platform used for binning. Reduced complexity of the binned cell populations may facilitate further genetic and/or cellular analysis of the target cells by reducing the number of non-target cells in an individual bin.

[0093] Analysis may be performed on individual bins to confirm the presence of target cells (e.g. fnRBC) in the individual bin. Such analysis may consist of any method known in the art including, but not limited to, FISH, PCR, STR detection, SNP analysis, biomarker detection, and sequence analysis **(Figures 34 &35)**.

[0094] For example, a peripheral maternal venous blood sample enriched by the methods herein can be analyzed to determine pregnancy or a condition of a fetus (e.g., sex of fetus or aneuploidy). The analysis step for fetal cells may further involves comparing the ratio of maternal to paternal genomic DNA on the identified fetal cells.

IV. Fetal Biomarkers

[0095] In some embodiments fetal biomarkers may be used to detect and/or isolate fetal cells, after enrichment or after detection of fetal abnormality or lack thereof. For example, this may be performed by distinguishing between fetal and maternal nRBCs based on relative expression of a gene (e.g., DYS1, DYZ, CD-71, ε - and ζ -globin) that is differentially expressed during fetal development. In preferred embodiments, biomarker genes are differentially expressed in the first and/or second trimester. "Differentially expressed," as applied to nucleotide sequences or polypeptide sequences in a cell or cell nuclei, refers to differences in over/under-expression of that sequence when compared to the level of expression of the same sequence in another sample, a control or a reference sample. In some embodiments, expression differences can be temporal and/or cell-specific. For example, for cell-specific expression of biomarkers, differential expression of one or more biomarkers in the cell(s) of interest can be higher or lower relative to background cell populations. Detection of such difference in expression of the biomarker may indicate the presence of a rare cell (e.g., fnRBC) versus other cells in a mixed sample (e.g., background cell populations). In other embodiments, a ratio of two or more such biomarkers that are differentially expressed can be measured and used to detect rare cells.

[0096] In one embodiment, fetal biomarkers comprise differentially expressed hemoglobins. Erythroblasts (nRBCs) are very abundant in the early fetal circulation, virtually absent in normal adult blood and by having a short finite lifespan, there is no risk of obtaining fnRBC which may persist from a previous pregnancy. Furthermore, unlike trophoblast cells, fetal erythroblasts are not prone to mosaic characteristics.

[0097] Yolk sac erythroblasts synthesize ε -, ζ -,γ- and α -globins, these combine to form the embryonic hemoglobins. Between six and eight weeks, the primary site of erythropoiesis shifts from the yolk sac to the liver, the three embryonic hemoglobins are replaced by fetal hemoglobin (HbF) as the predominant oxygen transport system, and ε - and ζ -globin production gives way to γ -, α - and β -globin production within definitive erythrocytes (Peschle et al., 1985). HbF remains the principal hemoglobin until birth, when the second globin switch occurs and β -globin production accelerates.

[0098] Hemoglobin (Hb) is a heterodimer composed of two identical α globin chains and two copies of a second globin. Due to differential gene expression during fetal development, the composition of the second chain changes from ϵ globin during early embryonic development (1 to 4 weeks of gestation) to γ globin during fetal development (6 to 8 weeks of gestation) to β globin in neonates and adults as illustrated in (Table 1).

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[0099] Table 1. Relative expression of ε, γ and β in maternal and fetal RBCs.

		ε	γ	B
1st trimester	Fetal	++	++	-
	Maternal	-	+/-	++
2nd trimester	Fetal	-	++	+/-
	Maternal	-	+/-	++

[00100] In the late-first trimester, the earliest time that fetal cells may be sampled by CVS, fnRBCs contain, in addition to α globin, primarily ε and γ globin. In the early to mid second trimester, when amniocentesis is typically performed, fnRBCs contain primarily γ globin with some adult β globin. Maternal cells contain almost exclusively α and β globin, with traces of γ detectable in some samples. Therefore, by measuring the relative expression of the ε, γ and β genes in RBCs purified from maternal blood samples, the presence of fetal cells in the sample can be determined. Furthermore, positive controls can be utilized to assess failure of the FISH analysis itself.

[00101] In various embodiments, fetal cells are distinguished from maternal cells based on the differential expression of hemoglobins β, γ or ε. Expression levels or RNA levels can be determined in the cytoplasm or in the nucleus of cells. Thus in some embodiments, the methods herein involve determining levels of messenger RNA (mRNA), ribosomal RNA (rRNA), or nuclear RNA (nRNA).

[00102] In some embodiments, identification of fnRBCs can be achieved by measuring the levels of at least two hemoglobins in the cytoplasm or nucleus of a cell. In various embodiments, identification and assay is from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 fetal nuclei. Furthermore, total nuclei arrayed on one or more slides can number from about 100, 200, 300, 400, 500, 700, 800, 5000, 10,000, 100,000, 1,000,000, 2,000,000 to about 3,000,000. In some embodiments, a ratio for γ/β or ε/β is used to determine the presence of fetal cells, where a number less than one indicates that a fnRBC(s) is not present. In some embodiments, the relative expression of γ/β or ε/β provides a fnRBC index ("FNI"), as measured by γ or ε relative to β. In some embodiments, a FNI for γ/β greater than 5, 10, 15, 20, 25, 30, 35, 40, 45, 90, 180, 360, 720, 975, 1020, 1024, 1250 to about 1250, indicate that a fnRBC(s) is present. In yet other embodiments, a FNI for γ/β of less than about 1 indicates that a fnRBC(s) is not present. Preferably, the above FNI is determined from a sample obtained during a first trimester. However, similar ratios can be used during second trimester and third trimester.

[00103] In some embodiments, the expression levels are determined by measuring nuclear RNA transcripts including, nascent or unprocessed transcripts. In another embodiment, expression levels are determined by measuring mRNA, including ribosomal RNA. There are many methods known in the art for imaging (e.g., measuring) nucleic acids or RNA including, but not limited to, using expression arrays from Affymetrix, Inc. or Illumina, Inc.

[00104] RT-PCR primers can be designed by targeting the globin variable regions, selecting the amplicon size, and adjusting the primers annealing temperature to achieve equal PCR amplification efficiency. Thus TaqMan probes can be designed for each of the amplicons with well-separated fluorescent dyes, Alexa fluor®-355 for ε, Alexa Fluor®-488 for γ, and Alexa Fluor-555 for β. The specificity of these primers can be first verified using ε, γ, and β cDNA as templates. The primer sets that give the best specificity can be selected for further assay development. As an alternative, the primers can be selected from two exons spanning an intron sequence to amplify only the mRNA to eliminate the genomic DNA contamination.

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[00105] The primers selected can be tested first in a duplex format to verify their specificity, limit of detection, and amplification efficiency using target cDNA templates. The best combinations of primers can be further tested in a triplex format for its amplification efficiency, detection dynamic range, and limit of detection.

[00106] Various commercially available reagents are available for RT-PCR, such as One-step RT-PCR reagents, including Qiagen One-Step RT-PCR Kit and Applied Biosytems TaqMan One-Step RT-PCR Master Mix Reagents kit. Such reagents can be used to establish the expression ratio of ε, γ, *and* β using purified RNA from enriched samples. Forward primers can be labeled for each of the targets, using Alexa fluor-355 for ε, Alexa fluor-488 for γ, and Alexa fluor-555 for β. Enriched cells can be deposited by cytospinning onto glass slides. Additionally, cytospinning the enriched cells can be performed after in situ RT-PCR. Thereafter, the presence of the fluorescent-labeled amplicons can be visualized by fluorescence microscopy. The reverse transcription time and PCR cycles can be optimized to maximize the amplicon signal:background ratio to have maximal separation of fetal over maternal signature. Preferably, signal:background ratio is greater than 5, 10, 50 or 100 and the overall cell loss during the process is less than 50, 10 or 5%.

V. Fetal Cell Analysis

[00107] **Figure 4** illustrates an overview of some embodiments of the present invention.

[00108] Aneuploidy means the condition of having less than or more than the normal diploid number of chromosomes. In other words, it is any deviation from euploidy. Aneuploidy includes conditions such as monosomy (the presence of only one chromosome of a pair in a cell's nucleus), trisomy (having three chromosomes of a particular type in a cell's nucleus), tetrasomy (having four chromosomes of a particular type in a cell's nucleus), pentasomy (having five chromosomes of a particular type in a cell's nucleus), triploidy (having three of every chromosome in a cell's nucleus), and tetraploidy (having four of every chromosome in a cell's nucleus). Birth of a live triploid is extraordinarily rare and such individuals are quite abnormal, however triploidy occurs in about 2-3% of all human pregnancies and appears to be a factor in about 15% of all miscarriages. Tetraploidy occurs in approximately 8% of all miscarriages. (http://www.emedicine.com/med/topic3241.htm).

[00109] In step **400**, a sample is obtained from an animal, such as a human. In some embodiments, animal or human is pregnant, suspected of being pregnant, or may have been pregnant, and, the systems and methods herein are used to diagnose pregnancy and/or conditions of the fetus (e.g. trisomy). In some embodiments, the animal or human is suspected of having a condition, has a condition, or had a condition (e.g., cancer) and, the systems and methods herein are used to diagnose the condition, determine appropriate therapy, and/or monitor for recurrence.

[00110] In both scenarios a sample obtained from the animal can be a blood sample e.g., of up to 50, 40, 30, 20, or 15 mL. In some cases multiple samples are obtained from the same animal at different points in time (e.g. before therapy, during therapy, and after therapy, or during 1st trimester, 2nd trimester, and 3rd trimester of pregnancy).

[00111] In optional step **402**, rare cells (e.g., fetal cells or epithelial cells) or DNA of such rare cells are enriched using one or more methods known in the art or described herein. For example, to enrich fetal cells from a maternal blood sample, the sample can be applied to a size-base separation module (e.g., two-dimensional array of obstacles) configured to direct cells or particles in the sample greater than 8 microns to a first outlet and cells or particles in the sample smaller than 8 microns to a second outlet. The fetal cells can subsequently be further enriched from maternal white blood cells (which are also greater than 8 microns) based on their potential magnetic property. For example, N_2 or anti-CD71 coated magnetic beads is added to the first enriched product to make the hemoglobin in the red blood cells (maternal and fetal) paramagnetic. The enriched sample is then flowed through a column coupled to an external magnet. This captures both the fnRBC's and mnRBC's creating a second enriched product. The sample can then be subjected to hyperbaric pressure or other stimulus to initiate apoptosis in the fetal cells. Fetal

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cells/nuclei can then be enriched using microdissection, for example. It should be noted that even an enriched product can be dominated (>50%) by cells not of interest (e.g. maternal red blood cells). In some cases an enriched sample has the rare cells (or rare genomes) consisting of up to 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, or 50% of all cells (or genomes) in the enriched sample. For example, using the systems herein, a maternal blood sample of 20 mL from a pregnant human can be enriched for fetal cells such that the enriched sample has a total of about 500 cells, 2% of which are fetal and the rest are maternal.

[00112] In step **404**, the enriched product is split between two or more discrete locations. In some embodiments, a sample is split into at least 2, 10, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3,000, 4,000, 5000, or 10,000 total different discrete sites or about 100, 200, 500, 1000, 1200, 1500 sites. In some embodiments, output from an enrichment module is serially divided into wells of a 1536 microwell plate (Figure 8). This can result in one cell or genome per location or 0 or 1 cell or genome per location. In some embodiments, cell splitting results in more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 10,000, 20,000, 50,000, 100,000, 200,000, or 500,000 cells or genomes per location. When splitting a sample enriched for epithelial cells, endothelial cells, or CTC's, the load at each discrete location (e.g., well) can include several leukocytes, while one only some of the loads includes one or more CTC's. When splitting a sample enriched for fetal cells preferably each site includes 0 or 1 fetal cells.

[00113] Examples of discrete locations which could be used as addressable locations include, but are not limited to, wells, bins, sieves, pores, geometric sites, slides, matrixes, membranes, electric traps, gaps, obstacles, or in-situ within a cell or nuclear membrane. In some embodiments, the discrete cells are addressable such that one can correlate a cell or cell sample with a particular location.

[00114] Examples of methods for splitting a sample into discrete addressable locations include, but are not limited to, fluorescent activated cell sorting (FACS) (Sherlock, JV et al. Ann. Hum. Genet. 62 (Pt. 1): 9-23 (1998)), micromanipulation (Samura, O., Ct al Hum. Genet. 107(1):28-32 (2000)) and dilution strategies (Findlay, I. et al. Mol. Cell. Endocrinol. 183 Suppl 1: S5-12 (2001)). Other methods for sample splitting cell sorting and splitting methods known in the art may also be used. For example, samples can be split by affinity sorting techniques using affinity agents (e.g. antibodies) bound to any immobilized or mobilized substrate (Samura O., et al., Hum. Genet. 107(1):28-32 (2000)). Such affinity agents can be specific to a cell type e.g. RBC's fetal cells epithelial cells including those specifically binding EpCAM, antigen-i, or CD-71.

[00115] In some embodiments, a sample or enriched sample is transferred to a cell sorting device that includes an array of discrete locations for capturing cells traveling along a fluid flow. The discrete locations can be arranged in a defined pattern across a surface such that the discrete sites are also addressable. In some embodiments, the sorting device is coupled to any of the enrichment devices known in the art or disclosed herein. Examples of cell sorting devices included are described in International Publication No. WO 01/35071. Examples of surfaces that may be used for creating arrays of cells in discrete addressable sites include, but are not limited to, cellulose, cellulose acetate, nitrocellulose, glass, quartz or other crystalline substrates such as gallium arsenide, silicones, metals, semiconductors, various plastics and plastic copolymers, cyclo-olefin polymers, various membranes and gels, microspheres, beads and paramagnetic or supramagnetic microparticles.

[00116] In some embodiments, a sorting device comprises an array of wells or discrete locations wherein each well or discrete location is configured to hold up to 1 cell. Each well or discrete addressable location may have a capture mechanism adapted for retention of such cell (e.g. gravity, suction, etc.) and optionally a release mechanism for selectively releasing a cell of interest from a specific well or site (e.g. bubble actuation). Figure B illustrates such an embodiment.

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[00117] In step **406**, nucleic acids of interest from each cell or nuclei arrayed are tagged by amplification. Preferably, the amplified/tagged nucleic acids include at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 90, 90 or 100 polymorphic genomic DNA regions such as short tandem repeats (STRs) or variable number of tandem repeats ("VNTR"). When the amplified DNA regions include one or more STR/s/, the STR/s/ are selected for high heterozygosity (variety of alleles) such that the paternal allele of any fetal cell is more likely to be distinct in length from the maternal allele. This results in improved power to detect the presence of fetal cells in a mixed sample and any potential of fetal abnormalities in such cells. In some embodiment, STR(s) amplified are selected for their association with a particular condition. For example, to determine fetal abnormality an STR sequence comprising a mutation associated with fetal abnormality or condition is amplified. Examples of STRs that can be amplified/analyzed by the methods herein include, but are not limited to D21S1414, D21S1411, D21S1412, D21S11 MBP, D13S634, D13S631, D18S535, AmgXY and XHPRT. Additional STRs that can be amplified/analyzed by the methods herein include, but are not limited to, those at locus F13B (1:q31-q32); TPOX (2:p23-2pter); FIBRA (FGA) (4:q28); CSFIPO (5:q33.3-q34); FI3A (6:p24-p25); THOI (11:p15-15.5); VWA (12:p12-pter); CDU (12p12-pter); D14S1434 (14:q32.13); CYAR04 (p450) (15:q21.1) D21S11 (21:q11-q21) and D22S1045 (22:q12.3). In some cases, STR loci are chosen on a chromosome suspected of trisomy and on a control chromosome. Examples of chromosomes that are often trisomic include chromosomes 21, 18, 13, and X. In some cases, 1 or more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 STRs are amplified per chromosome tested (Samura, O. et al., Clin. Chem. 47(9):1622-6 (2001)). For example amplification can be used to generate amplicons of up to 20, up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 150, up to 200, up to 300, up to 400, up to 500 or up to 1000 nucleotides in length. Di-, tri-, tetra- , or penta-nucleotide repeat STR loci can be used in the methods described herein.

[00118] To amplify and tag genomic DNA region(s) of interest, PCR primers can include: (i) a primer element, (ii) a sequencing element, and (iii) a locator element.

[00119] The primer element is configured to amplify the genomic DNA region of interest (e.g. STR). The primer element includes, when necessary, the upstream and downstream primers for the amplification reactions. Primer elements can be chosen which are multiplexible with other primer pairs from other tags in the same amplification reaction (e.g. fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primer element can have at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40 or 50 nucleotide bases, which are designed to specifically hybridize with and amplify the genomic DNA region of interest.

[00120] The sequencing element can be located on the 5' end of each primer element or nucleic acid tag. The sequencing element is adapted to cloning and/or sequencing of the amplicons. (Marguiles, M, Nature 437 (7057): 376-80) The sequencing element can be about 4, 6, 8, 10, 18, 20, 28, 36, 46 or 50 nucleotide bases in length.

[00121] The locator element (also known as a unique tag sequence), which is often incorporated into the middle part of the upstream primer, can include a short DNA or nucleic acid sequence between 4-20 bp in length (e.g., about 4, 6, 8, 10, or 20 nucleotide bases). The locator element makes it possible to pool the amplicons from all discrete addressable locations following the amplification step and analyze the amplicons in parallel. In some embodiments each locator element is specific for a single addressable location.

[00122] Tags are added to the cells/DNA at each discrete location using an amplification reaction. Amplification can be performed using PCR or by a variety of methods including, but not limited to, singleplex PCR, quantitative PCR, quantitative fluorescent PCR (QF-PCR), multiplex fluorescent PCR (MF-PCR), real time PCR (RT-PCR), single cell PCR, restriction fragment length polymorphism PCR (PCR-RFLP), PCR-RFLP/RT-PCR-RFLP, hot start

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PCR, nested PCR, in situ polonony PCR, in situ rolling circle amplification (RCA), bridge PCR, picotiter PCR, multiple strand displacement amplification (MDA), and emulsion PCR. Other suitable amplification methods include the ligase chain reaction (LCR), transcription amplification, self-sustained sequence replication, selective amplification of target polynucleotide sequences, consensus sequence primed polymerase chain reaction (CP-PCR), arbitrarily primed polymerase chain reaction (AP-PCR), degenerate oligonucleotide-primed PCR (DOP-PCR) and nucleic acid based sequence amplification (NABSA). Additional examples of amplification techniques using PCR primers are described in, U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617 and 6,582,938.

[00123] In some embodiments, a further PCR amplification is performed using nested primers for the one or more genomic DNA regions of interest to ensure optimal performance of the multiplex amplification. The nested PCR amplification generates sufficient genomic DNA starting material for further analysis such as in the parallel sequencing procedures below.

[00124] In step **408**, genomic DNA regions tagged/amplified are pooled and purified prior to further processing. Methods for pooling and purifying genomic DNA are known in the art.

[00125] In step **410**, pooled genomic DNA/amplicons are analyzed to measure, e.g. allele abundance of genomic DNA regions (e.g. STRs amplified). In some embodiments such analysis involves the use of capillary gel electrophoresis (CGE). In other embodiments, such analysis involves sequencing or ultra deep sequencing.

[00126] Sequencing can be performed using the classic Sanger sequencing method or any other method known in the art.

[00127] For example, sequencing can occur by sequencing-by-synthesis, which involves inferring the sequence of the template by synthesizing a strand complementary to the target nucleic acid sequence. Sequence-by-synthesis can be initiated using sequencing primers complementary to the sequencing element on the nucleic acid tags. The method involves detecting the identity of each nucleotide immediately after (substantially real-time) or upon (real-time) the incorporation of a labeled nucleotide or nucleotide analog into a growing strand of a complementary nucleic acid sequence in a polymerase reaction. After the successful incorporation of a label nucleotide, a signal is measured and then nulled by methods known in the art. Examples of sequence-by-synthesis methods are described in U.S. Application Publication Nos. 2003/0044781, 2006/0024711, 2006/0024678 and 2005/0100932. Examples of labels that can be used to label nucleotide or nucleotide analogs for sequencing-by-synthesis include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties. Sequencing-by-synthesis can generate at least 1,000, at least 5,000, at least 10,000, at least 20,000, 30,000, at least 40,000, at least 50,000, at least 100,000 or at least 500,000 reads per hour. Such reads can have at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 or at least 150 bases per read.

[00128] Another sequencing method involves hybridizing the amplified genomic region of interest to a primer complementary to it. This hybridization complex is incubated with a polymerase, ATP sulfurylase, luciferase, apyrase, and the substrates luciferin and adenosine 5' phosphosulfate. Next, deoxynucleotide triphosphates corresponding to the bases A, C, G, and T (U) are added sequentially. Each base incorporation is accompanied by release of pyrophosphate, converted to ATP by sulfurylase, which drives synthesis of oxyluciferin and the release of visible light. Since pyrophosphate release is equimolar with the number of incorporated bases, the light given off is proportional to the number of nucleotides adding in any one step. The process is repeated until the entire sequence is determined.

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[00129] Yet another sequencing method involves a four-color sequencing by ligation scheme (degenerate ligation), which involves hybridizing an anchor primer to one of four positions. Then an enzymatic ligation reaction of the anchor primer to a population of degenerate nonamers that are labeled with fluorescent dyes is performed. At any given cycle, the population of nonamers that is used is structure such that the identity of one of its positions is correlated with the identity of the fluorophore attached to that nonamer. To the extent that the ligase discriminates for complementarily at that queried position, the fluorescent signal allows the inference of the identity of the base. After performing the ligation and four-color imaging, the anchor primer:nonamer complexes are stripped and a new cycle begins. Methods to image sequence information after performing ligation are known in the art.

[00130] Preferably, analysis involves the use of ultra-deep sequencing, such as described in Marguiles et al., Nature 437 (7057): 376-80 (2005). Briefly, the amplicons are diluted and mixed with beads such that each bead captures a single molecule of the amplified material. The DNA molecule on each bead is then amplified to generate millions of copies of the sequence which all remain bound to the bead. Such amplification can occur by PCR. Each bead can be placed in a separate well, which can be a (optionally addressable) picolitre-sized well. In some embodiments, each bead is captured within a droplet of a PCR-reaction-mixture-in-oil-emulsion and PCR amplification occurs within each droplet. The amplification on the bead results in each bead carrying at least one million, at least 5 million, or at least 10 million copies of the original amplicon coupled to it. Finally, the beads are placed into a highly parallel sequencing by synthesis machine which generates over 400,000 reads (~100bp per read) in a single 4 hour run.

[00131] Other methods for ultra-deep sequencing that can be used are described in Hong, S. et al. Nat. Biotechnol. 22(4):435-9 (2004); Bennett, B. et al. Pharmacogenomics 6(4):373-82 (2005); Shendure, P. et al. Science 309 (5741):1728-32 (2005).

[00132] The role of the ultra-deep sequencing is to provide an accurate and quantitative way to measure the allele abundances for each of the STRs. The total required number of reads for each of the aliquot wells is determined by the number of STRs, the error rates of the multiplex PCR, and the Poisson sampling statistics associated with the sequencing procedures.

[00133] In one example, the enrichment output from step **402** results in approximately 500 cells of which 98% are maternal cells and 2% are fetal cells. Such enriched cells are subsequently split into 500 discrete locations (e.g., wells) in a microtiter plate such that each well contains 1 cell. PCR is used to amplify STR's (~3-10 STR loci) on each chromosome of interest. Based on the above example, as the fetal/maternal ratio goes down, the aneuploidy signal becomes diluted and more loci are needed to average out measurement errors associated with variable DNA amplification efficiencies from locus to locus. The sample division into wells containing ~1 cell proposed in the methods described herein achieves pure or highly enriched fetal/maternal ratios in some wells, alleviating the requirements for averaging of PCR errors over many loci.

[00134] In one example, let 'f' be the fetal/maternal DNA copy ratio in a particular PCR reaction. Trisomy increases the ratio of maternal to paternal alleles by a factor 1+f/2. PCR efficiencies vary from allele to allele within a locus by a mean square error in the logarithm given by σ_{allele}^2, and vary from locus to locus by σ_{locus}^2, where this second variance is apt to be larger due to differences in primer efficiency. N_a is the loci per suspected aneuploid chromosome and N_c is the control loci. If the mean of the two maternal allele strengths at any locus is 'm' and the paternal allele strength is 'p,' then the squared error expected is the mean of the ln(ratio(m/p)), where this mean is taken over N loci is given by $2(\sigma_{allele}^2)/N$. When taking the difference of this mean of ln(ratio(m/p)) between a suspected aneuploidy region and a control region, the error in the difference is given by

$$\sigma_{diff}^2 = 2(\sigma_{allele}^2)/N_a + 2(\sigma_{allele}^2)/N_c \quad (1)$$

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[00135] For a robust detection of aneuploidy we require

$$3\sigma_{diff} < f/2.$$

[00136] For simplicity, assuming $N_a = N_c = N$ in Equation 1, this gives the requirement

$$6\sigma_{allele}/N^{1/2} < f/2, \quad (3)$$

or a minimum N of

$$N = 144(\sigma_{allele}/f)^2 \quad (4)$$

[00137] In the context of trisomy detection, the suspected aneuploidy region is usually the entire chromosome and N denotes the number of loci per chromosome. For reference, Equation 3 is evaluated for N in the following **Table 2** for various values of σ_{allele} and f.

		f	
σ_{allele}	**0.1**	**0.3**	**1.0**
0.1	144	16	1
0.3	1296	144	13
1.0	14400	1600	144

Table 2. Required number of loci per chromosome as a function of σ_{allele} and f.

Since sample splitting decreases the number of starting genome copies which increases σ_{allele} at the same time that it increases the value of f in some wells, the methods herein are based on the assumption that the overall effect of splitting is favorable; i.e., that the PCR errors do not increase too fast with decreasing starting number of genome copies to offset the benefit of having some wells with large f. The required number of loci can be somewhat larger because for many loci the paternal allele is not distinct from the maternal alleles, and this incidence depends on the heterozygosity of the loci. In the case of highly polymorphic STRs, this amounts to an approximate doubling of N.

[00138] The role of the sequencing is to measure the allele abundances output from the amplification step. It is desirable to do this without adding significantly more error due to the Poisson statistics of selecting only a finite number of amplicons for sequencing. The rms error in the ln(abundance) due to Poisson statistics is approximately $(N_{reads})^{-1/2}$. It is desirable to keep this value less than or equal to the PCR error σ_{allele}. Thus, a typical paternal allele needs to be allocated at least $(\sigma_{allele})^{-2}$ reads. The maternal alleles, being more abundant, do not add appreciably to this error when forming the ratio estimate for m/p. The mixture input to sequencing contains amplicons from N_{loci} loci of which roughly an abundance fraction f/2 are paternal alleles. Thus, the total required number of reads for each of the aliquot wells is given approximately by $2N_{loci}/(f\,\sigma_{allele}^2)$. Combining this result with Equation 4, it is found a total number of reads over all the wells given approximately by

$$N_{reads} = 288\ N_{wells}\ f^{-3}. \quad (5)$$

[00139] When performing sample splitting, a rough approximation is to stipulate that the sample splitting causes f to approach unity in at least a few wells. If the sample splitting is to have advantages, then it must be these wells which dominate the information content in the final result. Therefore, Equation (5) with f=1 is adopted, which suggests a minimum of about 300 reads per well. For 500 wells, this gives a minimum requirement for ~150,000 sequence reads. Allowing for the limited heterozygosity of the loci tends to increase the requirements (by a factor of ~2 in the case of STRs), while the effect of reinforcement of data from multiple wells tends to relax the requirements with respect to this result (in the baseline case examined above it is assumed that ~10 wells have a pure fetal cell). Thus the required total number of reads per patient is expected to be in the range 100,000 – 300,000.

[00140] In step **412**, wells with rare cells/alleles (e.g., fetal alleles) are identified. The locator elements of each tag can be used to sort the reads (~200,000 sequence reads) into 'bins' which correspond to the individual wells of the

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microtiter plates (~500 bins). The sequence reads from each of the bins (~400 reads per bin) are then separated into the different genomic DNA region groups, (e.g. STR loci,) using standard sequence alignment algorithms. The aligned sequences from each of the bins are used to identify rare (e.g., non-maternal) alleles. It is estimated that on average a 15 ml blood sample from a pregnant human will result in ~10 bins having a single fetal cell each.

[00141] The following are two examples by which rare alleles can be identified. In a first approach, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above in order to obtain maternal alleles. This sample can be a white blood cell fraction or simply a dilution of the original sample before enrichment. In a second approach, the sequences or genotypes for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles determines which discrete location (e.g. well) contained fetal cells. Determining the number of bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original cell population or enriched sample. Bins containing fetal cells are identified with high levels of confidence because the non-maternal alleles are detected by multiple independent polymorphic DNA regions, e.g. STR loci.

[00142] In step **414**, condition of rare cells or DNA is determined. This can be accomplished by determining abundance of selected alleles (polymorphic genomic DNA regions) in bin(s) with rare cells/DNA. In some embodiments, allele abundance is used to determine aneuploidy, e.g. chromosomes 13, 18 and 21. Abundance of alleles can be determined by comparing ratio of maternal to paternal alleles for each genomic region amplified (e.g., ~12 STR's). For example, if 12 STRs are analyzed, for each bin there are 33 sequence reads for each of the STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker will be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). (Adinolfi, P. et al., Prenat. Diagn, 17(13):1299-311 (1997)). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. In some embodiments, the information from the different DNA regions on each chromosome are combined to increase the confidence of a given aneuploidy call. In some embodiments, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[00143] In some embodiments allele abundance is used to determine segmental anuepolidy. Normal diploid cells have two copies of each chromosome and thus two alleles of each gene or loci. Changes in the allele abundance for a particular chromosomal region may be indicative of a chromosomal rearrangement, such as a deletion, duplication or translocation event. In some embodiments, the information from the different DNA regions on each chromosome are combined to increase the confidence of a given segmental aneuploidy call. In some embodiments, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[00144] The determination of fetal trisomy can be used to diagnose conditions such as abnormal fetal genotypes, including, trisomy 13, trisomy 18, trisomy 21 (Down syndrome) and Klinefelter Syndrome (XXY). Other examples of abnormal fetal genotypes include, but are not limited to, aneuploidy such as, monosomy of one or more chromosomes (X chromosome monosomy, also known as Turner's syndrome), trisomy of one or more chromosomes (13, 18, 21, and X), tetrasomy and pentasomy of one or more chromosomes (which in humans is most commonly observed in the sex chromosomes, e.g. XXXX, XXYY, XXXY, XYYY, XXXXX, XXXXY, XXXYY, XYYYY and XXYYY), triploidy (three of every chromosome, e.g. 69 chromosomes in humans), tetraploidy (four of every chromosome, e.g. 92 chromosomes in humans) and multiploidy. In some embodiments, an abnormal fetal genotype

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is a segmental aneuploidy. Examples of segmental aneuploidy include, but are not limited to, 1p36 duplication, dup(17)(p11.2p11.2) syndrome, Down syndrome, Pelizaeus-Merzbacher disease, dup(22)(q11.2q11.2) syndrome, and cat-eye syndrome. In some cases, an abnormal fetal genotype is due to one or more deletions of sex or autosomal chromosomes, which may result in a condition such as Cri-du-chat syndrome, Wolf-Hirschhorn, Williams-Beuren syndrome, Charcot-Marie-Tooth disease, Hereditary neuropathy with liability to pressure palsies, Smith-Magenis syndrome, Neurofibromatosis, Alagille syndrome, Velocardiofacial syndrome, DiGeorge syndrome, Steroid sulfatase deficiency, Kallmann syndrome, Microphthalmia with linear skin defects, Adrenal hypoplasia, Glycerol kinase deficiency, Pelizaeus-Merzbacher disease, Testis-determining factor on Y, Azospermia (factor a), Azospermia (factor b), Azospermia (factor c), or 1p36 deletion. In some embodiments, a decrease in chromosomal number results in an XO syndrome.

[00145] In one embodiment, the methods of the invention allow for the determination of maternal or paternal trisomy. In some embodiments, the methods of the invention allow for the determination of trisomy or other conditions in fetal cells in a mixed maternal sample arising from more than one fetus.

[00146] In another aspect of the invention, standard quantitative genotyping technology is used to declare the presence of fetal cells and to determine the copy numbers (ploidies) of the fetal chromosomes. Several groups have demonstrated that quantitative genotyping approaches can be used to detect copy number changes (Wang, Moorhead et al. 2005). However, these approaches do not perform well on mixtures of cells and typically require a relatively large number of input cells (~10,000). The current invention addresses the complexity issue by performing the quantitative genotyping reactions on individual cells. In addition, multiplex PCR and DNA tags are used to perform the thousands of genotyping reaction on single cells in highly parallel fashion.

[00147] An overview of this embodiment is illustrated in **Figure 5**.

[00148] In step **500**, a sample (e.g., a mixed sample of rare and non-rare cells) is obtained from an animal or a human. See, e.g., step **400** of Figure 4. Preferably, the sample is a peripheral maternal blood sample.

[00149] In step **502**, the sample is enriched for rare cells (e.g., fetal cells) by any method known in the art or described herein. See, e.g., step **402** of **Figure 4**.

[00150] In step **504**, the enriched product is split into multiple distinct sites (e.g., wells). See, e.g., step 404 of Figure 4.

[00151] In step **506**, PCR primer pairs for amplifying multiple (e.g., 2 – 100) highly polymorphic genomic DNA regions (e.g., SNPs) are added to each discrete site or well in the array or microtiter plate. For example, PCR primer pairs for amplifying SNPs along chromosome 13, 18, 21 and/or X can be designed to detect the most frequent aneuoploidies. Other PCR primer pairs can be designed to amplify SNPs along control regions of the genome where aneuploidy is not expected. The genomic loci (e.g., SNPs) in the aneuploidy region or aneuploidy suspect region are selected for high polymorphism such that the paternal alleles of the fetal cells are more likely to be distinct from the maternal alleles. This improves the power to detect the presence of fetal cells in a mixed sample as well as fetal conditions or abnormalities. SNPs can also be selected for their association with a particular condition to be detected in a fetus. In some cases, one or more than one, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 SNPs are analyzed per target chromosome (e.g., 13, 18, 21, and/or X). The increase number of SNPs interrogated per chromosome ensures accurate results. PCR primers are chosen to be multiplexible with other pairs (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primers are designed to generate amplicons 10-200, 20-180, 40-160, 60-140 or 70-100 bp in size to increase the performance of the multiplex PCR.

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[00152] A second of round of PCR using nested primers may be performed to ensure optimal performance of the multiplex amplification. The multiplex amplification of single cells is helpful to generate sufficient starting material for the parallel genotyping procedure. Multiplex PCT can be performed on single cells with minimal levels of allele dropout and preferential amplification. See Sherlock, J., et al. Ann. Hum. Genet. 61 (Pt 1): 9-23 (1998); and Findlay, I., et al. Mol. Cell. Endocrinol. 183 Suppl. 1: S5-12 (2001).

[00153] In step **508**, amplified polymorphic DNA region(s) of interest (e.g., SNPs) are tagged e.g., with nucleic acid tags. Preferably, the nucleic acid tags serve two roles: to determine the identity of the different SNPs and to determine the identity of the bin from which the genotype was derived. Nucleic acid tags can comprise primers that allow for allele-specific amplification and/or detection. The nucleic acid tags can be of a variety of sizes including up to 10 base pairs, 10-40, 15-30, 18-25 or ~22 base pair long.

[00154] In some embodiments, a nucleic acid tag comprises a molecular inversion probe (MIP). Examples of MIPs and their uses are described in Hardenbol, P., et al., Nat. Biotechnol. 21(6):673-8 (2003); Hardenbol, P., et al., Genome Res. 15(2):269-75 (2005); and Wang, Y., et al., Nucleic Acids Res. 33(21):e183 (2005). **Figure 7A** illustrates one example of a MIP assay used herein. The MIP tag can include a locator element to determine the identity of the bin from which the genotype was derived. For example, when output from an enrichment procedure results in about 500 cells, the enriched product / cells can be split into a microliter plate containing 500 wells such that each cell is in a different distinct well. **Figure 7B** illustrates a microtiter plate with 500 wells each of which contains a single cell. Each cell is interrogated at 10 different SNPs per chromosome, on 4 chromosomes (e.g., chromosomes 13, 18, 21 and X). This analysis requires 40 MIPs per cell / well for a total of 20,000 tags per 500 wells (i.e., 4 chromosomes x 10 SNPs x 500 wells). The tagging step can also include amplification of the MIPs after their rearrangement or enzymatic "gap fill".

[00155] In one embodiment, a nucleic acid tag comprises a unique property, such as a difference in mass or chemical properties from other tags. In another embodiments a nucleic acid tag comprises a photoactivatable label, so that it crosslinks where it binds. In another embodiment a nucleic acid tag can be used as a linker for ultra deep sequencing. In another embodiment a nucleic acid tag can be used as a linker for arrays. In another embodiment a nucleic acid tag comprises a unique fluorescent label, (Such as FAM, JOE, ROX, NED, HEX, SYBR, PET, TAMRA, VIC, CY-3, CY-5, dR6G, DS-33, LIZ, DS-02, dR110, and Texas Red) which can be used to differentiate individual DNA fragments. In another embodiment a nucleic acid tag can serve as primer or hybridization site for a probe, to facilitate signal amplification or detection from a single cell by using a tractable marker. In some embodiments the labeled nucleic acid tag can be analyzed using a system coupled to a light source, such as an ABI 377, 310, 3700 or any other system which can detect fluorescently labeled DNA.

[00156] In step **510**, the tagged amplicons are pooled together for further analysis.

[00157] In step **512**, the genotype at each polymorphic site is determined and/or quantified using any technique known in the art. In one embodiment, genotyping occurs by hybridization of the MIP tags to a microarray containing probes complementary to the sequences of each MIP tag. See US Patent Nos. 6,858,412.

[00158] Using the example described above with the MIP probes, the 20,000 tags are hybridized to a single tag array containing complementary sequences to each of the tagged MIP probes. Microarrays (e.g. tag arrays) can include a plurality of nucleic acid probes immobilized to discrete spots (e.g., defined locations or assigned positions) on a substrate surface. For example, a microarray can have at least 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1,000, 5,000, 10,000, 15,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, or 100,000 different probes complementary to MIP tagged probes. Methods to prepare microarrays capable to monitor several genes according to the methods of the invention are well known in the art. Examples of microarrays that can be used in

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nucleic acid analysis that may be used are described in US Pat 6,300,063, US Pat 5,837,832, US Pat 6,969, 589, US Pat 6,040,138, US Pat 6,858, 412, US Publication No. 2005/0100893, US Publication No. 2004/0018491, US Publication No. 2003/0215821 and US Publication No. 2003/0207295.

[00159] In step **516**, bins with rare alleles (e.g., fetal alleles) are identified. Using the example described above, rare allele identification can be accomplished by first using the 22bp tags to sort the 20,000 genotypes into 500 bins which correspond to the individual wells of the original microtiter plates. Then, one can identify bins containing non-maternal alleles which correspond to wells that contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of its provides an accurate estimate of the number of fnRBCs that were present in the original enriched cell population. When a fetal cell is identified in a given bin, the non-maternal alleles can be detected by 40 independent SNPS s which provide an extremely high level of confidence in the result.

[00160] In step **518**, a condition such as trisomy is determined based on the rare cell polymorphism. For example, after identifying the ~10 bins that contain fetal cells, one can determine the ploidy of chromosomes 13, 18, 21 and X of such cells by comparing the ratio of maternal to paternal alleles for each of ~10 SNPs on each chromosome (X, 13, 18, 21). The ratios for the multiple SNPs on each chromosome can be combined (averaged) to increase the confidence of the aneuploidy call for that chromosome. In addition, the information from the ~10 independent bins containing fetal cells can also be combined to further increase the confidence of the call.

[00161] As described above, an enriched maternal sample with 500 cells can be split into 500 discrete locations such that each location contains one cell. If ten SNPs are analyzed in each of four different chromosomes, forty tagged MIP probes are added per discrete location to analyze forty different SNPs per cell. The forty SNPs are then amplified in each location using the primer element in the MIP probe as described above. All the amplicons from all the discrete locations are then pooled and analyzed using quantitative genotyping as describe above. In this example a total of 20,000 probes in a microarray are required to genotype the same 40 SNPs in each of the 500 discrete locations (4 chromosomes x 10 SNPs x 500 discrete locations).

[00162] The above embodiment can also be modified to provide for genotyping by hybridizing the nucleic acid tags to bead arrays as are commercially available by Illumina, Inc. and as described in US Patent Nos. 7,040,959; 7,035,740; 7033,754; 7,025,935, 6,998,274; 6, 942,968; 6,913,884; 6,890,764; 6,890,741; 6,858,394; 6,846,460; 6,812,005; 6,770,441; 6,663,832; 6,620,584; 6,544,732; 6,429,027; 6,396,995; 6,355,431 and US Publication Application Nos. 20060019258; 20050266432; 20050244870; 20050216207; 20050181394; 20050164246; 20040224353; 20040185482; 20030198573; 20030175773; 20030003490; 20020187515; and 20020177141; as well as Shen, R., et al. Mutation Research 573 70-82 (2005).

[00163] An overview of the use of nucleic acid tags is described in **Figure 7C**. After enrichment and amplification as described above, target genomic DNA regions are activated in **step 702** such that they may bind paramagnetic particles. In **step 703** assay oligonucleotides, hybridization buffer, and paramagnetic particles are combined with the activated DNA and allowed to hybridize (hybridization step). In some cases, three oligonucleotides are added for each SNP to be detected. Two of the three oligos are specific for each of the two alleles at a SNP position and are referred to as Allele-Specific Oligos (ASOs). A third oligo hybridizes several bases downstream from the SNP site and is referred to as the Locus-Specific Oligo (LSO). All three oligos contain regions of genomic complementarity (C1, C2, and C3) and universal PCR primer sites (P1, P2 and P3). The LSO also contains a unique address sequence (Address) that targets a particular bead type. In some cases, up to 1,536 SNPs may be interrogated in this manner. During the primer hybridization process, the assay oligonucleotides hybridize to the genomic DNA sample bound to paramagnetic particles. Because hybridization occurs prior to any amplification steps, no amplification bias is introduced into the assay. The above primers can further be modified to serve the two roles

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of determining the identity of the different SNPs and to determining the identity of the bin from which the genotype was derived. In **step 704**, following the hybridization step, several wash steps are performed reducing noise by removing excess and mis-hybridized oligonucleotides. Extension of the appropriate ASO and ligation of the extended product to the LSO joins information about the genotype present at the SNP site to the address sequence on the LSO. In **step 705**, the joined, full-length products provide a template for performing PCR reactions using universal PCR primers P1, P2, and P3. Universal primers P1 and P2 are labeled with two different labels (e.g., Cy3 and Cy5). Other labels that can be used include, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, scattering or fluorescent nanoparticles, Raman signal generating moieties, or electrochemical detection moieties. In **step 706**, the single-stranded, labeled DNAs are eluted and prepared for hybridization. In step **707**, the single-stranded, labeled DNAs are hybridized to their complement bead type through their unique address sequence. Hybridization of the GoldenGate Assay products onto the Array Matrix of Beadchip allows for separation of the assay products in solution, onto a solid surface for individual SNP genotype readout. In **step 708**, the array is washed and dried. In **step 709**, a reader such as the BeadArray Reader is used to analyze signals from the label. For example, when the labels are dye labels such as Cy3 and Cy5, the reader can analyze the fluorescence signal on the Sentrix Array Matrix or BeadChip. In **step 710**, a computer readable medium having a computer executable logic recorded on it can be used in a computer to perform receive data from one or more quantified DNA genomic regions to automate genotyping clusters and callings. Expression detection and analysis using microarrays is described in part in Valk, P. J. et al. New England Journal of Medicine 350(16), 1617-28, 2004; Modlich, O. et al. Clinical Cancer Research 10(10), 3410-21, 2004; Onken, Michael D. et al. Cancer Res. 64(20), 7205-7209, 2004; Gardian, et al. J. Biol. Chem. 280(1), 556-563, 2005; Becker, M. et al. Mol. Cancer Ther. 4(1), 151-170, 2005; and Flechner, SM et al. Am J Transplant 4(9), 1475-89, 2004; as well as in US Patent Nos. 5,445,934; 5,700,637; 5,744,305; 5,945,334; 6,054,270; 6,140,044; 6,261,776; 6,291,183; 6,346,413; 6,399,365; 6,420,169; 6,551,817; 6,610,482; 6,733,977; and EP 619 321; 323 203.

[00164] In any of the embodiments herein, preferably, more than 1000, 5,000, 10,000, 50,000, 100,000, 500,000, or 1,000,000 SNPs are interrogated in parallel.

[00165] In another aspect of the invention, illustrated in part by **Figure 6**, the systems and methods herein can be used to diagnose, prognose, and monitor neoplastic conditions such as cancer in a patient. Examples of neoplastic conditions contemplated herein include acute lymphoblastic leukemia, acute or chronic lymphocytic or granulocytic tumor, acute myeloid leukemia, acute promyelocytic leukemia, adenocarcinoma, adenoma, adrenal cancer, basal cell carcinoma, bone cancer, brain cancer, breast cancer, bronchi cancer, cervical dysplasia, chronic myelogenous leukemia, colon cancer, epidermoid carcinoma, Ewing's sarcoma, gallbladder cancer, gallstone tumor, giant cell tumor, glioblastoma multiforma, hairy-cell tumor, head cancer, hyperplasia, hyperplastic comeal nerve tumor, in situ carcinoma, intestinal ganglioneuroma, islet cell tumor, Kaposi's sarcoma, kidney cancer, larynx cancer, leiomyomater tumor, liver cancer, lung cancer, lymphomas, malignant carcinoid, malignant hypercalcemia, malignant melanomas, marfanoid habitus tumor, medullary carcinoma, metastatic skin carcinoma, mucosal neuromas, mycosis fungoide, myelodysplastic syndrome, myeloma, neck cancer, neural tissue cancer, neuroblastoma, osteogenic sarcoma, osteosarcoma, ovarian tumor, pancreas cancer, parathyroid cancer, pheochromocytoma, polycythemia vera, primary brain tumor, prostate cancer, rectum cancer, renal cell tumor, retinoblastoma, rhabdomyosarcoma, seminoma, skin cancer, small-cell lung tumor, soft tissue sarcoma, squamous cell carcinoma, stomach cancer, thyroid cancer, topical skin lesion, veticulum cell sarcoma, and Wilm's tumor.

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[00166] Cancers such as breast, colon, liver, ovary, prostate, and lung as well as other tumors exfoliate epithelial cells into the bloodstream. The presence of an increased number epithelial cells is associated with an active tumor or other neoplastic condition, tumor progression and spread, poor response to therapy, relapse of disease, and/or decreased survival over a period of several years. Therefore, enumerating and/or analyzing epithelial cells and CTC's in the bloodstream can be used to diagnose, prognose, and/or monitor neoplastic conditions.

[00167] In step **600,** a sample is obtained from an animal such as a human. The human can be suspected of having cancer or cancer recurrence or may have cancer and is in need of therapy selection. The sample obtained is a mixed sample comprising normal cells as well as one or more CTCs, epithelial cells, endothelial cells, stem cells, or other cells indicative of cancer. In some cases, the sample is a blood sample. In some cases multiple samples are obtained from the animal at different points in time (e.g., regular intervals such as daily, or every 2, 3 or 4 days, weekly, bimonthly, monthly, bi-yearly or yearly.

[00168] In step **602,** the mixed sample is then enriched for epithelial cells or CTC's or other cell indicative of cancer. Epithelial cells that are exfoliated from solid tumors have been found in very low concentrations in the circulation of patients with advanced cancers of the breast, colon, liver, ovary, prostate, and lung, and the presence or relative number of these cells in blood has been correlated with overall prognosis and response to therapy. These epithelial cells which are in fact CTCs can be used as an early indicator of tumor expansion or metastasis before the appearance of clinical symptoms.

[00169] CTCs are generally larger than most blood cells. Therefore, one useful approach for obtaining CTCs in blood is to enrich them based on size, resulting in a cell population enriched in CTCs. Another way to enrich CTCs is by affinity separation, using antibodies specific for particular cell surface markers may be used. Useful endothelial cell surface markers include CD105, CD106, CD144, and CD146; useful tumor endothelial cell surface markers include TEM1, TEM5, and TEM8 (see, e.g., Carson-Walter et al., Cancer Res. 61:6649-6655 (2001)); and useful mesenchymal cell surface markers include CD133. Antibodies to these or other markers may be obtained from, e.g., Chemicon, Abcam, and R&D Systems.

[00170] In one example, a size-based separation module that enriches CTC's from a fluid sample (e.g., blood) comprises an array of obstacles that selectively deflect particles having a hydrodynamic size larger than 10 μm into a first outlet and particles having a hydrodynamic size smaller than 10 μm into a second outlet is used to enrich epithelial cells and CTC's from the sample.

[00171] In step **603,** the enriched product is split into a plurality of discrete sites, such as mirowells. Examplary microwells that can be used in the present invention include microplates having 1536 wells as well as those of lesser density (e.g., 96 and 384 wells). Microwell plate design contemplated herein include those have 14 outputs that can be automatically dispensed at the same time, as well as those with 16, 24, or 32 outputs such that e.g., 32 outputs can be dispenses simultaneously. **Figure 9** illustrates one embodiments of a microwell plate contemplated herein.

[00172] Dispensing of the cells into the various discrete sites is preferably automated. In some cases, about 1, 5, 10, or 15 μL of enriched sample is dispensed into each well. Preferably, the size of the well and volume dispensed into each well is such that only 1 cell is dispensed per well and only 1-5 or less than 3 cells can fit in each well.

[00173] An exemplary array for sample splitting is illustrated in **Figure 8A. Figure 8B** illustrates an isometric view and **Figure 8B** illustrates a top view and cross sectional view of such an array. A square array of wells is arranged such that each subsequent row or column of wells is identical to the previous row or column of wells, respectively. In some embodiments, an array of wells is configured in a substrate or plate that about 2.0 cm^2, 2.5 cm^2, 3 cm^2 or larger. The wells can be of any shape, e.g., round, square, or oval. The height or width of each well can be between 5-50 μm, 10-40 μm, or about 25 μm. The depth of each well can be up to 100, 80, 60, or 40 μm; and the radius

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between the centers of two wells in one column is between 10-60 μm, 20-50 μm, or about 35 μm. Using these configurations, an array of wells of area 2.5 cm^2 can have a at least 0.1 x 10^6 wells, 0.2 x 10^6 wells, 0.3 x 10^6 wells, 0.4 x 10^6 wells, or 0.5 x 10^6 wells.

[00174] In some embodiments, such as those illustrated in **Figure 8C** each well may have an opening at the bottom. The bottom opening is preferably smaller in size than the cells of interest. In this case, if the average radius of a CTC is about 10 μm, the bottom opening of each well can have a radius of up to 8, 7, 6, 5, 4, 3, 2 or 1 μm. The bottom opening allows for cells non-of interest and other components smaller than the cell of interest to be removed from the well using flow pressure, leaving the cells of interest behind in the well for further processing. Methods and systems for actuating removal of cells from discrete predetermined sites are disclosed in US Patent No. 6,692,952 and US Application Serial No. 11/146,581.

[00175] In some cases, the array of wells can be a micro-electro-mechanical system (MEMS) such that it integrates mechanical elements, sensors, actuators, and electronics on a common silicon substrate through microfabrication technology. Any electronics in the system can be fabricated using integrated circuit (IC) process sequences (e.g., CMOS, Bipolar, or BICMOS processes), while the micromechanical components are fabricated using compatible micromachining processes that selectively etch away parts of the silicon wafer or add new structural layers to form the mechanical and electromechanical devices. One example of a MEMS array of wells includes a MEMS isolation element within each well. The MEMS isolation element can create a flow using pressure and/or vacuum to increase pressure on cells and particles not of interest to escape the well through the well opening. In any of the embodiments herein, the array of wells can be coupled to a microscope slide or other substrate that allows for convenient and rapid optical scanning of all chambers (i.e. discrete sites) under a microscope. In some embodiments, a 1536-well microtiter plate is used for enhanced convenience of reagent addition and other manipulations.

[00176] In some cases, the enriched product can be split into wells such that each well is loaded with a plurality of leukocytes (e.g., more than 100, 200, 500, 1000, 2000, or 5000). In some cases, about 2500 leukocytes are dispensed per well, while random wells will have a single epithelial CTC or up to 2, 3, 4, or 5 epithelial cells or CTC's. Preferably, the probability of getting a single epithelial cell or CTC into a well is calculated such that no more than 1 CTC is loaded per well. The probability of dispensing CTC's from a sample into wells can be calculated using Poisson statistics. When dispensing a 15 mL sample into 1536 wellplate at 10 μL per well, it is not until the number of CTC's in the sample is > 100 that there is more than negligible probability of two or more CTC's being loaded into the sample well. **Figure 9** illustrates the probability density function of loading two CTC's into the same plate.

[00177] In step **604**, rare cells (e.g. epithelial cells or CTC's) or rare DNA is detected and/or analyzed in each well.

[00178] In some embodiments, detection and analysis includes enumerating epithelial cells and/or CTC's. CTCs typically have a short half-life of approximately one day, and their presence generally indicates a recent influx from a proliferating tumor. Therefore, CTCs represent a dynamic process that may reflect the current clinical status of patient disease and therapeutic response. Thus, in some embodiments, step **604** involves enumerating CTC and/or epithelial cells in a sample (array of wells) and determining based on their number if a patient has cancer, severity of condition, therapy to be used, or effectiveness of therapy administered.

[00179] In some cases, the method herein involve making a series of measurements, optionally made at regular intervals such as one day, two days, three days, one week, two weeks, one month, two months, three months, six months, or one year, one may track the level of epithelial cells present in a patient's bloodstream as a function of time. In the case of existing cancer patients, this provides a useful indication of the progression of the disease and assists medical practitioners in making appropriate therapeutic choices based on the increase, decrease, or lack of

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change in epithelial cells, e.g., CTCs, in the patient's bloodstream. For those at risk of cancer, a sudden increase in the number of cells detected may provide an early warning that the patient has developed a tumor. This early diagnosis, coupled with subsequent therapeutic intervention, is likely to result in an improved patient outcome in comparison to an absence of diagnostic information.

[00180] In some cases, more than one type of cell (e.g., epithelial, endothelial, etc.) can be enumerated and a determination of a ratio of numbers of cells or profile of various cells can be obtained to generate the diagnosis or prognosis.

[00181] Alternatively, detection of rare cells or rare DNA (e.g. epithelial cells or CTC's) can be made by detecting one or more cancer biomarkers, e.g., any of those listed in **Figure 10** in one or more cells in the array. Detection of cancer biomarkers can be accomplished using, e.g., an antibody specific to the marker or by detecting a nucleic acid encoding a cancer biomarker, e.g., listed in Figure 9.

[00182] In some cases single cell analysis techniques are used to analyze individual cells in each well. For example, single cell PCR may be performed on a single cell in a discrete location to detect one or more mutant alleles in the cell (Thornhill AR, J. Mol. Diag; (4) 11-29 (2002)) or a mutation in a gene listed in Figure 9. In-cell PCR, gene expression analysis can be performed even when the number of cells per well is very low (e.g. 1 cell per well) using techniques known in the art. (Giordano et al., Am. J. Pathol. 159:1231-1238 (2001), and Buckhaults et al., Cancer Res. 63:4144-4149 (2003). In some cases, single cell expression analysis can be performed to detection expression of one or more genes of interest (Liss B., Nucleic Acids Res., 30 (2002)) including those listed in Figure 9. Furthermore, ultra-deep sequencing can be performed on single cells using methods such as those described in Marguiles M., et al. Nature, "Genome sequencing in microfabricated high-density picolitre reactors." DOI 10.1038, in which whole genomes are fragmented, fragments are captured using common adapters on their own beads and within droplets of an emulsion, clonally amplified. Such ultra-deep sequending can also be used to detect mutations in genes associated with cancer, such as those listed in Figure 9. In addition, fluorescence in-situ hybridization can be used, e.g., to determine the tissue or tissues of origin of the cells being analyzed.

[00183] In some cases, morphological analyses are performed on the cells in each well. Morphological analyses include identification, quantification and characterization of mitochondrial DNA, telomerase, or nuclear matrix proteins. Parrella et al., Cancer Res. 61:7623-7626 (2001); Jones et al., Cancer Res. 61:1299-1304 (2001); Fliss et al., Science 287:2017-2019 (2000); and Soria et al., Clin. Cancer Res. 5:971-975 (1999). In particular, in some cases, the molecular analyses involves determining whether any mitochrondial abnormalities or whether perinuclear compartments are present. Carew et al., Mol. Cancer 1:9 (2002); and Wallace, Science 283:1482-1488 (1999).

[00184] A variety of cellular characteristics may be measured using any technique known in the art, including: protein phosphorylation, protein glycosylation, DNA methylation (Das et al., J. Clin. Oncol. 22:4632-4642 (2004)), microRNA levels (He et al., Nature 435:828-833 (2005), Lu et al., Nature 435:834-838 (2005), O'Donnell et al., Nature 435:839-843 (2005), and Calin et al., N. Engl. J. Med. 353:1793-1801 (2005)), cell morphology or other structural characteristics, e.g., pleomorphisms, adhesion, migration, binding, division, level of gene expression, and presence of a somatic mutation. This analysis may be performed on any number of cells, including a single cell of interest, e.g., a cancer cell.

[00185] In one embodiment, the cell(s) (such as fetal, maternal, epithelial or CTCs) in each well are lysed and RNA is extracted using any means known in the art. For example, The Quiagen RNeasy™ 96 bioRobot™ 8000 system can be used to automate high-throughput isolation of total RNA from each discrete site. Once the RNA is extracted reverse transcriptase reactions can be performed to generate cDNA sequences, which can then be used for performing multiplex PCR reactions on target genes. For example, 1 or more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or

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20 target genes can be amplified in the same reaction. When more than one target genes are used in the same amplification reaction, primers are chosen to be multiplexable (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis) with other pairs of primers. Multiple dyes and multi-color fluorescence readout may be used to increase the multiplexing capacity. Examples of dyes that can be used to label primers for amplification include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties.

[00186] In another embodiment, fetal or maternal cells or nuclei are enriched using one or more methods disclosed herein. Preferably, fetal cells are enriched by flowing the sample through an array of obstacles that selectively directs particles or cells of different hydrodynamic sizes into different outlets such that fetal cells and cells larger than fetal cells are directed into a first outlet and one or more cells or particles smaller than the rare cells are directed into a second outlet.

[00187] Total RNA or poly-A mRNA is then obtained from enriched cell(s) (fetal or maternal cells) using purification techniques known in the art. Generally, about 1 μg – 2 μg of total RNA is sufficient. Next, a first-strand complementary DNA (cDNA) is synthesized using reverse transcriptase and a single T7-oligo(dT) primer. Next, a second-strand cDNA is synthesized using DNA ligase, DNA polymerase, and RNase enzyme. Next, the double stranded cDNA (ds-cDNA) is purified.

[00188] In another embodiment , total RNA is extracted from enriched cells (fetal cells or maternal cells). Next a, two one-quarter scale Message Amp II reactions (Ambion, Austin, Texas) are performed for each RNA extraction using 200 ng of total RNA. MessageAmp is a procedure based on antisense RNA (aRNA) amplification, and involves a series of enzymatic reactions resulting in linear amplification of exceedingly small amounts of RNA for use in array analysis. Unlike exponential RNA amplification methods, such as NASBA and RT-PCR, aRNA amplification maintains representation of the starting mRNA population. The procedure begins with total or poly(A) RNA that is reverse transcribed using a primer containing both oligo(dT) and a T7 RNA polymerase promoter sequence. After first-strand synthesis, the reaction is treated with RNase H to cleave the mRNA into small fragments. These small RNA fragments serve as primers during a second-strand synthesis reaction that produces a double-stranded cDNA template.

[00189] In some embodiments, cDNAs, which are reverse transcribed from mRNAs obtained from fetal or maternal cells, are tagged and sequenced. The type and abundance of the cDNAs can be used to determine whether a cell is a fetal cell (such as by the presence of Y chromosome specific transcripts) or whether the fetal cell has a genetic abnormality (such as aneuploidy, abundance or type of alternative transcripts or problems with DNA methylation or imprinting).

[00190] In one embodiment, PCR amplification can be performed on genes that are expressed in epithelial cells and not in normal cells, e.g., white blood cells or other cells remaining in an enriched product. Exemplary genes that can be analyzed according to the methods herein include EGFR, EpCAM, GA733-2, MUC-1, HER-2, Claudin-7 and any other gene identified in **Figure 10**.

[00191] For example, analysis of the expression level or pattern of such a polypeptide or nucleic acid, e.g., cell surface markers, genomic DNA, mRNA, or microRNA, may result in a diagnosis or prognosis of cancer.

[00192] In some embodiments, cDNAs, which are reverse transcribed from mRNAs obtained from fetal or maternal cells, are tagged and sequenced. The type and abundance of the cDNAs can be used to determine whether a cell is a

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fetal cell (such as by the presence of Y chromsome specific transcripts) or whether the fetal cell has a genetic abnormality (such as anueploidy, or problems with DNA methylation or imprinting).

[00193] In some embodiments, analysis step **604** involves identifying cells from a mixed sample that express genes which are not expressed in the non-rare cells (e.g. EGFR or EpCAM). For example, an important indicator for circulating tumor cells is the presence/expression of EGFR or EGF at high levels wherein non-cancerous epithelial cells will express EGFR or EGF at smaller amounts if at all.

[00194] In addition, for lung cancer and other cancers, the presence or absence of certain mutations in EGFR can be associated with diagnosis and/or prognosis of the cancer as well and can also be used to select a more effective treatment (see, e.g., International Publication WO 2005/094357). For example, many non-small cell lung tumors with EGFR mutations respond to small molecule EGFR inhibitors, such as gefitinib (Iressa; AstraZeneca), but often eventually acquire secondary mutations that make them drug resistant. In some embodiments, one can determine a therapy treatment for a patient by enriching epithelial cells and/or CTC's using the methods herein, splitting sample of cells (preferably so no more than 1 CTC is in a discrete location), and detecting one or more mutations in the EGFR gene of such cells. Exemplary mutations that can be analyzed include those clustered around the ATP-binding pocket of the EGFR TK domain, which are known to make cells susceptible to gefitinib inhibition. Thus, presence of such mutations supports a diagnosis of cancer that is likely to respond to treatment using gefitinib.

[00195] Many patients who respond to gefitinib eventually develop a second mutation, often a methionine-to-threonine substitution at position 790 in exon 20 of the TK domain. This type of mutation renders such patients resistant to gefitinib. Therefore, the present invention contemplates testing for this mutation as well to provide further diagnostic information.

[00196] Since many EGFR mutations, including all EGFR mutations in NSC lung cancer reported to date that are known to confer sensitivity or resistance to gefitinib, lie within the coding regions of exons 18 to 21, this region of the EGFR gene may be emphasized in the development of assays for the presence of mutations. Examples of primers that can be used to detect mutations in EGFR include those listed in **Figure 11.**

[00197] In step **605**, a determination is made as to the condition of a patient based on analysis made above. In some cases the patient can be diagnosed with cancer or lack thereof. In some cases, the patient can be prognosed with a particular type of cancer. In cases where the patient has cancer, therapy may be determined based on the types of mutations detected.

[00198] In another embodiment, cancer cells may be detected in a mixed sample (e.g. circulating tumor cells and circulating normal cells) using one or more of the sequencing methods described herein. Briefly, RNA is extracted from cells in each location and converted to cDNA as described above. Target genes are then amplified and high throughput ultra deep sequencing is performed to detect a mutation expression level associated with cancer.

VI. Computer Executable Logic

[00199] Any of the steps herein can be performed using computer program product that comprises a computer executable logic recorded on a computer readable medium. For example, the computer program can use data from target genomic DNA regions to determine the presence or absence of fetal cells in a sample and to determine fetal abnormalit(ies) in cells detected. In some embodiments, computer executable logic uses data input on STR or SNP intensities to determine the presence of fetal cells in a test sample and determine fetal abnormalities and/or conditions in said cells.

[00200] The computer program may be specially designed and configured to support and execute some or all of the functions for determining the presence of rare cells such as fetal cells or epithelial/CTC's in a mixed sample and abnormalities and/or conditions associated with such rare cells or their DNA including the acts of (i) controlling the

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splitting or sorting of cells or DNA into discrete locations (ii) amplifying one or more regions of genomic DNA e.g. trisomic region(s) and non-trisomic region(s) (particularly DNA polymorphisms such as STR and SNP) in cells from a mixed sample and optionally control sample, (iii) receiving data from the one or more genomic DNA regions analyzed (e.g. sequencing or genotyping data); (iv) identifying bins with rare (e.g. non-maternal) alleles, (v) identifying bins with rare (e.g. non-maternal) alleles as bins containing fetal cells or epithelial cells, (vi) determining number of rare cells (e.g. fetal cells or epithelial cells) in the mixed sample, (vii) detecting the levels of maternal and non-maternal alleles in identified fetal cells, (viii) detecting a fetal abnormality or condition in said fetal cells and/or (ix) detecting a neoplastic condition and information concerning such condition such as its prevalence, origin, susceptibility to drug treatment(s), etc. In particular, the program can fit data of the quantity of allele abundance for each polymorphism into one or more data models. One example of a data model provides for a determination of the presence or absence of aneuploidy using data of amplified polymorphisms present at loci in DNA from samples that are highly enriched for fetal cells. The determination of presence of fetal cells in the mixed sample and fetal abnormalities and/or conditions in said cells can be made by the computer program or by a user.

[00201] In one example, let 'f' be the fetal/maternal DNA copy ratio in a particular PCR reaction. Trisomy increases the ratio of maternal to paternal alleles by a factor 1+f/2. PCR efficiencies vary from allele to allele within a locus by a mean square error in the logarithm given by σ_{allele}^2, and vary from locus to locus by σ_{locus}^2, where this second variance is apt to be larger due to differences in primer efficiency. N_a is the loci per suspected aneuploid chromosome and N_c is the control loci. If the mean of the two maternal allele strengths at any locus is 'm' and the paternal allele strength is 'p,' then the squared error expected is the mean of the ln(ratio(m/p)), where this mean is taken over N loci is given by $2(\sigma_{allele}^2)/N$. When taking the difference of this mean of ln(ratio(m/p)) between a suspected aneuploidy region and a control region, the error in the difference is given by

$$\sigma_{diff}^2 = 2(\sigma_{allele}^2)/N_a + 2(\sigma_{allele}^2)/N_c \quad (1)$$

[00202] For a robust detection of aneuploidy we require

$$3\sigma_{diff} < f/2.$$

[00203] For simplicity, assuming $N_a = N_c = N$ in Equation 1, this gives the requirement

$$6\sigma_{allele}/N^{1/2} < f/2, \quad (3)$$

or a minimum N of

$$N = 144(\sigma_{allele}/f)^2 \quad (4)$$

[00204] In the context of trisomy detection, the suspected aneuploidy region is usually the entire chromosome and N denotes the number of loci per chromosome. For reference, Equation 3 is evaluated for N in **Table 2** for various values of σ_{allele} and f.

[00205] The role of the sequencing is to measure the allele abundances output from the amplification step. It is desirable to do this without adding significantly more error due to the Poisson statistics of selecting only a finite number of amplicons for sequencing. The rms error in the ln(abundance) due to Poisson statistics is approximately $(N_{reads})^{-1/2}$. It is desirable to keep this value less than or equal to the PCR error σ_{allele}. Thus, a typical paternal allele needs to be allocated at least $(\sigma_{allele})^{-2}$ reads. The maternal alleles, being more abundant, do not add appreciably to this error when forming the ratio estimate for m/p. The mixture input to sequencing contains amplicons from N_{loci} loci of which roughly an abundance fraction f/2 are paternal alleles. Thus, the total required number of reads for each of the aliquot wells is given approximately by $2N_{loci}/(f\,\sigma_{allele}^2)$. Combining this result with Equation 4, it is found a total number of reads over all the wells given approximately by

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$N_{reads} = 288\ N_{wells}\ f^3$. Thus, the program can determine the total number of reads that need to be obtained for determining the presence or absence of aneuploidy in a patient sample.

[00206] The computer program can work in any computer that may be any of a variety of types of general-purpose computers such as a personal computer, network server, workstation, or other computer platform now or later developed. In some embodiments, a computer program product is described comprising a computer usable medium having the computer executable logic (computer software program, including program code) stored therein. The computer executable logic can be executed by a processor, causing the processor to perform functions described herein. In other embodiments, some functions are implemented primarily in hardware using, for example, a hardware state machine. Implementation of the hardware state machine so as to perform the functions described herein will be apparent to those skilled in the relevant arts.

[00207] In one embodiment, the computer executing the computer logic of the invention may also include a digital input device such as a scanner. The digital input device can provide an image of the target genomic DNA regions (e.g. DNA polymorphism, preferably STRs or SNPs) according to method of the invention. For instance, the scanner can provide an image by detecting fluorescent, radioactive, or other emissions; by detecting transmitted, reflected, or scattered radiation; by detecting electromagnetic properties or characteristics; or by other techniques. Various detection schemes are employed depending on the type of emissions and other factors. The data typically are stored in a memory device, such as the system memory described above, in the form of a data file.

[00208] In one embodiment, the scanner may identify one or more labeled targets. For instance, in the genotyping analysis described herein a first DNA polymorphism may be labeled with a first dye that fluoresces at a particular characteristic frequency, or narrow band of frequencies, in response to an excitation source of a particular frequency. A second DNA polymorphisms may be labeled with a second dye that fluoresces at a different characteristic frequency. The excitation sources for the second dye may, but need not, have a different excitation frequency than the source that excites the first dye, e.g., the excitation sources could be the same, or different, lasers.

[00209] In one embodiment, a human being may inspect a printed or displayed image constructed from the data in an image file and may identify the data (e.g. fluorescence from microarray) that are suitable for analysis according to the method of the invention. In another embodiment, the information is provided in an automated, quantifiable, and repeatable way that is compatible with various image processing and/or analysis techniques.

[00210] Another aspect of the invention is kits which permit the enrichment and analysis of the rare cells present in small qualities in the samples. Such kits may include any materials or combination of materials described for the individual steps or the combination of steps ranging from the enrichment through the genetic analysis of the genomic material. Thus, the kits may include the arrays used for size-based separation or enrichment, labels for uniquely labeling each cell, the devices utilized for splitting the cells into individual addressable locations and the reagents for the genetic analysis. For example, a kit might contain the arrays for size-based separation, unique labels for the cells and reagents for detecting polymorphisms including STRs or SNPs, such as reagents for performing PCR.

[00211] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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EXAMPLES

Example 1. Separation of Fetal Cord Blood

[00212] Figure 1E shows a schematic of the device used to separate nucleated cells from fetal cord blood.

[00213] *Dimensions:* 100 mm × 28 mm × 1mm

[00214] *Array design*: 3 stages, gap size = 18, 12 and 8 μm for the first, second and third stage, respectively.

[00215] *Device fabrication*: The arrays and channels were fabricated in silicon using standard photolithography and deep silicon reactive etching techniques. The etch depth is 140 μm. Through holes for fluid access are made using KOH wet etching. The silicon substrate was sealed on the etched face to form enclosed fluidic channels using a blood compatible pressure sensitive adhesive (9795, 3M, St Paul, MN).

[00216] *Device packaging*: The device was mechanically mated to a plastic manifold with external fluidic reservoirs to deliver blood and buffer to the device and extract the generated fractions.

[00217] *Device operation*: An external pressure source was used to apply a pressure of 2.0 PSI to the buffer and blood reservoirs to modulate fluidic delivery and extraction from the packaged device.

[00218] *Experimental conditions*: Human fetal cord blood was drawn into phosphate buffered saline containing Acid Citrate Dextrose anticoagulants. 1mL of blood was processed at 3 mL/hr using the device described above at room temperature and within 48 hrs of draw. Nucleated cells from the blood were separated from enucleated cells (red blood cells and platelets), and plasma delivered into a buffer stream of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (14190-144, Invitrogen, Carlsbad, CA) containing 1% Bovine Serum Albumin (BSA) (A8412-100ML, Sigma-Aldrich, St Louis, MO) and 2 mM EDTA (15575-020, Invitrogen, Carlsbad, CA).

[00219] *Measurement techniques*: Cell smears of the product and waste fractions (Figure 12A-12B) were prepared and stained with modified Wright-Giemsa (WG16, Sigma Aldrich, St. Louis, MO).

[00220] *Performance*: Fetal nucleated red blood cells were observed in the product fraction (Figure 12A) and absent from the waste fraction (Figure 12B).

Example 2. Isolation of Fetal Cells from Maternal blood

[00221] The device and process described in detail in Example 1 were used in combination with immunomagnetic affinity enrichment techniques to demonstrate the feasibility of isolating fetal cells from maternal blood.

[00222] *Experimental conditions*: blood from consenting maternal donors carrying male fetuses was collected into K_2EDTA vacutainers (366643, Becton Dickinson, Franklin Lakes, NJ) immediately following elective termination of pregnancy. The undiluted blood was processed using the device described in Example 1 at room temperature and within 9 hrs of draw. Nucleated cells from the blood were separated from enucleated cells (red blood cells and platelets), and plasma delivered into a buffer stream of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (14190-144, Invitrogen, Carlsbad, CA) containing 1% Bovine Serum Albumin (BSA) (A8412-100ML, Sigma-Aldrich, St Louis, MO). Subsequently, the nucleated cell fraction was labeled with anti-CD71 microbeads (130-046-201, Miltenyi Biotech Inc., Auburn, CA) and enriched using the MiniMACS™ MS column (130-042-201, Miltenyi Biotech Inc., Auburn, CA) according to the manufacturer's specifications. Finally, the CD71-positive fraction was spotted onto glass slides.

[00223] *Measurement techniques*: Spotted slides were stained using fluorescence *in situ* hybridization (FISH) techniques according to the manufacturer's specifications using Vysis probes (Abbott Laboratories, Downer's Grove, IL). Samples were stained from the presence of X and Y chromosomes. In one case, a sample prepared from a known Trisomy 21 pregnancy was also stained for chromosome 21.

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[00224] *Performance*: Isolation of fetal cells was confirmed by the reliable presence of male cells in the CD71-positive population prepared from the nucleated cell fractions (Figures 13A–13F). In the single abnormal case tested, the trisomy 21 pathology was also identified (Figure 14).

Example 3. Confirmation of the Presence of Male Fetal Cells in Enriched Samples.

[00225] Confirmation of the presence of a male fetal cell in an enriched sample is performed using qPCR with primers specific for DYZ, a marker repeated in high copy number on the Y chromosome. After enrichment of fnRBC by any of the methods described herein, the resulting enriched fnRBC are binned by dividing the sample into 100 PCR wells. Prior to binning, enriched samples may be screened by FISH to determine the presence of any fnRBC containing an aneuploidy of interest. Because of the low number of fnRBC in maternal blood, only a portion of the wells will contain a single fnRBC (the other wells are expected to be negative for fnRBC). The cells are fixed in 2% Paraformaldehyde and stored at 4°C. Cells in each bin are pelleted and resuspended in 5 µl PBS plus 1 µl 20 mg/ml Proteinase K (Sigma #P-2308). Cells are lysed by incubation at 65°C for 60 minutes followed by inactivation of the Proteinase K by incubation for 15 minutes at 95°C. For each reaction, primer sets (DYZ forward primer TCGAGTGCATTCCATTCCG; DYZ reverse primer ATGGAATGGCATCAAACGGAA; and DYZ Taqman Probe 6FAM-TGGCTGTCCATTCCA-MGBNFQ), TaqMan Universal PCR master mix, No AmpErase and water are added. The samples are run and analysis is performed on an ABI 7300: 2 minutes at 50°C, 10 minutes 95°C followed by 40 cycles of 95°C (15 seconds) and 60°C (1 minute). Following confirmation of the presence of male fetal cells, further analysis of bins containing fnRBC is performed. Positive bins may be pooled prior to further analysis.

[00226] **Figure 30** shows the results expected from such an experiment. The data in **Figure 30** was collected by the following protocol. Nucleated red blood cells were enriched from cord cell blood of a male fetus by sucrose gradient two Heme Extractions (HE). The cells were fixed in 2% paraformaldehyde and stored at 4°C. Approximately 10 x 1000 cells were pelleted and resuspended each in 5 µl PBS plus 1 µl 20 mg/ml Proteinase K (Sigma #P-2308). Cells were lysed by incubation at 65°C for 60 minutes followed by a inactivation of the Proteinase K by 15 minute at 95°C. Cells were combined and serially diluted 10-fold in PBS for 100, 10 and 1 cell per 6 µl final concentration were obtained. Six µl of each dilution was assayed in quadruplicate in 96 well format. For each reaction, primer sets (0.9uM DYZ forward primer TCGAGTGCATTCCATTCCG; 0.9uM DYZ reverse primer ATGGAATGGCATCAAACGGAA; and 0.5uM DYZ TaqMan Probe 6FAM-TGGCTGTCCATTCCA-MGBNFQ), TaqMan Universal PCR master mix, No AmpErase and water were added to a final volume of 25 µl per reaction. Plates were run and analyzed on an ABI 7300: 2 minutes at 50°C, 10 minutes 95°C followed by 40 cycles of 95°C (15 seconds) and 60°C (1 minute). These results show that detection of a single fnRBC in a bin is possible using this method.

Example 4. Confirmation of the presence of fetal cells in enriched samples by STR analysis.

[00227] Maternal blood is processed through a size-based separation module, with or without subsequent MHEM enhancement of fnRBCs. The enhanced sample is then subjected to FISH analysis using probes specific to the aneuploidy of interest (e.g., trisomy 13, trisomy 18, and XYY). Individual positive cells are isolated by "plucking" individual positive cells from the enhanced sample using standard micromanipulation techniques. Using a nested PCR protocol, STR marker sets are amplified and analyzed to confirm that the FISH-positive aneuploid cell(s) are of fetal origin. For this analysis, comparison to the maternal genotype is typical. An example of a potential resulting data set is shown in **Table 3**. Non-maternal alleles may be proven to be paternal alleles by paternal genotyping or genotyping of known fetal tissue samples. As can be seen, the presence of paternal alleles in the resulting cells,

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demonstrates that the cell is of fetal origin (cells # 1, 2, 9, and 10). Positive cells may be pooled for further analysis to diagnose aneuploidy of the fetus, or may be further analyzed individually.

Table 3. STR locus alleles in maternal and fetal cells

DNA Source	STR locus D14S	STR locus D16S	STR locus D8S	STR locus F13B	STR locus vWA
Maternal alleles	14, 17	11, 12	12, 14	9, 9	16, 17
Cell #1 alleles		8			19
Cell #2 alleles	17		15		
Cell #3 alleles			14		
Cell #4 alleles					
Cell #5 alleles	17	12		9	
Cell #6 alleles					
Cell #7 alleles					19
Cell #8 alleles					
Cell #9 alleles	17		14	7, 9	17, 19
Cell #10 alleles			15		

Example 5. Confirmation of the presence of fetal cells in enriched samples by SNP analysis.

[00228] Maternal blood is processed through a size-based separation module, with or without subsequent MHEM enhancement of fnRBCs. The enhanced sample is then subjected to FISH analysis using probes specific to the aneuploidy of interest (e.g., triploidy 13, triploidy 18, and XYY). Samples testing positive with FISH analysis are then binned into 96 microtiter wells, each well containing 15 µl of the enhanced sample. Of the 96 wells, 5-10 are expected to contain a single fnRBC and each well should contain approximately 1000 nucleated maternal cells (both WBC and mnRBC). Cells are pelleted and resuspended in 5µl PBS plus 1µl 20 mg/ml Proteinase K (Sigma #P-2308). Cells are lysed by incubation at 65°C for 60 minutes followed by a inactivation of the Proteinase K by 15 minute at 95°C.

[00229] In this example, the maternal genotype (BB) and fetal genotype (AB) for a particular set of SNPs is known. The genotypes A and B encompass all three SNPs and differ from each other at all three SNPs. The following sequence from chromosome 7 contains these three SNPs (rs7795605, rs7795611 and rs7795233 indicated in brackets, respectively):

(ATGCAGCAAGGCACAGACTAA[G/A]CAAGGAGA[G/C]GCAAAATTTTC[A/G]TAGGGGAGAGAAATGG GTCATT).

[00230] In the first round of PCR, genomic DNA from binned enriched cells is amplified using primers specific to the outer portion of the fetal-specific allele A and which flank the interior SNP (forward primer ATGCAGCAAGGCACAGACTACG; reverse primer AGAGGGGAGAGAAATGGGTCATT). In the second round of PCR, amplification using real time SYBR Green PCR is performed with primers specific to the inner portion of allele A and which encompass the interior SNP (forward primer CAAGGCACAGACTAAGCAAGGAGAG; reverse primer GGCAAAATTTTCATAGGGGAGAGAAATGGGTCATT).

[00231] Expected results are shown in **Figure 31**. Here, six of the 96 wells test positive for allele A, confirming the presence of cells of fetal origin, because the maternal genotype (BB) is known and cannot be positive for allele A. DNA from positive wells may be pooled for further analysis or analyzed individually.

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Example 6. Quantitative Genotyping Using Molecular Inversion Probes for Trisomy Diagnosis on Fetal Cells

[00232] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1. Quantitative genotyping can then be used to detect chromosome copy number changes. Figure 5 depicts a flow chart depicting the major steps involved in detecting chromosome copy number changes using the methods described herein. For example, the enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[00233] *Perform multiplex PCR and nested PCR:* PCR primer pairs for multiple (40 – 100) highly polymorphic SNPs can then be added to each well in the microtiter plate. For example, SNPs primers can be designed along chromosomes 13, 18, 21 and X to detect the most frequent aneuploidies, and along control regions of the genome where aneuploidy is not expected. Multiple (~10) SNPs would be designed for each chromosome of interest to allow for non-informative genotypes and to ensure accurate results. The SNPs listed in the Table below can be used to performed analysis and associated PCR primers can be designed as described below.

SNPs that can be used for fetal cell analysis			
Chromosome 13	Chromosome 18	Chromosome 21	Chromosome X/Y
refSNP rs9510053	refSNP rs584853	refSNP rs469000	refSNP rs6608727
refSNP rs7339372	refSNP rs2345588	refSNP rs7278903	refSNP rs2015487
refSNP rs9580269	refSNP rs9973072	refSNP rs1004044	refSNP rs5953330
refSNP rs724946	refSNP rs7504787	refSNP rs11910419	refSNP rs5953330
refSNP rs11842845	refSNP rs4303617	refSNP rs2832890	refSNP rs1984695
refSNP rs7490040	refSNP rs9947441	refSNP rs1785477	refSNP rs5906775
refSNP rs12430585	refSNP rs2912334	refSNP rs2250226	refSNP rs5951325
refSNP rs713280	refSNP rs11659665	refSNP rs2243594	refSNP rs11798710
refSNP rs202090	refSNP rs8098249	refSNP rs10483087	refSNP rs4898352
refSNP rs5000966	refSNP rs12968582	refSNP rs855262	refSNP rs5987079

[00234] PCR primers would be chosen to be multiplexible with other pairs (fairly uniform melting temperature, absence of cross-priming on the human genome, and absence of primer-primer interaction based on sequence analysis). The primers would be designed to generate amplicons 70 - 100 bp in size to increase the performance of the multiplex PCR. The primers would contain a 22 bp tag on the 5' which is used in the genotyping analysis. Multiplex PCR protocols can be performed as described in Findlay et al. Molecular Cell Endocrinology 183 (2001) S5-S12. Primer concentrations can vary from 0.7 pmoles to 60 pmoles per reaction. Briefly, PCRs are performed in a total volume of 25 µl per well, Taq polymerase buffer (Perkin-Elmer), 200 µM dNTPs, primer, 1.5 mM MgCl2 and 0.6 units AmpliTaq (Perkin-Elmer). After denaturation at 95 °C for 5 min, 41 cycles at 94, 60 and 72 °C for 45 s are performed in a MJ DNA engine thermal cycler. The amplification can be run with an annealing temperature different that 60 °C depending on the primer pair being amplified. Final extension can be for 10 min.

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[00235] A second of round of PCR using nested primers may be performed to ensure optimal performance of the multiplex amplification. Two ul aliquot of each PCR reaction is diluted 40 fold (to 80 ul total) with nuclease free water from the PCR kit. A no template or negative control is generated to test for contamination. The amplification with the nested PCR primers is run with an annealing temperature of 60° C - 68° C depending on the primer pair being amplified.

Nested PCR cycle		
Step	Temp (C)	Time (mins)
1.0	95	0.5
2.0	94	0.5
3.0	X	1.5
4.0	72	1.5
5.0	cycle to step 2, 44 times	
6.0	72	10

[00236] [illegible]ology developed by Affymetrix (Santa Clara, CA) can genotype 20,000 SNPs or more in a single reaction. In the typical MIP assay, each SNP would be assigned a 22bp DNA tag which allows the SNP to be uniquely identified during the highly parallel genotyping assay. In this example, the DNA tags serve two roles: (1) determine the identity of the different SNPs and (2) determine the identity of the well from which the genotype was derived. For example, a total of 20,000 tags would be required to genotype the same 40 SNPs in 500 wells different wells (4 chromosomes x 10 SNPs x 500 wells)

[00237] The tagged MIP probes would be combined with the amplicons from the initial multiplex single-cell PCR (or nested PCR) and the genotyping reactions would be performed. The probe/template mix would be divided into 4 tubes each containing a different nucleotide (e.g. G, A, T or C). Following an extension and ligation step, the mixture would be treated with exonuclease to remove all linear molecules and the tags of the surviving circular molecules would be amplified using PCR. The amplified tags form all of the bins would then be pooled and hybridized to a single DNA microarray containing the complementary sequences to each of the 20,000 tags.

[00238] *Identify bins with non-maternal alleles (e.g. fetal cells):* The first step in the data analysis procedure would be to use the 22bp tags to sort the 20,000 genotypes into bins which correspond to the individual wells of the original microtiter plates. The second step would be to identify bins contain non-maternal alleles which correspond to wells that contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins would provide an accurate estimate of the number of fnRBCs that were present in the original enriched cell population. When a fetal cell is identified in a given bin, the non-maternal alleles would be detected by 40 independent SNPs which provide an extremely high level of confidence in the result.

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[00239] *Detect ploidy for chromosomes 13, 18, and 21*: After identifying approximately 10 bins that contain fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18, 21 and X by comparing ratio of maternal to paternal alleles for each of the 10 SNPs on each chromosome. The ratios for the multiple SNPs on each chromosome can be combined (averaged) to increase the confidence of the aneuploidy call for that chromosome. In addition, the information from the approximate 10 independent bins containing fetal cells can also be combined to further increase the confidence of the call.

Example 7. Ultra-deep Sequencing for Trisomy Diagnosis on Fetal Cells

[00240] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1. The enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[00241] *Perform multiplex PCR and Ultra-Deep Sequencing with bin specific tags:* PCR primer pairs for highly polymorphic STR loci (multiple loci per chromosome of interest) are then added to each well in the microtiter plate. The polymorphic STRs listed in the Table below can be used to performed analysis and associated PCR primers can be designed.

STR loci that can be used for fetal cell analysis	
MARKER	CHROMOSOME LOCATION
D21S1414	21q21
MBP	18q23-ter
D13S634	13q14.3-22
D13S631	13q31-32
D18S535	18q12.2-12.3
D21S1412	21(S171-S198)
D21S1411	21q22.3
D21S11	21q21
D18S386	18q22.1-18q22.2
D13S258	13q21.2-13q31
D13S303	13q22-13q31
D18S1002	18q11

[00242] The primers for each STR will have two important features. First, each of the primers will contain a common ~18bp sequence on the 5' end which is used for the subsequent DNA cloning and sequencing procedures. Second, each well in the microtiter plate is assigned a unique ~6bp DNA tag sequence which is incorporated into the middle part of the upstream primer for each of the different STRs. The DNA tags make it possible to pool all of the STR amplicons following the multiplex PCR which makes it possible to analyze the amplicons in parallel more cost effectively during the ultra-deep sequencing procedure. DNA tags of length ~ 6 bp provide a compromise between information content (4096 potential bins) and the cost of synthesizing primers.

[00243] Multiplex PCR protocols can be performed as described in Findlay et al. Molecular Cell Endocrinology 183 (2001) S5-S12. Primer concentrations can vary from 0.7 pmoles to 60 pmoles per reaction. Briefly, PCRs are performed in a total volume of 25 µl per well, Taq polymerase buffer (Perkin-Elmer), 200 µM dNTPs, primer, 1.5 mM MgCl2 and 0.6 units AmpliTaq (Perkin-Elmer). After denaturation at 95 °C for 5 min, 41 cycles at 94, 60 and

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72 °C for 45 s are performed in a MJ DNA engine thermal cycler. The amplification can be run with an annealing temperature different that 60 °C depending on the primer pair being amplified. Final extension can be for 10 min.

[00244] Following PCR, the amplicons from each of the wells in the microtiter plate are pooled, purified and analyzed using a single-molecule sequencing strategy as described in Margulies et al. Nature 437(2005) 376-380. Briefly, the amplicons are diluted and mixed with beads such that each bead captures a single molecule of the amplified material. The DNA-carrying beads are isolated in separate 100 um aqueous droplets made through the creation of a PCR-reation-mixture-in-oil emulsion. The DNA molecule on each bead is then amplified to generate millions of copies of the sequence, which all remain bound to the bead. Finally, the beads are placed into a highly parallel sequencing-by-synthesis machine which can generate over 400,000 sequence reads (~100bp per read) in a single 4 hour run.

[00245] Ultra-deep sequencing provides an accurate and quantitative way to measure the allele abundances for each of the STRs. The total required number of reads for each of the aliquot wells is determined by the number of STRs and the error rates of the multiplex PCR and the Poisson sampling statistics associated with the sequencing procedures. Statistical models which may account for variables in amplification can be used to detect ploidy changes with high levels of confidence. Using this statistical model it can be predicted that ~100,000 to 300,000 sequence reads will be required to analyze each patient, with ~3 to 10 STR loci per chromosome. Specifically, ~33 reads for each of 12 STRs in each of the individual wells of the microtiter plate will be read (33 reads x 12 STRs per well x 500 wells = 200,000 reads).

[00246] *Identify bins with non-maternal alleles (e.g. fetal cells)*: The first step in the data analysis procedure would be to use the 6bp DNA tags to sort the 200,000 sequence reads into bins which correspond to the individual wells of the microtiter plates. The ~400 sequence reads from each of the bins would then be separated into the different STR groups using standard sequence alignment algorithms. The aligned sequences from each of the bins would then be analyzed to identify non-maternal alleles. These can be identified in one of two ways. First, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above. This sample can be a white blood cell fraction (which will contain only negligible numbers of fetal cells), or simply a dilution of the original sample before enrichment. Alternatively, the genotype profiles for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles then determines which wells in the initial microtiter plate contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original enriched cell population. Bins containing fetal cells would be identified with high levels of confidence because the non-maternal alleles are detected by multiple independent STRs.

[00247] *Detect ploidy for chromosomes 13, 18, and 21:* After identifying the bins that contained fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18 and 21 by comparing the ratio of maternal to paternal alleles for each of the STRs. Again, for each bin there will be ~33 sequence reads for each of the 12 STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker can be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. The information from the different STRs on each chromosome can be combined to increase the confidence of a given aneuploidy call. In addition, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

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Example 8. Sequencing for Trisomy Diagnosis on Fetal Cells

[00248] Fetal cells or nuclei can be isolated as described in the enrichment section or as described in example 1 and 2. Sequencing methods can then be used to detect chromosome copy number changes. **Figure 4** depicts a flow chart depicting the major steps involved in detecting chromosome copy number changes using the methods described herein. For example, the enrichment process described in example 1 may generate a final mixture containing approximately 500 maternal white blood cells (WBCs), approximately 100 maternal nuclear red blood cells (mnBCs), and a minimum of approximately 10 fetal nucleated red blood cells (fnRBCs) starting from an initial 20 ml blood sample taken late in the first trimester. The output of the enrichment procedure would be divided into separate wells of a microtiter plate with the number of wells chosen so no more than one cell or genome copy is located per well, and where some wells may have no cell or genome copy at all.

[00249] *Perform multiplex PCR and Sequencing with bin specific tags:*

[00250] PCR primer pairs for highly polymorphic STR loci (multiple loci per chromosome of interest) can be added to each well in the microtiter plate. For example, STRs could be designed along chromosomes 13, 18, 21 and X to detect the most frequent aneuploidies, and along control regions of the genome where aneuploidy is not expected. Typically, four or more STRs should be analyzed per chromosome of interest to ensure accurate detection of aneuploidy.

[00251] The primers for each STR can be designed with two important features. First, each primer can contain a common ~18bp sequence on the 5' end which can be used for the subsequent DNA cloning and sequencing procedures. Second, each well in the microtiter plate can be assigned a unique ~6bp DNA tag sequence which can be incorporated into the middle part of the upstream primer for each of the different STRs. The DNA tags make it possible to pool all of the STR amplicons following the multiplex PCR, which makes possible to analyze the amplicons in parallel during the ultra-deep sequencing procedure. Furthermore, nested PCR strategies for the STR amplification can achieve higher reliability of amplification from single cells.

[00252] Sequencing can be performed using the classic Sanger sequencing method or any other method known in the art.

[00253] For example, sequencing can occur by sequencing-by-synthesis, which involves inferring the sequence of the template by synthesizing a strand complementary to the target nucleic acid sequence. Sequence-by-synthesis can be initiated using sequencing primers complementary to the sequencing element on the nucleic acid tags. The method involves detecting the identity of each nucleotide immediately after (substantially real-time) or upon (real-time) the incorporation of a labeled nucleotide or nucleotide analog into a growing strand of a complementary nucleic acid sequence in a polymerase reaction. After the successful incorporation of a label nucleotide, a signal is measured and then nulled by methods known in the art. Examples of sequence-by-synthesis methods are described in U.S. Application Publication Nos. 2003/0044781, 2006/0024711, 2006/0024678 and 2005/0100932. Examples of labels that can be used to label nucleotide or nucleotide analogs for sequencing-by-synthesis include, but are not limited to, chromophores, fluorescent moieties, enzymes, antigens, heavy metal, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moeities, scattering or fluorescent nanoparticles, Raman signal generating moieties, and electrochemical detection moieties. Sequencing-by-synthesis can generate at least 1,000, at least 5,000, at least 10,000, at least 20,000, 30,000, at least 40,000, at least 50,000, at least 100,000 or at least 500,000 reads per hour. Such reads can have at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 or at least 150 bases per read.

[00254] Another sequencing method involves hybridizing the amplified genomic region of interest to a primer complementary to it. This hybridization complex is incubated with a polymerase, ATP sulfurylase, luciferase,

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apyrase, and the substrates luciferin and adenosine 5' phosphosulfate. Next, deoxynucleotide triphosphates corresponding to the bases A, C, G, and T (U) are added sequentially. Each base incorporation is accompanied by release of pyrophosphate, converted to ATP by sulfurylase, which drives synthesis of oxyluciferin and the release of visible light. Since pyrophosphate release is equimolar with the number of incorporated bases, the light given off is proportional to the number of nucleotides adding in any one step. The process is repeated until the entire sequence is determined.

[00255] Yet another sequencing method involves a four-color sequencing by ligation scheme (degenerate ligation), which involves hybridizing an anchor primer to one of four positions. Then an enzymatic ligation reaction of the anchor primer to a population of degenerate nonamers that are labeled with fluorescent dyes is performed. At any given cycle, the population of nonamers that is used is structure such that the identity of one of its positions is correlated with the identity of the fluorophore attached to that nonamer. To the extent that the ligase discriminates for complementarily at that queried position, the fluorescent signal allows the inference of the identity of the base. After performing the ligation and four-color imaging, the anchor primer:nonamer complexes are stripped and a new cycle begins.

[00256] *Identify bins with non-maternal alleles (e.g. fetal cells)*: The first step in the data analysis procedure would be to use the 6bp DNA tags to sort the 200,000 sequence reads into bins which correspond to the individual wells of the microtiter plates. The ~400 sequence reads from each of the bins would then be separated into the different STR groups using standard sequence alignment algorithms. The aligned sequences from each of the bins would then be analyzed to identify non-maternal alleles. These can be identified in one of two ways. First, an independent blood sample fraction known to contain only maternal cells can be analyzed as described above. This sample can be a white blood cell fraction (which will contain only negligible numbers of fetal cells), or simply a dilution of the original sample before enrichment. Alternatively, the genotype profiles for all the wells can be similarity-clustered to identify the dominant pattern associated with maternal cells. In either approach, the detection of non-maternal alleles then determines which wells in the initial microtiter plate contained fetal cells. Determining the number bins with non-maternal alleles relative to the total number of bins provides an estimate of the number of fetal cells that were present in the original enriched cell population. Bins containing fetal cells would be identified with high levels of confidence because the non-maternal alleles are detected by multiple independent STRs.

[00257] *Detect ploidy for chromosomes 13, 18, and 21:* After identifying the bins that contained fetal cells, the next step would be to determine the ploidy of chromosomes 13, 18 and 21 by comparing the ratio of maternal to paternal alleles for each of the STRs. Again, for each bin there will be ~33 sequence reads for each of the 12 STRs. In a normal fetus, a given STR will have 1:1 ratio of the maternal to paternal alleles with approximately 16 sequence reads corresponding to each allele (normal diallelic). In a trisomic fetus, three doses of an STR marker can be detected either as three alleles with a 1:1:1 ratio (trisomic triallelic) or two alleles with a ratio of 2:1 (trisomic diallelic). In rare instances all three alleles may coincide and the locus will not be informative for that individual patient. The information from the different STRs on each chromosome can be combined to increase the confidence of a given aneuploidy call. In addition, the information from the independent bins containing fetal cells can also be combined to further increase the confidence of the call.

Example 9. Device Embodiment

[00258] Microfluidic devices of the invention were designed by computer-aided design (CAD) and microfabricated by photolithography. A two-step process was developed in which a blood sample is first debulked to remove the large population of small cells, and then the rare target epithelial cells target cells are recovered by immunoaffinity capture. The devices were defined by photolithography and etched into a silicon substrate based on the CAD-

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generated design. The cell enrichment module, which is approximately the size of a standard microscope slide, contains 14 parallel sample processing sections and associated sample handling channels that connect to common sample and buffer inlets and product and waste outlets. Each section contains an array of microfabricated obstacles that is optimized to enrich the target cell type by hydrodynamic size via displacement of the larger cells into the product stream. In this example, the microchip was designed to separate red blood cells (RBCs) and platelets from the larger leukocytes and CTCs. Enriched populations of target cells were recovered from whole blood passed through the device. Performance of the cell enrichment microchip was evaluated by separating RBCs and platelets from white blood cells (WBCs) in normal whole blood (**Figure 15**). In cancer patients, CTCs are found in the larger WBC fraction. Blood was minimally diluted (30%), and a 6 ml sample was processed at a flow rate of up to 6 ml/hr. The product and waste stream were evaluated in a Coulter Model "A^C-T diff" clinical blood analyzer, which automatically distinguishes, sizes, and counts different blood cell populations. The enrichment chip achieved separation of RBCs from WBCs, in which the WBC fraction had >99% retention of nucleated cells, >99% depletion of RBCs, and >97% depletion of platelets. Representative histograms of these cell fractions are shown in **Figure16**. Routine cytology confirmed the high degree of enrichment of the WBC and RBC fractions (**Figure 17**).

[00259] Next, epithelial cells were recovered by affinity capture in a microfluidic module that is functionalized with immobilized antibody. A capture module with a single chamber containing a regular array of antibody-coated microfabricated obstacles was designed. These obstacles are disposed to maximize cell capture by increasing the capture area approximately four-fold, and by slowing the flow of cells under laminar flow adjacent to the obstacles to increase the contact time between the cells and the immobilized antibody. The capture modules may be operated under conditions of relatively high flow rate but low shear to protect cells against damage. The surface of the capture module was functionalized by sequential treatment with 10% silane, 0.5% gluteraldehyde, and avidin, followed by biotinylated anti-EpCAM. Active sites were blocked with 3% bovine serum albumin in PBS, quenched with dilute Tris HCl, and stabilized with dilute L-histidine. Modules were washed in PBS after each stage and finally dried and stored at room temperature. Capture performance was measured with the human advanced lung cancer cell line NCI-H1650 (ATCC Number CRL-5883). This cell line has a heterozygous 15 bp in-frame deletion in exon 19 of EGFR that renders it susceptible to gefitinib. Cells from confluent cultures were harvested with trypsin, stained with the vital dye Cell Tracker Orange (CMRA reagent, Molecular Probes, Eugene, OR), resuspended in fresh whole blood, and fractionated in the microfluidic chip at various flow rates. In these initial feasibility experiments, cell suspensions were processed directly in the capture modules without prior fractionation in the cell enrichment module to debulk the red blood cells; hence, the sample stream contained normal blood red cells and leukocytes as well as tumor cells. After the cells were processed in the capture module, the device was washed with buffer at a higher flow rate (3ml/hr) to remove the nonspecifically bound cells. The adhesive top was removed and the adherent cells were fixed on the chip with paraformaldehyde and observed by fluorescence microscopy. Cell recovery was calculated from hemacytometer counts; representative capture results are shown in **Table 4**. Initial yields in reconstitution studies with unfractionated blood were greater than 60% with less than 5% of non-specific binding.

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Table 4

Run number	Avg. flow rate	Length of run	No. cells processed	No. cells captured	Yield
1	3.0	1 hr	150,000	38,012	25%
2	1.5	2 hr	150,000	30,000/ml	60%
3	1.08	2 hr	108,000	68,661	64%
4	1.21	2 hr	121,000	75,491	62%

[00260] Next, NCI-H1650 cells that were spiked into whole blood and recovered by size fractionation and affinity capture as described above were successfully analyzed in situ. In a trial run to distinguish epithelial cells from leukocytes, 0.5 ml of a stock solution of fluorescein-labeled CD45 pan-leukocyte monoclonal antibody were passed into the capture module and incubated at room temperature for 30 minutes. The module was washed with buffer to remove unbound antibody, and the cells were fixed on the chip with 1% paraformaldehyde and observed by fluorescence microscopy. As shown in **Figure 18**, the epithelial cells were bound to the obstacles and floor of the capture module. Background staining of the flow passages with CD45 pan-leukocyte antibody is visible, as are several stained leukocytes, apparently because of a low level of non-specific capture.

Example 10: Device embodiments

[00261] A design for preferred device embodiments of the invention is shown in **Figure 19A**, and parameters corresponding to three preferred device embodiments associated with this design are shown in **Figures 19B** and **19C**. These embodiments are particularly useful for enrich epithelial cells from blood.

Example 11: Determining counts for large cell types

[00262] Using the methods of the invention, a diagnosis of the absence, presence, or progression of cancer may be based on the number of cells in a cellular sample that are larger than a particular cutoff size. For example, cells with a hydrodynamic size of 14 microns or larger may be selected. This cutoff size would eliminate most leukocytes. The nature of these cells may then be determined by downstream molecular or cytological analysis.

[00263] Cell types other than epithelial cells that would be useful to analyze include endothelial cells, endothelial progenitor cells, endometrial cells, or trophoblasts indicative of a disease state. Furthermore, determining separate counts for epithelial cells, e.g., cancer cells, and other cell types, e.g., endothelial cells, followed by a determination of the ratios between the number of epithelial cells and the number of other cell types, may provide useful diagnostic information.

[00264] A device of the invention may be configured to isolate targeted subpopulations of cells such as those described above, as shown in **Figures 20A-D**. A size cutoff may be selected such that most native blood cells, including red blood cells, white blood cells, and platelets, flow to waste, while non-native cells, which could include endothelial cells, endothelial progenitor cells, endometrial cells, or trophoblasts, are collected in an enriched sample. This enriched sample may be further analyzed.

[00265] Using a device of the invention, therefore, it is possible to isolate a subpopulation of cells from blood or other bodily fluids based on size, which conveniently allows for the elimination of a large proportion of native blood cells when large cell types are targeted. As shown schematically in **Figure 21**, a device of the invention may include counting means to determine the number of cells in the enriched sample, or the number of cells of a particular type, e.g., cancer cells, within the enriched sample, and further analysis of the cells in the enriched sample may provide additional information that is useful for diagnostic or other purposes.

Example 12: Method for detection of EGFR mutations

[00266] A blood sample from a cancer patient is processed and analyzed using the devices and methods of the invention, resulting in an enriched sample of epithelial cells containing CTCs. This sample is then analyzed to

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identify potential EGFR mutations. The method permits both identification of known, clinically relevant EGFR mutations as well as discovery of novel mutations. An overview of this process is shown in **Figure 22**.

[00267] Below is an outline of the strategy for detection and confirmation of EGFR mutations:

1) Sequence CTC EGFR mRNA

a) Purify CTCs from blood sample;

b) Purify total RNA from CTCs;

c) Convert RNA to cDNA using reverse transcriptase;

d) Use resultant cDNA to perform first and second PCR reactions for generating sequencing templates; and

e) Purify the nested PCR amplicon and use as a sequencing template to sequence EGFR exons 18-21.

2) Confirm RNA sequence using CTC genomic DNA

a) Purify CTCs from blood sample;

b) Purify genomic DNA (gDNA) from CTCs;

c) Amplify exons 18, 19, 20, and/or 21 via PCR reactions; and

d) Use the resulting PCR amplicon(s) in real-time quantitative allele-specific PCR reactions in order to confirm the sequence of mutations discovered via RNA sequencing.

[00268] Further details for each step outlined above are as follows.

1) Sequence CTC EGFR mRNA

a) Purify CTCs from blood sample. CTCs are isolated using any of the size-based enrichment and/or affinity purification devices of the invention.

b) Purify total RNA from CTCs. Total RNA is then purified from isolated CTC populations using, e.g., the Qiagen Micro RNeasy kit, or a similar total RNA purification protocol from another manufacturer; alternatively, standard RNA purification protocols such as guanidium isothiocyanate homogenization followed by phenol/chloroform extraction and ethanol precipitation may be used. One such method is described in "Molecular Cloning – A Laboratory Manual, Second Edition" (1989) by J. Sambrook, E.F. Fritch and T. Maniatis, p. 7.24.

c) Convert RNA to cDNA using reverse transcriptase. cDNA reactions are carried out based on the protocols of the supplier of reverse transcriptase. Typically, the amount of input RNA into the cDNA reactions is in the range of 10 picograms (pg) to 2 micrograms (μg) total RNA. First-strand DNA synthesis is carried out by hybridizing random 7mer DNA primers, or oligo-dT primers, or gene-specific primers, to RNA templates at 65°C followed by snap-chilling on ice. cDNA synthesis is initiated by the addition of iScript Reverse Transcriptase (BioRad) or SuperScript Reverse Transcriptase (Invitrogen) or a reverse transcriptase from another commercial vendor along with the appropriate enzyme reaction buffer. For iScript, reverse transcriptase reactions are carried out at 42°C for 30-45 minutes, followed by enzyme inactivation for 5 minutes at 85°C. cDNA is stored at -20°C until use or used immediately in PCR reactions. Typically, cDNA reactions are carried out in a final volume of 20 μl, and 10% (2 μl) of the resultant cDNA is used in subsequent PCR reactions.

d) Use resultant cDNA to perform first and second PCR reactions for generating sequencing templates. cDNA from the reverse transcriptase reactions is mixed with DNA primers specific for the region of interest (**Figure 23**). See **Table 5** for sets of primers that may be used for amplification of exons 18-21. In **Table 5**, primer set M13(+)/M12(-) is internal to primer set M11(+)/M14(-). Thus primers M13(+) and M12(-) may be used in the nested round of amplification, if primers M11(+) and M14(-) were used in the first round of expansion. Similarly, primer set M11(+)/M14(-) is internal to primer set M15(+)/M16(-), and primer set

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M23(+)/M24(-) is internal to primer set M21(+)/M22(-). Hot Start PCR reactions are performed using Qiagen Hot-Star Taq Polymerase kit, or Applied Biosystems HotStart TaqMan polymerase, or other Hot Start thermostable polymerase, or without a hot start using Promega GoTaq Green Taq Polymerase master mix, TaqMan DNA polymerase, or other thermostable DNA polymerase. Typically, reaction volumes are 50 µl, nucleotide triphosphates are present at a final concentration of 200 µM for each nucleotide, $MgCl_2$ is present at a final concentration of 1-4 mM, and oligo primers are at a final concentration of 0.5 µM. Hot start protocols begin with a 10-15 minute incubation at 95°C, followed by 40 cycles of 94°C for one minute (denaturation), 52°C for one minute (annealing), and 72°C for one minute (extension). A 10 minute terminal extension at 72°C is performed before samples are stored at 4°C until they are either used as template in the second (nested) round of PCRs, or purified using QiaQuick Spin Columns (Qiagen) prior to sequencing. If a hot-start protocol is not used, the initial incubation at 95°C is omitted. If a PCR product is to be used in a second round of PCRs, 2 µl (4%) of the initial PCR product is used as template in the second round reactions, and the identical reagent concentrations and cycling parameters are used.

Table 5. Primer Sets for expanding EGFR mRNA around Exons 18-21

Name	SEQ ID NO	Sequence (5' to 3')	cDNA Coordinates	Amplicon Size
NXK-M11(+)	1	TTGCTGCTGGTGGTGGC	(+) 1966-1982	813
NXK-M14(-)	2	CAGGGATTCCGTCATATGGC	(-) 2778-2759	
NXK-M13(+)	3	GATCGGCCTCTTCATGCG	(+) 1989-2006	747
NXK M12(-)	4	GATCCAAAGGTCATCAACTCCC	(-) 2735-2714	
NXK-M15(+)	5	GCTGTCCAACGAATGGGC	(+) 1904-1921	894
NXK-M16(-)	6	GGCGTTCTCCTTTCTCCAGG	(-) 2797-2778	
NXK-M21(+)	7	ATGCACTGGGCCAGGTCTT	(+) 1881-1899	944
NXK-M22(-)	8	CGATGGTACATATGGGTGGCT	(-) 2824-2804	
NXK-M23(+)	9	AGGCTGTCCAACGAATGGG	(+) 1902-1920	904
NXK-M24(-)	10	CTGAGGGAGGCGTTCTCCT	(-) 2805-2787	

e) Purify the *nested PCR amplicon and use as a sequencing template to sequence EGFR exons 18-21.* Sequencing is performed by ABI automated fluorescent sequencing machines and fluorescence-labeled DNA sequencing ladders generated via Sanger-style sequencing reactions using fluorescent dideoxynucleotide mixtures. PCR products are purified using Qiagen QuickSpin columns, the Agencourt AMPure PCR Purification System, or PCR product purification kits obtained from other vendors. After PCR products are purified, the nucleotide concentration and purity is determined with a Nanodrop 7000 spectrophotometer, and the PCR product concentration is brought to a concentration of 25 ng/µl. As a quality control measure, only PCR products that have a UV-light absorbance ratio (A_{260}/A_{280}) greater than 1.8 are used for sequencing. Sequencing primers are brought to a concentration of 3.2 pmol/µl.

2) Confirm RNA sequence using CTC genomic DNA

a) Purify *CTCs from blood sample.* As above, CTCs are isolated using any of the size-based enrichment and/or affinity purification devices of the invention.

b) Purify genomic DNA (gDNA) from CTCs. Genomic DNA is purified using the Qiagen DNeasy Mini kit, the Invitrogen ChargeSwitch gDNA kit, or another commercial kit, or via the following protocol:

1. Cell pellets are either lysed fresh or stored at -80°C and are thawed immediately before lysis.

2. Add 500 µl 50mM Tris pH 7.9/100mM EDTA/0.5%SDS (TES buffer).

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3. Add 12.5 µl Proteinase K (IB15406, 20mg/ml), generating a final [ProtK] = 0.5 mg/ml.

4. Incubate at 55°C overnight in rotating incubator.

5. Add 20 µl of RNase cocktail (500 U/ml RNase A + 20,000 U/ml RNase T1, Ambion #2288) and incubate four hours at 37°C.

6. Extract with Phenol (Kodak, Tris pH 8 equilibrated), shake to mix, spin 5 min. in tabletop centrifuge.

7. Transfer aqueous phase to fresh tube.

8. Extract with Phenol/Chloroform/Isoamyl alcohol (EMD, 25:24:1 ratio, Tris pH 8 equilibrated), shake to mix, spin five minutes in tabletop centrifuge.

9. Add 50 µl 3M NaOAc pH = 6.

10. Add 500 µl EtOH.

11. Shake to mix. Strings of precipitated DNA may be visible. If anticipated DNA concentration is very low, add carrier nucleotide (usually yeast tRNA).

12. Spin one minute at max speed in tabletop centrifuge.

13. Remove supernatant.

14. Add 500 µl 70% EtOH, Room Temperature (RT)

15. Shake to mix.

16. Spin one minute at max speed in tabletop centrifuge.

17. Air dry 10-20 minutes before adding TE.

18. Resuspend in 400 µl TE. Incubate at 65°C for 10 minutes, then leave at RT overnight before quantitation on Nanodrop.

c) Amplify exons 18, 19, 20, and/or 21 via PCR reactions. Hot start nested PCR amplification is carried out as described above in step **1d**, except that there is no nested round of amplification. The initial PCR step may be stopped during the log phase in order to minimize possible loss of allele-specific information during amplification. The primer sets used for expansion of EGFR exons 18-21 are listed in **Table 6** (see also Paez et al., Science 304:1497-1500 (Supplementary Material) (2004)).

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Table 6. Primer sets for expanding EGFR genomic DNA

Name	SEQ ID NO	Sequence (5' to 3')	Exon	Amplicon Size
NXK-ex18.1(+)	11	TCAGAGCCTGTGTTTCTACCAA	18	534
NXK-ex18.2(-)	12	TGGTCTCACAGGACCACTGATT	18	
NXK-ex18.3(+)	13	TCCAAATGAGCTGGCAAGTG	18	397
NXK-ex18.4(-)	14	TCCCAAACACTCAGTGAAACAAA	18	
NXK-ex19.1(+)	15	AAATAATCAGTGTGATTCGTGGAG	19	495
NXK-ex19.2(-)	16	GAGGCCAGTGCTGTCTCTAAGG	19	
NXK-ex19.3(+)	17	GTGCATCGCTGGTAACATCC	19	298
NXK-ex19.4(-)	18	TGTGGAGATGAGCAGGGTCT	19	
NXK-ex20.1(+)	19	ACTTCACAGCCCTGCGTAAAC	20	555
NXK-ex20.2(-)	20	ATGGGACAGGCACTGATTTGT	20	
NXK-ex20.3(+)	21	ATCGCATTCATGCGTCTTCA	20	379
NXK-ex20.4(-)	22	ATCCCCATGGCAAACTCTTG	20	
NXK-ex21.1(+)	23	GCAGCGGGTTACATCTTCTTTC	21	526
NXK-ex21.2(-)	24	CAGCTCTGGCTCACACTACCAG	21	
NXK-ex21.3(+)	25	GCAGCGGGTTACATCTTCTTTC	21	349
NXK-ex21.4(-)	26	CATCCTCCCCTGCATGTGT	21	

d) Use the resulting PCR amplicon(s) in real-time quantitative allele-specific PCR reactions in order to confirm the sequence of mutations discovered via RNA sequencing. An aliquot of the PCR amplicons is used as template in a multiplexed allele-specific quantitative PCR reaction using TaqMan PCR 5' Nuclease assays with an Applied Biosystems model 7500 Real Time PCR machine (**Figure 24**). This round of PCR amplifies subregions of the initial PCR product specific to each mutation of interest. Given the very high sensitivity of Real Time PCR, it is possible to obtain complete information on the mutation status of the EGFR gene even if as few as 10 CTCs are isolated. Real Time PCR provides quantification of allelic *sequences* over 8 logs of input DNA concentrations; thus, even heterozygous mutations in impure populations are easily detected using this method.

[00269] Probe and primer sets are designed for all known mutations that affect gefitinib responsiveness in NSCLC patients, including over 40 such somatic mutations, including point mutations, deletions, and insertions, that have been reported in the medical literature. For illustrative purposes, examples of primer and probe sets for five of the point mutations are listed in **Table 7**. In general, oligonucleotides may be designed using the primer optimization software program Primer Express (Applied Biosystems), with hybridization conditions optimized to distinguish the wild type EGFR DNA sequence from mutant alleles. EGFR genomic DNA amplified from lung cancer cell lines that are known to carry EGFR mutations, such as H358 (wild type), H1650 (15-bp deletion, Δ2235-2249), and H1975 (two point mutations, 2369 C→ T, 2573 T→ G), is used to optimize the allele-specific Real Time PCR reactions. Using the TaqMan 5' nuclease assay, allele-specific labeled probes specific for wild type sequence or for known EGFR mutations are developed. The oligonucleotides are designed to have melting temperatures that easily distinguish a match from a mismatch, and the Real Time PCR conditions are optimized to distinguish wild type and mutant alleles. All Real Time PCR reactions are carried out in triplicate.

[00270] Initially, labeled probes containing wild type sequence are multiplexed in the same reaction with a single mutant probe. Expressing the results as a ratio of one mutant allele sequence versus wild type sequence may identify samples containing or lacking a given mutation. After conditions are optimized for a given probe set, it is then possible to multiplex probes for all of the mutant alleles within a given exon within the same Real Time PCR assay, increasing the ease of use of this analytical tool in clinical settings.

[00271] A unique probe is designed for each wild type allele and mutant allele sequence. Wild-type sequences are marked with the fluorescent dye VIC at the 5' end, and mutant sequences with the fluorophore FAM. A

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fluorescence quencher and Minor Groove Binding moiety are attached to the 3' ends of the probes. ROX is used as a passive reference dye for normalization purposes. A standard curve is generated for wild type sequences and is used for relative quantitation. Precise quantitation of mutant signal is not required, as the input cell population is of unknown, and varying, purity. The assay is set up as described by ABI product literature, and the presence of a mutation is confirmed when the signal from a mutant allele probe rises above the background level of fluorescence (**Figure 25**), and this threshold cycle gives the relative frequency of the mutant allele in the input sample.

Table 7. Probes and Primers for Allele-Specific qPCR

Name	SEQ ID NO	Sequence (5' to 3', mutated position in bold)	EMBL Chromosome 7 Genomic Coordinates	Description	Mut
NXK-M01	27	CCGCAGCATGTCAAGATCAC	(+)55,033,694-55,033,713	(+) primer	
NXK-M02	28	TCCTTCTGCATGGTATTCTTTCTCT	(-)55,033,769-55,033,745	(-) primer	
Pwt-L858R	29	VIC-TTTGGGCTGGCCAA-MGB	(+)55,033,699-55,033,712	WT allele probe	L858
Pmut-L858R	30	FAM-TTTTGGGCGGGCCA-MGB	(+)55,033,698-55,033,711	Mutant allele probe	
NXK-M03	31	ATGGCCAGCGTGGACAA	(+)55,023,207-55,023,224	(+) primer	
NXK-M04	32	AGCAGGTACTGGGAGCCAATATT	(-)55,023,355-55,023,333	(-) primer	
Pwt-T790M	33	VIC-ATGAGCTGCGTGATGA-MGB	(-)55,023,290-55,023,275	WT allele probe	T790
Pmut-T790M	34	FAM-ATGAGCTGCATGATGA-MGB	(-)55,023,290-55,023,275	Mutant allele probe	
NXK-M05	35	GCCTCTTACACCCAGTGGAGAA	(+)55,015,831-55,015,852	(+) primer	
NXK-ex18.5	36	GCCTGTGCCAGGGACCTT	(-)55,015,965-55,015,948	(-) primer	
Pwt-G719SC	37	VIC-ACCGGAGCCCAGCA-MGB	(-)55,015,924-55,015,911	WT allele probe	G71
Pmut-G719S	38	FAM-ACCGGAGCTCAGCA-MGB	(-)55,015,924-55,015,911	Mutant allele probe	
mut-G719C	39	FAM-ACCGGAGCACAGCA-MGB	(-)55,015,924-55,015,911	Mutant allele probe	
NXK-ex21.5	40	ACAGCAGGGTCTTCTCTGTTTCAG	(+)55,033,597-55,033,620	(+) primer	
NXK-M10	41	ATCTTGACATGCTGCGGTGTT	(-)55,033,710 55,033,690	(-) primer	
Pwt-H835L	42	VIC-TTGGTGCACCGCGA-MGB	(+)55,033,803-55,033,816	WT allele probe	H8
Pmut-H835L	43	FAM-TGGTGCTCCGCGAC-MGB	(+)55,033,803-55,033,816	Mutant allele probe	
NXK-M07	101	TGGATCCCAGAAGGTGAGAAA	(+)55,016,630-55,016,650	(+) primer	
NXK-ex19.5	102	AGCAGAAACTCACATCGAGGATTT	(-)55,016,735-55,016,712	(-) primer	
Pwt-delE746-A750	103	AAGGAATTAAGAGAAGCAA	(+)55,016,681-55,016,699	WT allele probe	delE A7
Pmut-delE746-A750var1	104	CTATCAAAACATCTCC	(+)55,016,676-55,016,691	Mutant allele probe, variant 1	
Pmut-delE746-A750var1	105	CTATCAAGACATCTCC	(+)55,016,676-55,016,691	Mutant allele probe, variant 2	

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Example 13: Absence of EGFR expression in leukocytes

[00272] To test whether EGFR mRNA is present in leukocytes, several PCR experiments were performed. Four sets of primers, shown in **Table 8**, were designed to amplify four corresponding genes:

1) BCKDK (branched-chain a-ketoacid dehydrogenase complex kinase) – a "housekeeping" gene expressed in all types of cells, a positive control for both leukocytes and tumor cells;

2) CD45 – specifically expressed in leukocytes, a positive control for leukocytes and a negative control for tumor cells;

3) EpCaM – specifically expressed in epithelial cells, a negative control for leukocytes and a positive control for tumor cells; and

4) EGFR – the target mRNA to be examined.

Table 8

Name	SEQ ID NO	Sequence (5' to 3')	Description	Amplicon Size
BCKD_1	44	AGTCAGGACCCATGCACGG	BCKDK (+) primer	273
BCKD_2	45	ACCCAAGATGCAGCAGTGTG	BCKDK (-) primer	
CD45_1	46	GATGTCCTCCTTGTTCTACTC	CD45 (+) primer	263
CD45_2	47	TACAGGGAATAATCGAGCATGC	CD45 (-) primer	
EpCAM_1	48	GAAGGGAAATAGCAAATGGACA	EpCAM (+) primer	222
EpCAM_2	49	CGATGGAGTCCAAGTTCTGG	EpCAM (-) primer	
EGFR_1	50	AGCACTTACAGCTCTGGCCA	EGFR (+) primer	371
EGFR_2	51	GACTGAACATAACTGTAGGCTG	EGFR (-) primer	

[00273] Total RNAs of approximately 9×10^6 leukocytes isolated using a cell enrichment device of the invention (cutoff size 4 μm) and 5×10^6 H1650 cells were isolated by using RNeasy mini kit (Qiagen). Two micrograms of total RNAs from leukocytes and H1650 cells were reverse transcribed to obtain first strand cDNAs using 100 pmol random hexamer (Roche) and 200 U Superscript II (Invitrogen) in a 20 μl reaction. The subsequent PCR was carried out using 0.5 μl of the first strand cDNA reaction and 10 pmol of forward and reverse primers in total 25 μl of mixture. The PCR was run for 40 cycles of 95°C for 20 seconds, 56°C for 20 seconds, and 70°C for 30 seconds. The amplified products were separated on a 1% agarose gel. As shown in **Figure 26A**, BCKDK was found to be expressed in both leukocytes and H1650 cells; CD45 was expressed only in leukocytes; and both EpCAM and EGFR were expressed only in H1650 cells. These results, which are fully consistent with the profile of EGFR expression shown in **Figure 26B**, confirmed that EGFR is a particularly useful target for assaying mixtures of cells that include both leukocytes and cancer cells, because only the cancer cells will be expected to produce a signal.

Example 14: EGFR assay with low quantities of target RNA or high quantities of background RNA

[00274] In order to determine the sensitivity of the assay described in **Example 12**, various quantities of input NSCLC cell line total RNA were tested, ranging from 100 pg to 50 ng. The results of the first and second EGFR PCR reactions (**step** 1d, **Example 12**) are shown in **Figure 27**. The first PCR reaction was shown to be sufficiently sensitive to detect 1 ng of input RNA, while the second round increased the sensitivity to 100 pg or less of input RNA. This corresponds to 7-10 cells, demonstrating that even extremely dilute samples may generate detectable signals using this assay.

[00275] Next, samples containing 1 ng of NCI-H1975 RNA were mixed with varying quantities of peripheral blood mononuclear cell (PBMC) RNA ranging from 1 ng to 1 μg and used in PCR reactions as before. As shown in **Figure 28A**, the first set of PCR reactions demonstrated that, while amplification occurred in all cases, spurious bands appeared at the highest contamination level. However, as shown in **Figure 28B**, after the second, nested set

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of PCR reactions, the desired specific amplicon was produced without spurious bands even at the highest contamination level. Therefore, this example demonstrates that the EGFR PCR assays described herein are effective even when the target RNA occupies a tiny fraction of the total RNA in the sample being tested.

[00276] **Table 8** lists the RNA yield in a variety of cells and shows that the yield per cell is widely variable, depending on the cell type. This information is useful in order to estimate the amount of target and background RNA in a sample based on cell counts. For example, 1 ng of NCL-H1975 RNA corresponds to approximately 100 cells, while 1 μg of PBMC RNA corresponds to approximately 10^6 cells. Thus, the highest contamination level in the above-described experiment, 1,000:1 of PBMC RNA to NCL-H1975 RNA, actually corresponds to a 10,000:1 ratio of PBMCs to NCL-H1975 cells. Thus, these data indicate that EGFR may be sequenced from as few as 100 CTCs contaminated by as many as 10^6 leukocytes.

Table 8. RNA Yield versus Cell Type

Cells	Count	RNA Yield	[RNA]/Cell
NCI-H1975	2×10^6	26.9 μg	13.5 pg
NCI-H1650	2×10^6	26.1 μg	13.0 pg
H358	2×10^6	26.0 μg	13.0 pg
HT29	2×10^6	21.4 μg	10.7 pg
MCF7	2×10^6	25.4 μg	12.7 pg
PBMC #1	19×10^6	10.2 μg	0.5 pg
PBMC #2	16.5×10^6	18.4 μg	1.1 pg

[00277] Next, whole blood spiked with 1,000 cells/ml of Cell Tracker (Invitrogen)-labeled H1650 cells was run through the capture module chip of Figure 19C. To avoid inefficiency in RNA extraction from fixed samples, the captured H1650 cells were immediately counted after running and subsequently lysed for RNA extraction without formaldehyde fixation. Approximately 800 captured H1650 cells and >10,000 contaminated leukocytes were lysed on the chip with 0.5 ml of 4M guanidine thiocyanate solution. The lysate was extracted with 0.5 ml of phenol/chloroform and precipitated with 1ml of ethanol in the presence of 10 μg of yeast tRNA as carrier. The precipitated RNAs were DNase I-treated for 30 minutes and then extracted with phenol/chloroform and precipitated with ethanol prior to first strand cDNA synthesis and subsequent PCR amplification. These steps were repeated with a second blood sample and a second chip. The cDNA synthesized from chip1 and chip2 RNAs along with H1650 and leukocyte cDNAs were PCR amplified using two sets of primers, CD45_1 and CD45_2 (**Table 7**) as well as EGFR_5 (forward primer, 5'-GTTCGGCACGGTGTATAAGG-3') (SEQ ID NO: ___) and EGFR_6 (reverse primer, 5'-CTGGCCATCACGTAGGCTTC-3') (SEQ ID NO: ___). EGFR_5 and EGFR_6 produce a 138 bp wild type amplified fragment and a 123 bp mutant amplified fragment in H1650 cells. The PCR products were separated on a 2.5% agarose gel. As shown in **Figure 29**, EGFR wild type and mutant amplified fragments were readily detected, despite the high leukocyte background, demonstrating that the EGFR assay is robust and does not require a highly purified sample.

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WHAT IS CLAIMED IS:

1. A method for fetal diagnosis comprising:

labeling one or more genomic DNA regions in fetal and non-fetal cells, enriched from a maternal blood sample, using labels adapted to distinguish between individual cells, and

determining the presence or absence of a fetal abnormality by analyzing said labeled genomic DNA regions.

2. A method for fetal diagnosis comprising:

labeling one or more cDNA sequences generated from fetal and non-fetal cells, wherein said fetal and non-fetal cells are enriched from a maternal blood sample, using labels adapted to distinguish between individual cells, and

determining the presence or absence of a fetal abnormality by analyzing said labeled cDNA regions.

3. The method of claim 1, further comprising splitting said fetal and non-fetal cells such that individual cells are in addressable locations.

4. The method of claim 2,further comprising splitting said fetal and non-fetal cells such that individual cells are in addressable locations.

5. The method of claim 3, further comprising amplifying said one or more genomic DNA regions from each cell to generate a plurality of amplicons.

6. . The method of claim 5, further comprising pooling said amplicons.

7. The method of claim 5 wherein said regions of genomic DNA comprise one or more polymorphisms.

8. The method of claim 7, wherein said polymorphisms are STRs or SNPs.

9. The method of claim 5 wherein said amplification is performed by multiplex PCR.

10. The method of claim 9 wherein said amplification also comprises nested PCR.

11. The method of claim 3, wherein said labels each comprise a unique tag sequence specific for each addressable location.

12. The method of claim 1 or 2 wherein said label comprises a nucleic acid sequence between 4-20 bp long that is unique to each cell

13. The method of claim 1, wherein at least two genomic DNA regions on a chromosome of interest are labeled.

14. The method of claim 1, wherein said genomic DNA regions are located on two or more chromosomes of interest.

15. The method of claim 1 or 2, wherein said analyzing comprises performing ultra deep sequencing or quantitative genotyping.

16. The method of claim 1 or 2, wherein said fetal abnormality comprises an aneuploidy selected from the group consisting of: trisomy 13, trisomy 18, trisomy 21 (Down Syndrome), Klinefelter Syndrome (XXY),

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monosomy, triploidy, tetraploidy, or other irregular number of sex or autosomal chromosomes, and a combination thereof.

17. The method of claim 1 or 2, wherein said fetal abnormality comprises an aneuploidy that is maternally or paternally derived.

18. The method of claim 1 or 2, wherein the fetal abnormality is a segmental aneuploidy.

19. The method of claim 15, wherein said quantitative genotyping is performed by using molecular inversion probe technology.

20. A method for fetal diagnosis comprising:

amplifying a plurality of genomic DNA regions from one or more fetal cells enriched from maternal blood; wherein the resulting amplicons comprise a cell specific label, and

determining the presence or absence of a fetal abnormality by analyzing said amplicons.

21. A method for fetal diagnosis comprising:

amplifying a plurality of cDNA sequence generated from one or more fetal cells enriched from maternal blood; wherein the resulting amplicons comprise a cell specific label, and

determining the presence or absence of a fetal abnormality by analyzing said amplicons.

22. The method of claim 20 or 21, further comprising splitting said one or more fetal cells such that each individual cell is in an addressable location.

23. The method of claim 20 or 21, further comprising pooling said amplicons.

24. The method of claim 20 or 21, wherein said regions of genomic DNA comprise one or more polymorphisms.

25. The method of claim 24, wherein said polymorphisms are STRs or SNPs.

26. The method of claim 20 or 21, wherein said amplification is performed by multiplex PCR, singleplex PCR, quantitative PCR, quantitative fluorescent PCR (QF-PCR), multiplex fluorescent PCR (MF-PCR), real time PCR (RT-PCR), single cell PCR, restriction fragment length polymorphism PCR (PCR-RFLP), PCR-RFLP/RT-PCR-RFLP, hot start PCR, in situ polonony PCR, in situ rolling circle amplification (RCA), bridge PCR, picotiter PCR, emulsion PCR, ligase chain reaction (LCR), transcription amplification, self-sustained sequence replication, selective amplification of target polynucleotide sequences, consensus sequence primed polymerase chain reaction (CP-PCR), arbitrarily primed polymerase chain reaction (AP-PCR), degenerate oligonucleotide-primed PCR (DOP-PCR), MDA and nucleic acid based sequence amplification (NABSA).

27. The method of claim 26 wherein said amplification also comprises nested PCR.

28. The method of claim 20 or 21, wherein said labels each comprise a unique tag sequence specific for each addressable location.

29. The method of claim 20 or 21, wherein said label comprises a nucleic acid sequence between 4-20 bp long that is unique to each cell

30. The method of claim 20 wherein at least two genomic DNA regions on a chromosome of interest are labeled.

31. The method of claim 20 wherein said genomic DNA regions are located on two or more chromosomes of interest.

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32. The method of claim 20 or 21, wherein said analyzing comprises performing ultra deep sequencing or quantitative genotyping.

33. The method of claim 20 or 21, wherein said fetal abnormality comprises an aneuploidy is selected from the group consisting of: trisomy 13, trisomy 18, trisomy 21 (Down Syndrome), Klinefelter Syndrome (XXY), other irregular number of sex or autosomal chromosomes, and a combination thereof.

34. The method of claim 20 or 21, wherein said fetal abnormality comprises an aneuploidy that is maternally or paternally derived.

35. The method of claim 20 or 21, wherein the fetal abnormality is a segmental aneuploidy.

36. The method of claim 32, wherein said quantitative genotyping is performed by using molecular inversion probe technology.

37. A computer readable medium comprising logic for analyzing a plurality of amplicons, from one or more genomic DNA regions on one or more chromosomes from at least one fetal cell and at least one maternal cell, enriched from maternal blood.

38. A computer readable medium comprising logic for analyzing a plurality of amplicons, from one or more cDNA sequences generated from at least one fetal cell and at least one maternal cell, enriched from maternal blood.

39. The computer readable medium of claim 37 or 39, wherein said logic analyzes the ratio of maternal to fetal chromosome copy number.

40. The method of claim 37 or 39, wherein said amplicons comprise a unique tag sequence

41. The method of claim 37 or 39, wherein said amplicons comprise one or more polymorphisms.

42. The method of claim 41, wherein said polymorphisms are STRs or SNPs.

43. The method of claim 37, wherein said plurality of amplicons are from at least two genomic DNA regions on a chromosome of interest.

44. The method of claim 37, wherein said plurality of amplicons are from genomic DNA regions located on two or more chromosomes of interest.

45. The method of claim 37 or 39, wherein said plurality of amplicons comprises at least 6,000 amplicons.

46. The method of claim 1, 2, 20 or 21, further comprising binning at least a portion of said enriched cells

47. The method of claim 46, wherein said binning is performed using a platform selected from the group consisting of: microtiter plates, PCR wells, a nanofludic system, microencapsulation and a combination thereof.

48. The method of claim 46, wherein said binning is performed using serial dilution.

49. The method of claim 46, further comprising to negative selection of non-target cells or positive selection of target cells, prior to said binning.

50. The method of claim 46 wherein said binning is performed using a number of bins less than 100.

51. The method of claim 46 wherein said binning is performed using a number of bins greater than 100.

52. The methods of claim 50 wherein said bins are analyzed for the presence of at least one fetal cell.

53. The methods of claim 51 wherein said bins are analyzed for the presence of at least one fetal cell.

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54. The method of claim 52 wherein said analysis is performed using an analytical method selected from the group consisting of: FISH, fetal biomarker labels, SNP detection, PCR, and STR detection.

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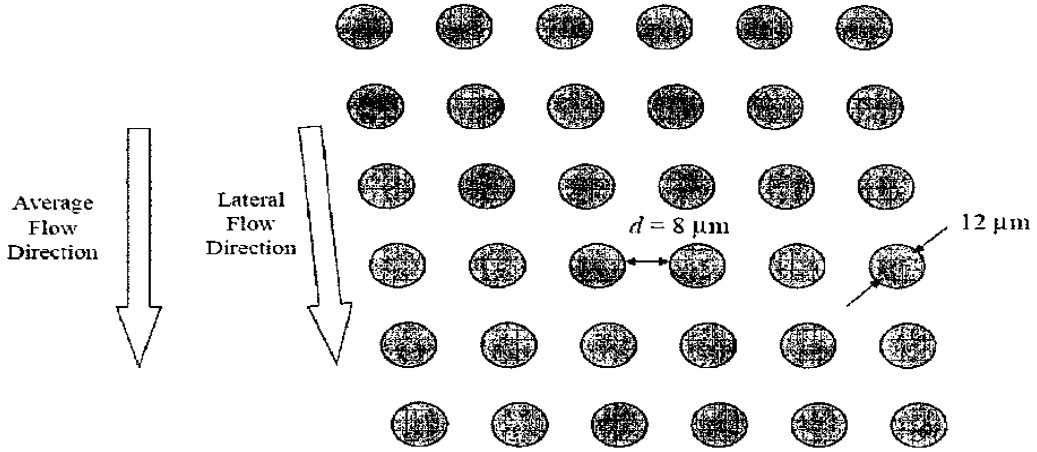


Figure 1A

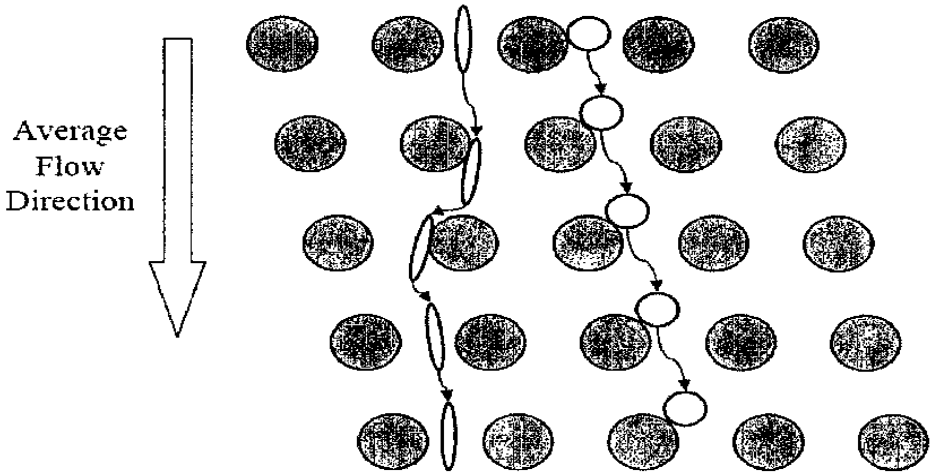


Figure 1B

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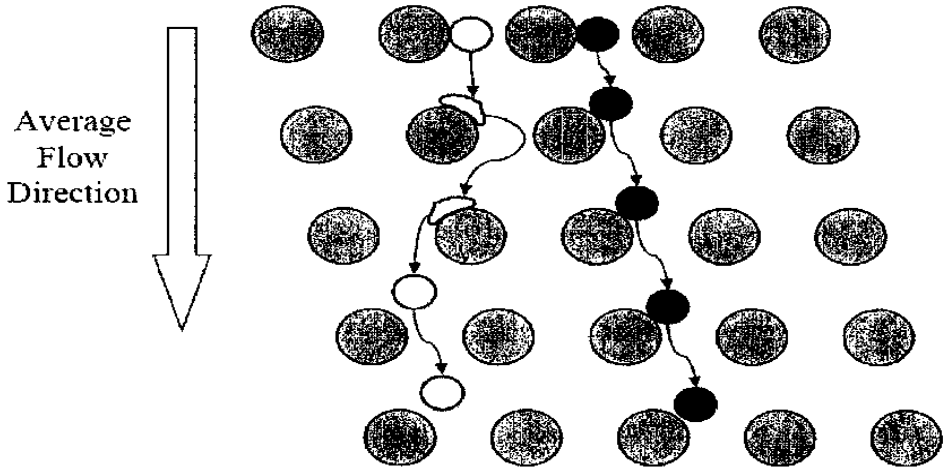


Figure 1C

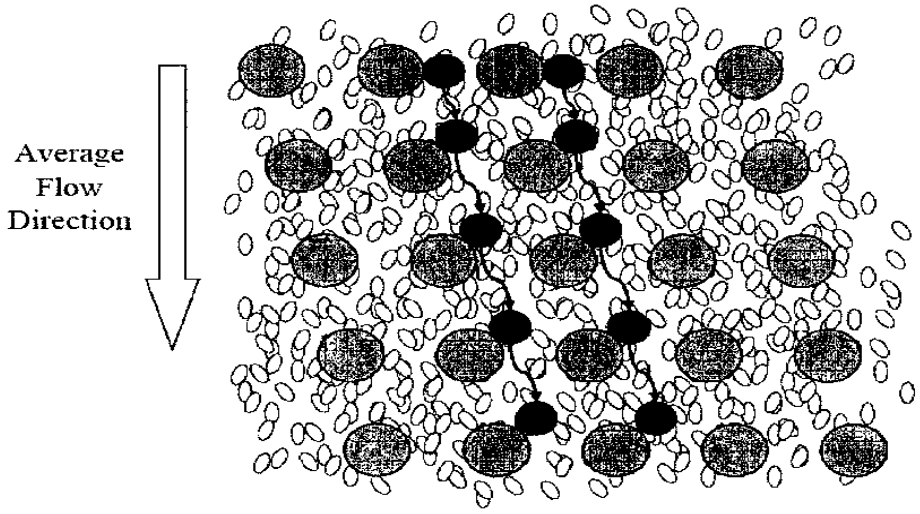


Figure 1D

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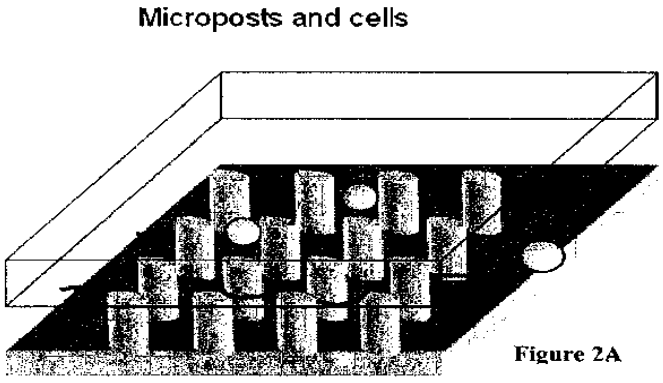


Figure 2A



Figure 2B

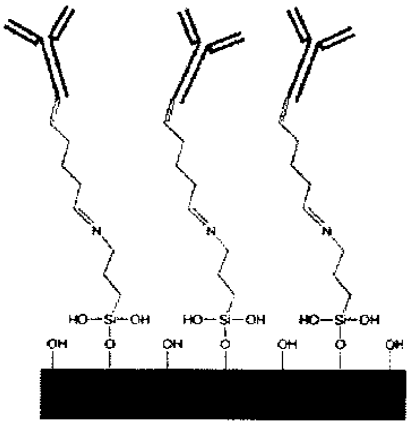


Figure 2C

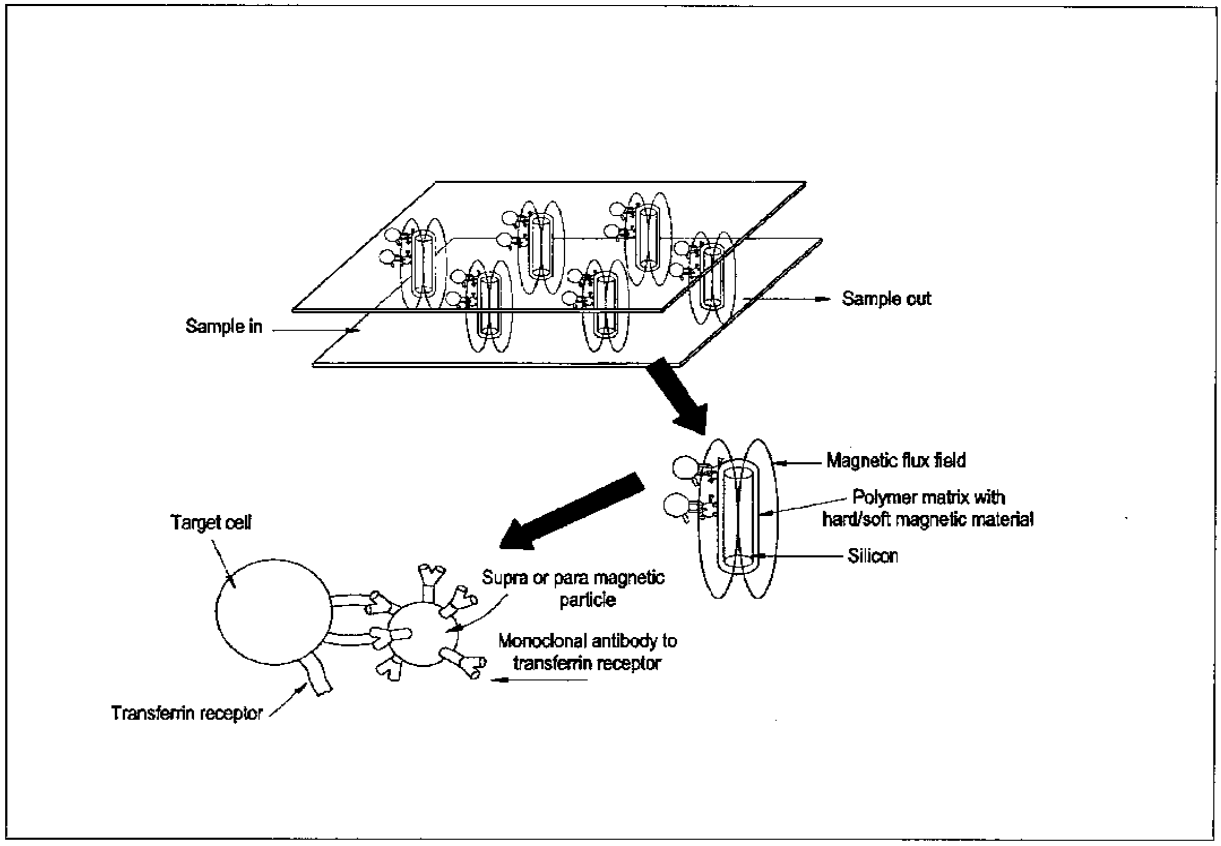

Sample in
Sample out
Magnetic flux field
Polymer matrix with
hard/soft magnetic material
Silicon
Target cell
Supra or para magnetic
particle
Monoclonal antibody to
transferrin receptor
Transferrin receptor

Figure 3

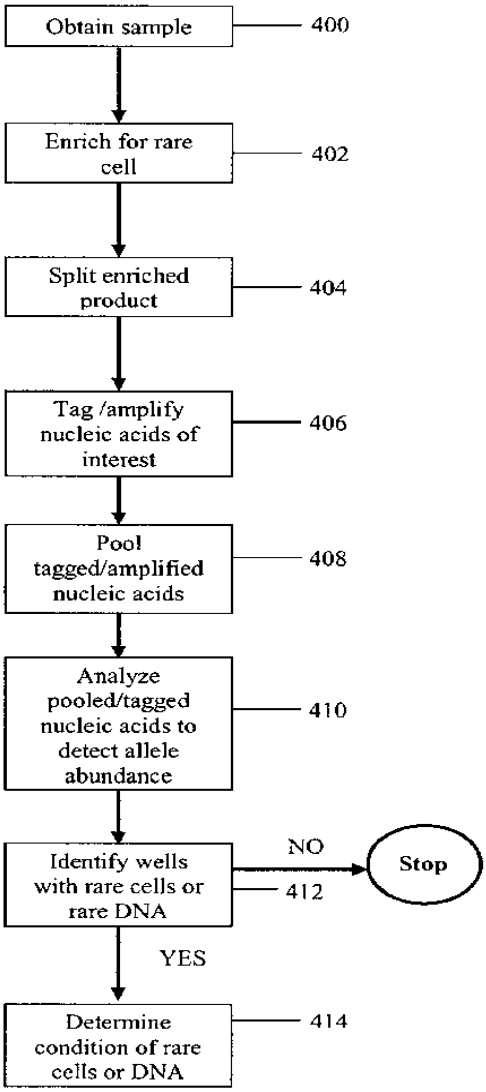

Obtain sample
400
Enrich for rare cell
402
Split enriched product
404
Tag /amplify nucleic acids of interest
406
Pool tagged/amplified nucleic acids
408
Analyze pooled/tagged nucleic acids to detect allele abundance
410
Identify wells with rare cells or rare DNA
NO
Stop
412
YES
Determine condition of rare cells or DNA
414

Figure 4

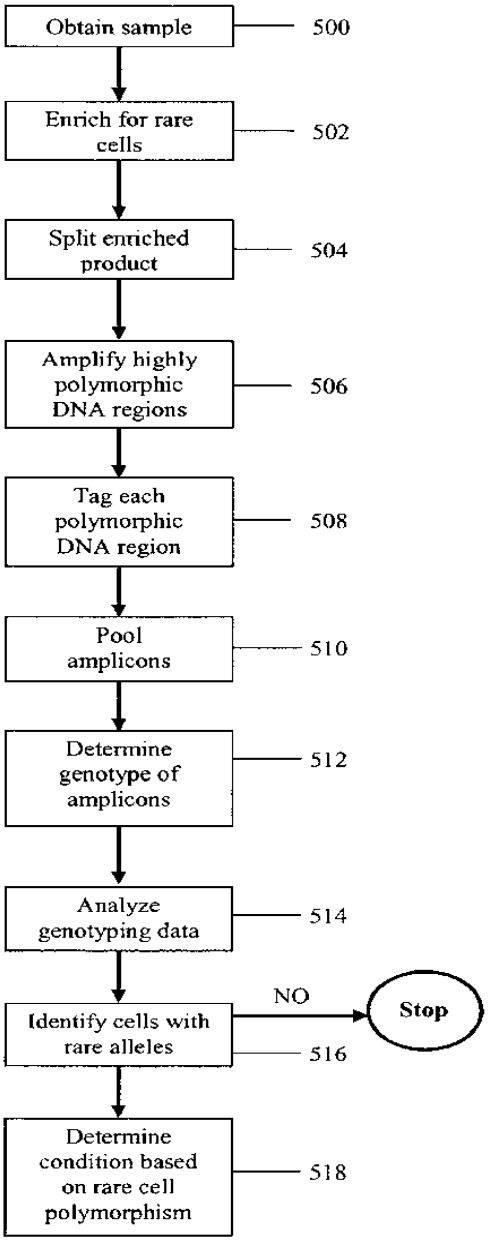

Obtain sample
500
Enrich for rare cells
502
Split enriched product
504
Amplify highly polymorphic DNA regions
506
Tag each polymorphic DNA region
508
Pool amplicons
510
Determine genotype of amplicons
512
Analyze genotyping data
514
Identify cells with rare alleles
NO
Stop
516
Determine condition based on rare cell polymorphism
518

Figure 5

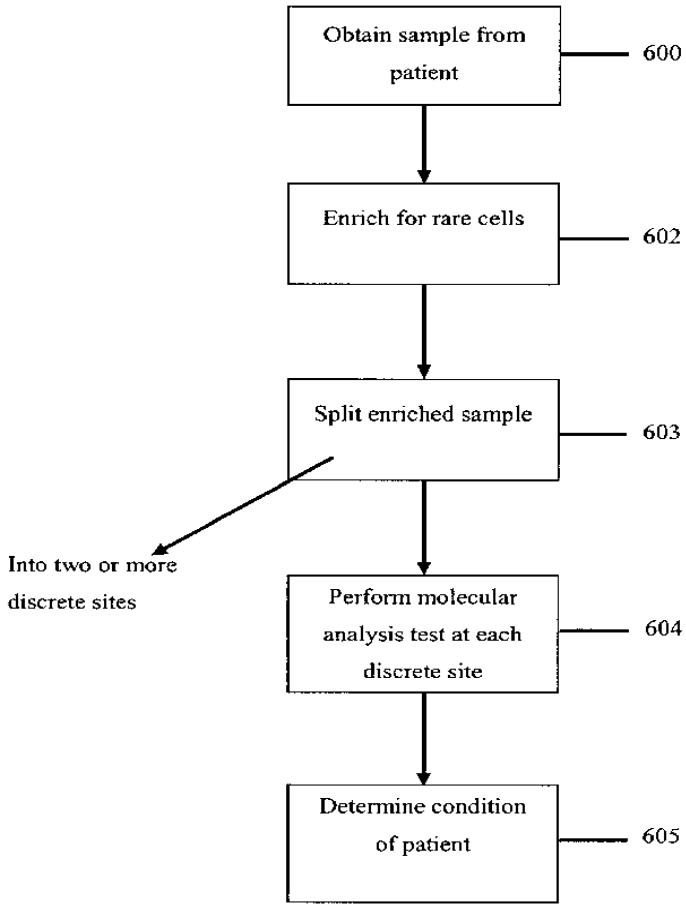
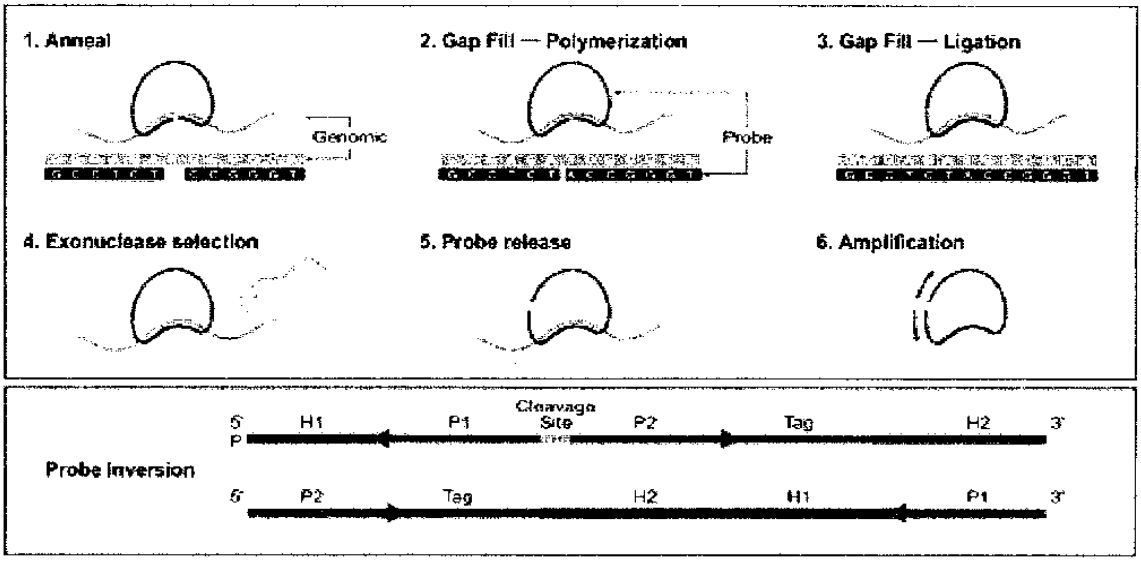

Obtain sample from patient
600
Enrich for rare cells
602
Split enriched sample
603
Into two or more discrete sites
Perform molecular analysis test at each discrete site
604
Determine condition of patient
605

Figure 6



Molecular Inversion Probes are so named because the oligonucleotide probe central to the process undergoes a unimolecular rearrangement from a molecule that cannot be amplified (step 1), into a molecule that can be amplified (step 6). This rearrangement is mediated by hybridization to genomic DNA (step 2) and an enzymatic "gap fill" process that occurs in an allele-specific manner (step 3). The resulting circularized probe can be separated from cross-reacted or unreacted probes by a simple exonuclease reaction (step 4). Figure 1 shows these steps.

Figure 7A

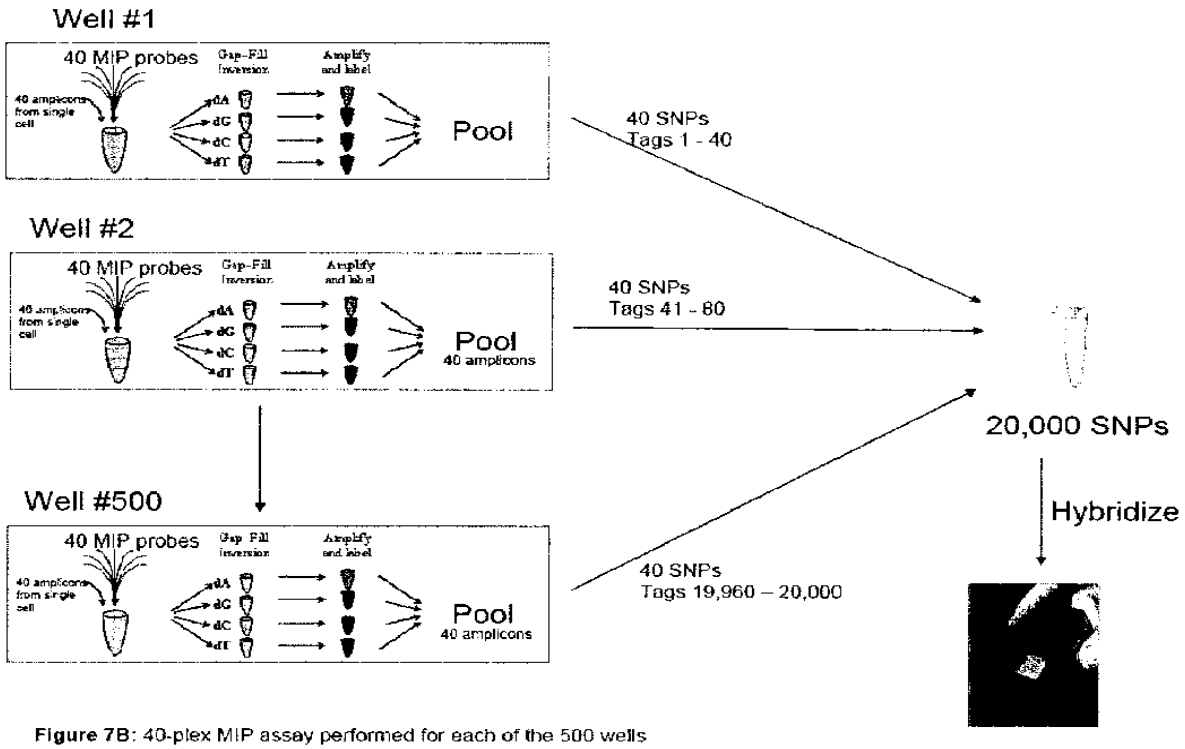


Figure 7B: 40-plex MIP assay performed for each of the 500 wells

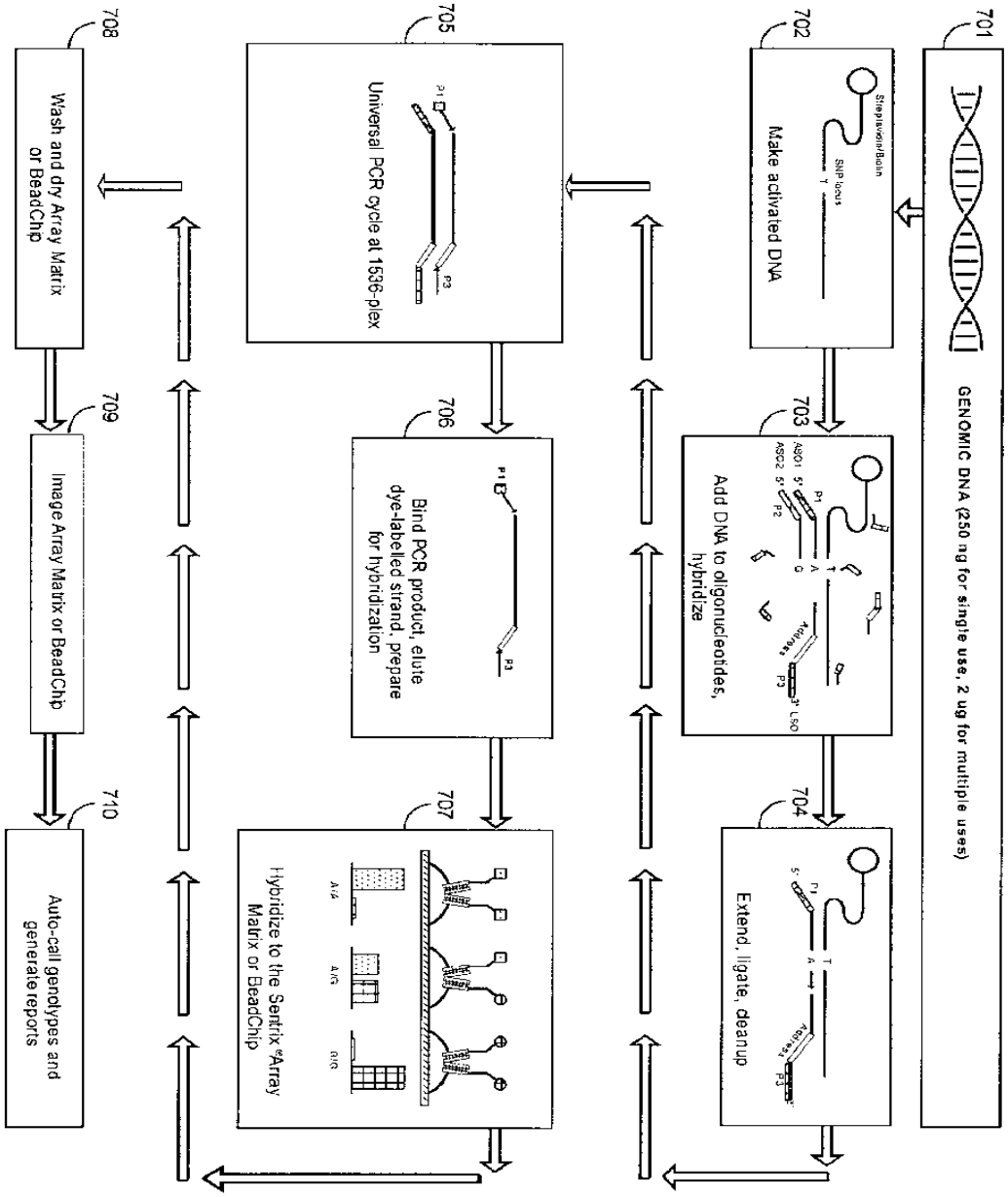

701
GENOMIC DNA (250 ng for single use, 2 ug for multiple uses)
702
Make activated DNA
703
Add DNA to oligonucleotides, hybridize
704
Extend, ligate, cleanup
705
Universal PCR cycle at 1536-plex
706
Bind PCR product, elute dye-labeled strand, prepare for hybridization
707
Hybridize to the Sentrix ®Array Matrix or BeadChip
708
Wash and dry Array Matrix or BeadChip
709
Image Array Matrix or BeadChip
710
Auto-call genotypes and generate reports

Figure 7C

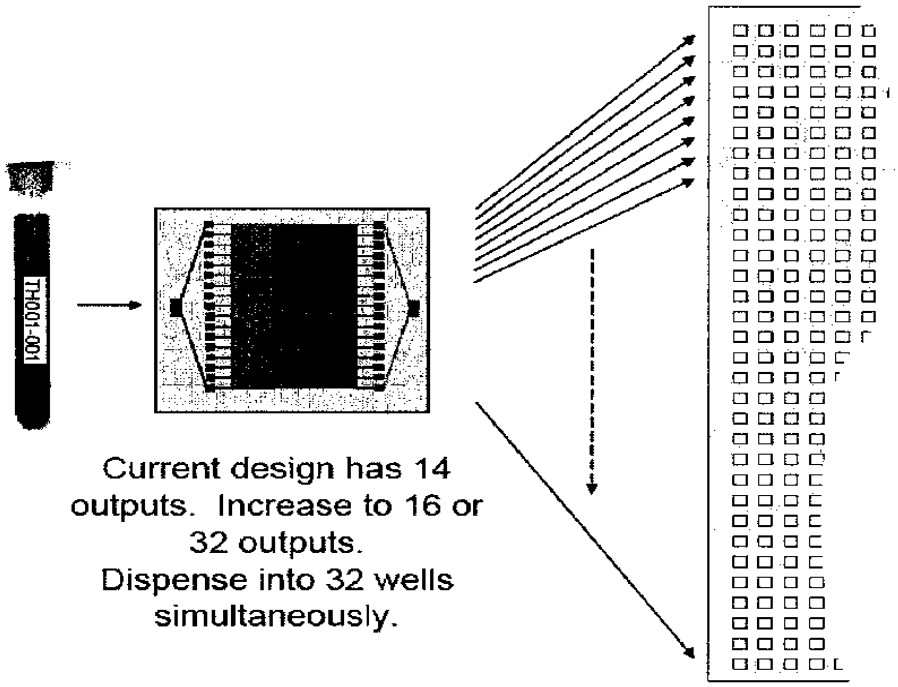
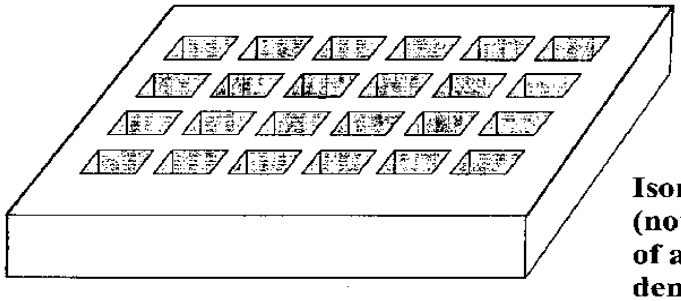

1536 well plate
32 x 48 wells
Current design has 14 outputs. Increase to 16 or 32 outputs.
Dispense into 32 wells simultaneously.

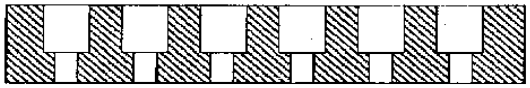
Figure 8A

Figure 8B

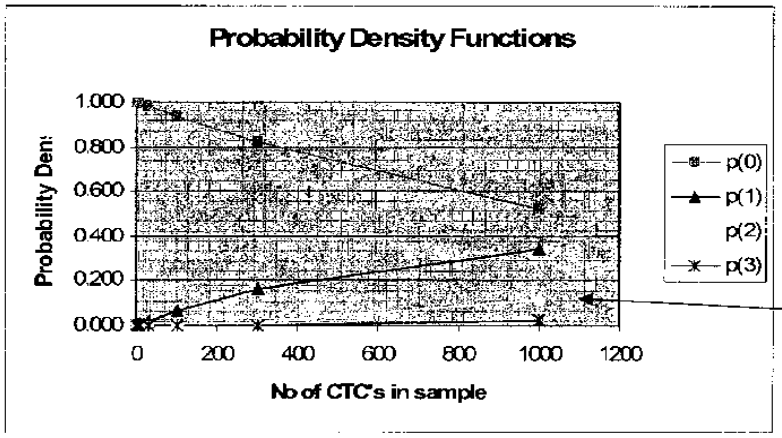


Isometric View (not illustrative of actual well density)

Figure 8C



Cross-section



Probability of two CTCs in same well

Figure 9

Figure 10

2AR
A DISINTEGRIN
ACTIVATOR OF THYROID AND RETINOIC ACID RECEPTOR (ACTR)
ADAM 11
ADIPOGENESIS INHIBITORY FACTOR (ADIF)
ALPHA 6 INTEGRIN SUBUNIT
ALPHA V INTEGRIN SUBUNIT
ALPHA-CATENIN
AMPLIFIED IN BREAST CANCER 1 (AIB1)
AMPLIFIED IN BREAST CANCER 3 (AIB3)
AMPLIFIED IN BREAST CANCER 4 (AIB4)
AMYLOID PRECURSOR PROTEIN SECRETASE (APPS)
AP-2 GAMMA
APPS
ATP-BINDING CASSETTE TRANSPORTER (ABCT)
PLACENTA-SPECIFIC (ABCP)
ATP-BINDING CASSETTE SUBFAMILY C MEMBER (ABCC1)
BAG-1
BASIGIN (BSG)
BCEI
B-CELL DIFFERENTIATION FACTOR (BCDF)
B-CELL LEUKEMIA 2 (BCL-2)
B-CELL STIMULATORY FACTOR-2 (BSF-2)
BCL-1
BCL-2-ASSOCIATED X PROTEIN (BAX)
BCRP
BETA 1 INTEGRIN SUBUNIT
BETA 3 INTEGRIN SUBUNIT
BETA 5 INTEGRIN SUBUNIT
BETA-2 INTERFERON
BETA-CATENIN
BETA-CATENIN
BONE SIALOPROTEIN (BSP)
BREAST CANCER ESTROGEN-INDUCIBLE SEQUENCE (BCEI)
BREAST CANCER RESISTANCE PROTEIN (BCRP)
BREAST CANCER TYPE 1 (BRCA1)
BREAST CANCER TYPE 2 (BRCA2)
BREAST CARCINOMA AMPLIFIED SEQUENCE 2 (BCAS2)
CADHERIN
EPITHELIAL CADHERIN-11
CADHERIN-ASSOCIATED PROTEIN
CALCITONIN RECEPTOR (CTR)
CALCIUM PLACENTAL PROTEIN (CAPL)
CALCYCLIN
CALLA
CAM5
CAPL
CARCINOEMBRYONIC ANTIGEN (CEA)
CATENIN
ALPHA 1
CATHEPSIN B
CATHEPSIN D
CATHEPSIN K
CATHEPSIN L2
CATHEPSIN O
CATHEPSIN O1
CATHEPSIN V
CD10
CD146
CD147
CD24
CD29
CD44
CD51
CD54
CD61
CD66e
CD82
CD87
CD9
CEA
CELLULAR RETINOL-BINDING PROTEIN 1 (CRBP1)
c-ERBB-2
CK7
CK8
CK18
CK19
CK20
CLAUDIN-7
c-MET
COLLAGENASE
FIBROBLAST
COLLAGENASE
INTERSTITIAL
COLLAGENASE-3
COMMON ACUTE LYMPHOCYTIC LEUKEMIA ANTIGEN (CALLA)
CONNEXIN 26 (Cx26)
CONNEXIN 43 (Cx43)
CORTACTIN
COX-2
CTLA-8
CTR
CTSD
CYCLIN D1
CYCLOOXYGENASE-2
CYTOKERATIN 18
CYTOKERATIN 19
CYTOKERATIN 8
CYTOTOXIC T-LYMPHOCYTE-ASSOCIATED SERINE ESTERASE 8 (CTLA-8)

DIFFERENTIATION-INHIBITING ACTIVITY (DIA)
DNA AMPLIFIED IN MAMMARY CARCINOMA 1 (DAM1)
DNA TOPOISOMERASE II ALPHA
DR-NM23
E-CADHERIN
EMMPRIN
EMS1
ENDOTHELIAL CELL GROWTH FACTOR (ECGR)
PLATELET-DERIVED (PD-ECGF)
ENKEPHALINASE
EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)
EPISIALIN
EPITHELIAL MEMBRANE ANTIGEN (EMA)
ER-ALPHA
ERBB2
ERBB4
ER-BETA
ERF-1
ERYTHROID-POTENTIATING ACTIVITY (EPA)
ESR1
ESTROGEN RECEPTOR-ALPHA
ESTROGEN RECEPTOR-BETA
ETS-1
EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER (EMMPRIN)
FIBRONECTIN RECEPTOR
BETA POLYPEPTIDE (FNRB)
FIBRONECTIN RECEPTOR BETA SUBUNIT (FNRB)
FLK-1
GA15.3
GA733.2
GALECTIN-3
GAMMA-CATENIN
GAP JUNCTION PROTEIN (26 kDa)
GAP JUNCTION PROTEIN (43 kDa)
GAP JUNCTION PROTEIN ALPHA-1 (GJA1)
GAP JUNCTION PROTEIN BETA-2 (GJB2)
GCP1
GELATINASE A
GELATINASE B
GELATINASE (72 kDa)
GELATINASE (92 kDa)
GLIOSTATIN
GLUCOCORTICOID RECEPTOR INTERACTING PROTEIN 1 (GRIP1)
GLUTATHIONE S-TRANSFERASE p
GM-CSF
GRANULOCYTE CHEMOTACTIC PROTEIN 1 (GCP1)
GRANULOCYTE-MACROPHAGE-COLONY STIMULATING FACTOR
GROWTH FACTOR RECEPTOR BOUND-7 (GRB-7)
GSTp
HAP
HEAT-SHOCK COGNATE PROTEIN 70 (HSC70)
HEAT-STABLE ANTIGEN
HEPATOCYTE GROWTH FACTOR (HGF)
HEPATOCYTE GROWTH FACTOR RECEPTOR (HGFR)
HEPATOCYTE-STIMULATING FACTOR III (HSF III)
HER-2
HER2/NEU
HERMES ANTIGEN
HET
HHM
HUMORAL HYPERCALCEMIA OF MALIGNANCY (HHM)
ICERE-I
INT-1
INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1)
INTERFERON-GAMMA-INDUCING FACTOR (IGIF)
INTERLEUKIN-1 ALPHA (IL-1A)
INTERLEUKIN-1 BETA (IL-1B)
INTERLEUKIN-11 (IL-11)
INTERLEUKIN-17 (IL-17)
INTERLEUKIN-18 (IL-18)
INTERLEUKIN-6 (IL-6)
INTERLEUKIN-8 (IL-8)
INVERSELY CORRELATED WITH ESTROGEN RECEPTOR EXPRESSION-1 (ICERE-1)
KAI1
KDR
KERATIN 8
KERATIN 18
KERATIN 19
KISS-1
LEUKEMIA INHIBITORY FACTOR (LIF)
LIF
LOST IN INFLAMMATORY BREAST CANCER (LIBC)
LOT ("LOST ON TRANSFORMATION")
LYMPHOCYTE HOMING RECEPTOR
MACROPHAGE-COLONY STIMULATING FACTOR
MAGE-3
MAMMAGLOBIN
MASPIN
MC56
M-CSF
MDC
MDNCF
MDR
MELANOMA CELL ADHESION MOLECULE (MCAM)
MEMBRANE METALLOENDOPEPTIDASE (MME)

MEMBRANE-ASSOCIATED NEUTRAL ENDOPEPTIDASE (NEP)
CYSTEINE-RICH PROTEIN (MDC)
METASTASIN (MTS-1)
MLN64
MMP1
MMP2
MMP3
MMP7
MMP9
MMP11
MMP13
MMP14
MMP15
MMP16
MMP17
MOESIN
MONOCYTE ARGININE-SERPIN
MONOCYTE-DERIVED NEUTROPHIL CHEMOTACTIC FACTOR
MONOCYTE-DERIVED PLASMINOGEN ACTIVATOR INHIBITOR
MTS-1
MUC-1
MUC18
MUCIN LIKE CANCER ASSOCIATED ANTIGEN (MCA)
MUCIN
MUC-1
MULTIDRUG RESISTANCE PROTEIN 1 (MDR, MDR1)
MULTIDRUG RESISTANCE RELATED PROTEIN-1 (MRP, MRP-1)
N-CADHERIN
NEP
NEU
NEUTRAL ENDOPEPTIDASE
NEUTROPHIL-ACTIVATING PEPTIDE 1 (NAP1)
NM23-H1
NM23-H2
NME1
NME2
NUCLEAR RECEPTOR COACTIVATOR-1 (NCoA-1)
NUCLEAR RECEPTOR COACTIVATOR-2 (NCoA-2)
NUCLEAR RECEPTOR COACTIVATOR-3 (NCoA-3)
NUCLEOSIDE DIPHOSPHATE KINASE A (NDPKA)
NUCLEOSIDE DIPHOSPHATE KINASE B (NDPKB)
ONCOSTATIN M (OSM)
ORNITHINE DECARBOXYLASE (ODC)
OSTEOCLAST DIFFERENTIATION FACTOR (ODF)
OSTEOCLAST DIFFERENTIATION FACTOR RECEPTOR (ODFR)
OSTEONECTIN (OSN, ON)
OSTEOPONTIN (OPN)
OXYTOCIN RECEPTOR (OXTR)
p27/kip1
p300/CBP COINTEGRATOR ASSOCIATE PROTEIN (p/CIP)
p53
p9Ka
PAI-1
PAI-2
PARATHYROID ADENOMATOSIS 1 (PRAD1)
PARATHYROID HORMONE-LIKE HORMONE (PTHLH)
PARATHYROID HORMONE-RELATED PEPTIDE (PTHrP)
P-CADHERIN
PD-ECGF
PDGF-β
PEANUT-REACTIVE URINARY MUCIN (PUM)
P-GLYCOPROTEIN (P-GP)
PGP-1
PHGS-2
PHS-2
PIP
PLAKOGLOBIN
PLASMINOGEN ACTIVATOR INHIBITOR (TYPE 1)
PLASMINOGEN ACTIVATOR INHIBITOR (TYPE 2)
PLASMINOGEN ACTIVATOR (TISSUE-TYPE)
PLASMINOGEN ACTIVATOR (UROKINASE-TYPE)
PLATELET GLYCOPROTEIN IIIa (GP3A)
PLAU
PLEOMORPHIC ADENOMA GENE-LIKE 1 (PLAGL1)
POLYMORPHIC EPITHELIAL MUCIN (PEM)
PRAD1
PROGESTERONE RECEPTOR (PgR)
PROGESTERONE RESISTANCE
PROSTAGLANDIN ENDOPEROXIDE SYNTHASE-2
PROSTAGLANDIN G/H SYNTHASE-2
PROSTAGLANDIN H SYNTHASE-2
pS2
PS6K
PSORIASIN
PTHLH
PTHrP
RAD51
RAD52
RAD54
RAP46
RECEPTOR-ASSOCIATED COACTIVATOR 3 (RAC3)
REPRESSOR OF ESTROGEN RECEPTOR ACTIVITY (REA)
S100A4

S100A6
S100A7
S6K
SART-1
SCAFFOLD ATTACHMENT FACTOR B (SAF-B)
SCATTER FACTOR (SF)
SECRETED PHOSPHOPROTEIN-1 (SPP-1)
SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE (SPARC)
STANNICALCIN
STEROID RECEPTOR COACTIVATOR-1 (SRC-1)
STEROID RECEPTOR COACTIVATOR-2 (SRC-2)
STEROID RECEPTOR COACTIVATOR-3 (SRC-3)
STEROID RECEPTOR RNA ACTIVATOR (SRA)
STROMELYSIN-1
STROMELYSIN-3
TENASCIN-C (TN-C)
TESTES-SPECIFIC PROTEASE 50
THROMBOSPONDIN I
THROMBOSPONDIN II
THYMIDINE PHOSPHORYLASE (TP)
THYROID HORMONE RECEPTOR ACTIVATOR MOLECULE 1 (TRAM-1)
TIGHT JUNCTION PROTEIN 1 (TJP1)
TIMP1
TIMP2
TIMP3
TIMP4
TISSUE-TYPE PLASMINOGEN ACTIVATOR
TN-C
TP53
tPA
TRANSCRIPTIONAL INTERMEDIARY FACTOR 2 (TIF2)
TREFOIL FACTOR 1 (TFF1)
TSG101
TSP-1
TSP1
TSP-2
TSP2
TSP50
TUMOR CELL COLLAGENASE STIMULATING FACTOR (TCSF)
TUMOR-ASSOCIATED EPITHELIAL MUCIN
uPA
uPAR
UROKINASE
UROKINASE-TYPE PLASMINOGEN ACTIVATOR
UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (uPAR)
UVOMORULIN
VASCULAR ENDOTHELIAL GROWTH FACTOR
VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2 (VEGFR2)
VASCULAR ENDOTHELIAL GROWTH FACTOR-A
VASCULAR PERMEABILITY FACTOR
VEGFR2
VERY LATE T-CELL ANTIGEN BETA (VLA-BETA)
VIMENTIN
VITRONECTIN RECEPTOR ALPHA POLYPEPTIDE (VNRA)
VITRONECTIN RECEPTOR
VON WILLEBRAND FACTOR
VPF
VWF
WNT-1
ZAC
ZO-1
ZONULA OCCLUDENS-1

Name	SEQ ID NO	Sequence (5' to 3')	Exon	Amplicon Size
NXK-ex18.1(+)	11	TCAGAGCCTGTGTTTCTACCAA	18	534
NXK-ex18.2(-)	12	TGGTCTCACAGGACCACTGATT	18	
NXK-ex18.3(+)	13	TCCAAATGAGCTGGCAAGTG	18	397
NXK-ex18.4(-)	14	TCCCAAACACTCAGTGAAACAAA	18	
NXK-ex19.1(+)	15	AAATAATCAGTGTGATTCGTGGAG	19	495
NXK-ex19.2(-)	16	GAGGCCAGTGCTGTCTCTAAGG	19	
NXK-ex19.3(+)	17	GTGCATCGCTGGTAACATCC	19	298
NXK-ex19.4(-)	18	TGTGGAGATGAGCAGGGTCT	19	
NXK-ex20.1(+)	19	ACTTCACAGCCCTGCGTAAAC	20	555
NXK-ex20.2(-)	20	ATGGGACAGGCACTGATTTGT	20	
NXK-ex20.3(+)	21	ATCGCATTCATGCGTCTTCA	20	379
NXK-ex20.4(-)	22	ATCCCCATGGCAAACTCTTG	20	
NXK-ex21.1(+)	23	GCAGCGGGTTACATCTTCTTTC	21	526
NXK-ex21.2(-)	24	CAGCTCTGGCTCACACTACCAG	21	
NXK-ex21.3(+)	25	GCAGCGGGTTACATCTTCTTTC	21	349
NXK-ex21.4(-)	26	CATCCTCCCCTGCATGTGT	21	

Figure 11

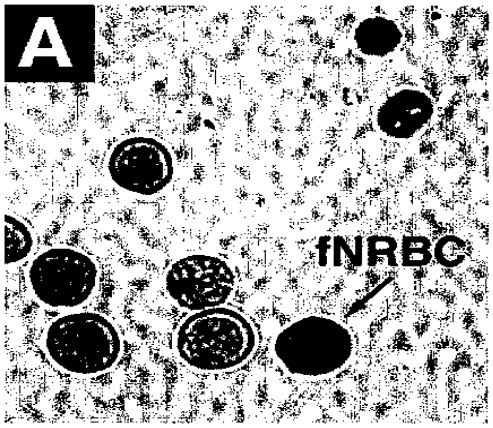


Figure 12 A

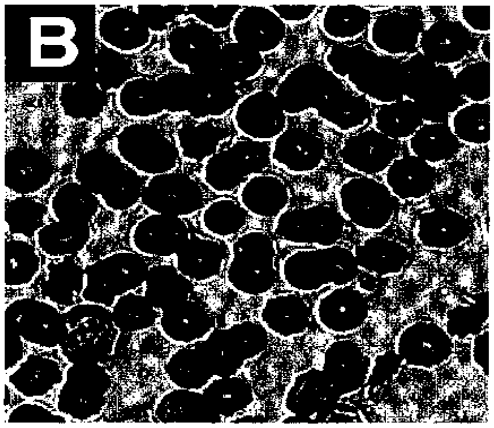


Figure 12 B

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00938

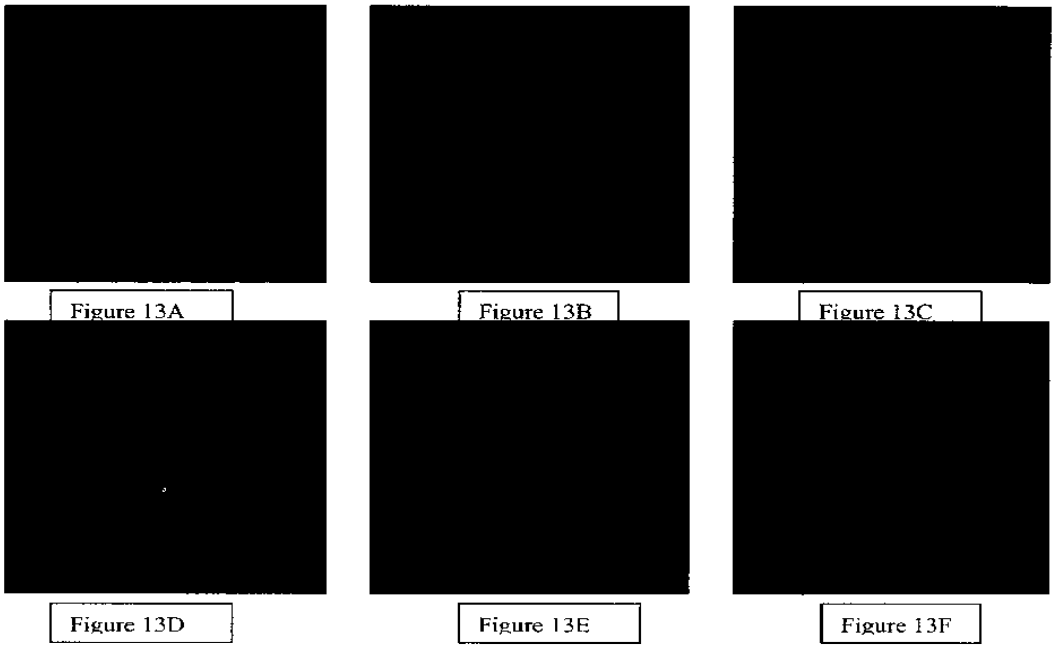


Figure 13A-F (Blue= nucleus, Red = X chromosome, Green = Y chromosome)

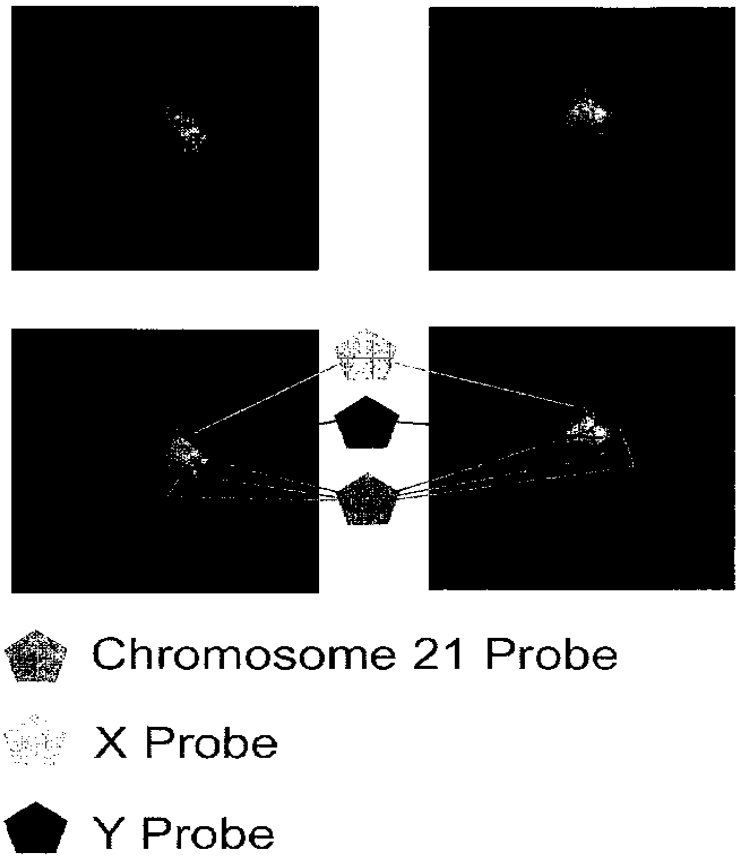


Figure 14

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00940

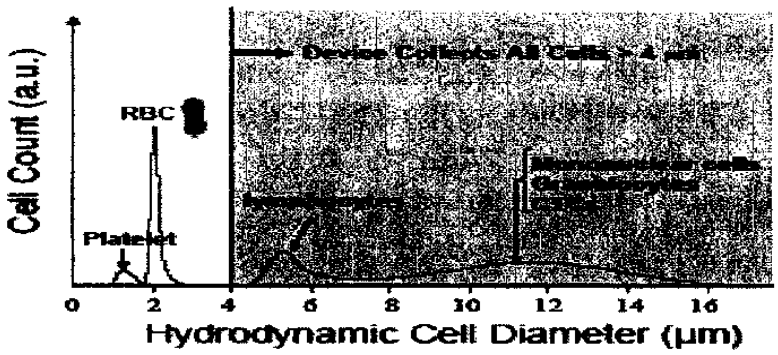


Figure 15

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00941

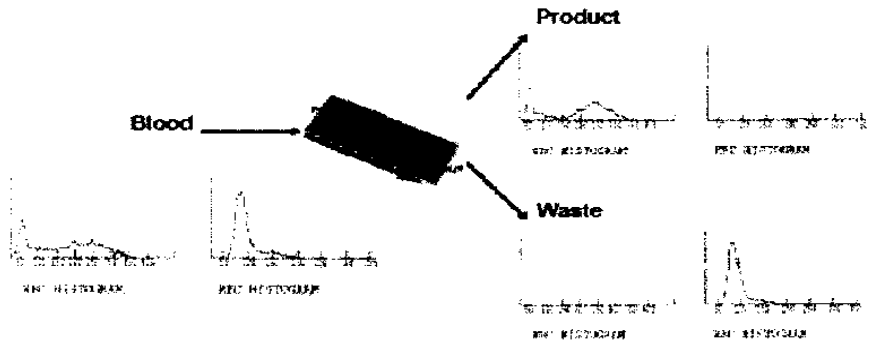


Figure 16

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00942

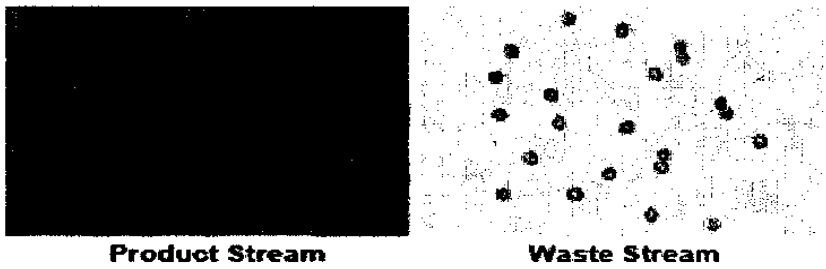


Figure 17

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00943



Figure 18

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 00944

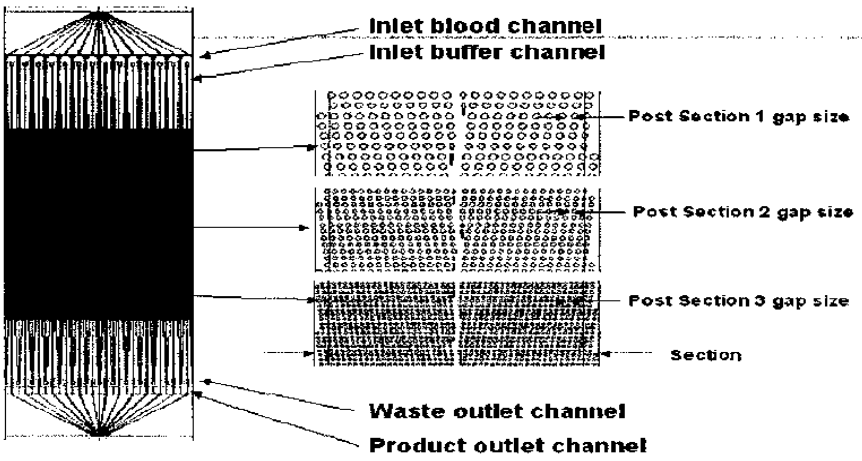
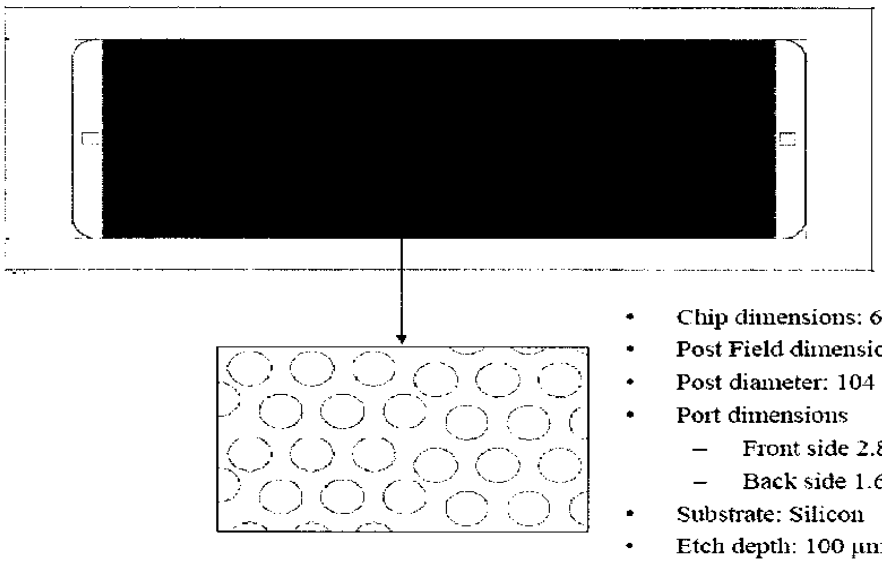

Inlet blood channel
Inlet buffer channel
Post Section 1 gap size
Post Section 2 gap size
Post Section 3 gap size
Section
Waste outlet channel
Product outlet channel

Figure 19A

		Version 1	Version 2	Version 3
Inlet channel width (blood)	50	50	100	100
Inlet channel width (buffer)	55	55	110	110
Outlet channel width (product)	49	49	98	98
Outlet channel width (waste)	50	50	100	100
Gap size / Deflect cell size				
Post section 1	18/9	36/18	44/22	50/25
Post section 2	12/6	24/12	30/15	36/18
Post section 3	8/4	16/8	20/10	24/12
Number of parallel sections	14	14	14	14
Etch depth	150	150	150	150
Product cell size (cut off)	4	8	10	12
Estimated Flow rate, ml/hr	5	10	20	30

Figure 19B



- Chip dimensions: 66.0 x 24.9 mm
- Post Field dimensions: 51.3 x 18.9 mm
- Post diameter: 104 μm
- Port dimensions
  - Front side 2.83 x2.83 mm
  - Back side 1.66 x 1.66 mm
- Substrate: Silicon
- Etch depth: 100 μm

Figure 19C

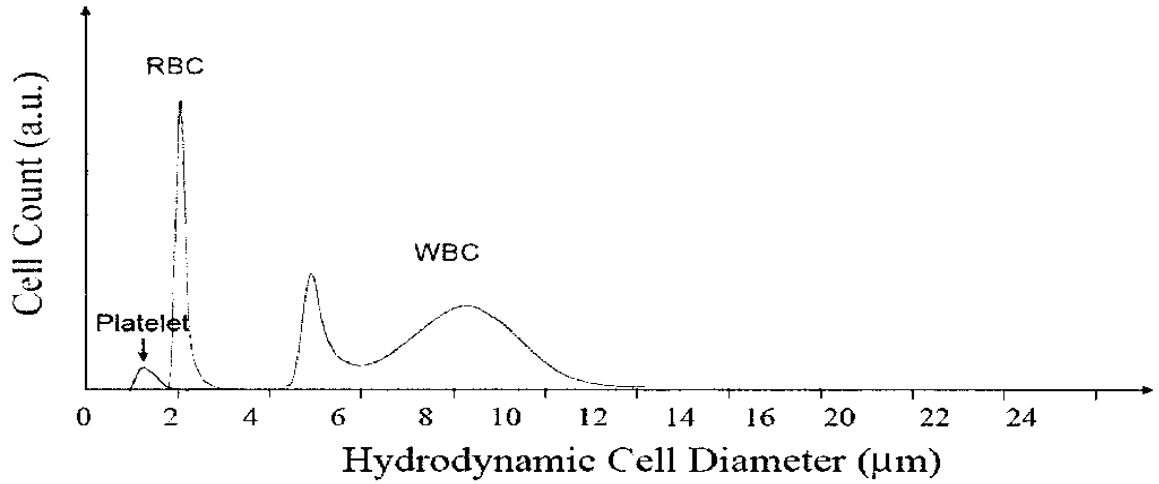


Figure 20A

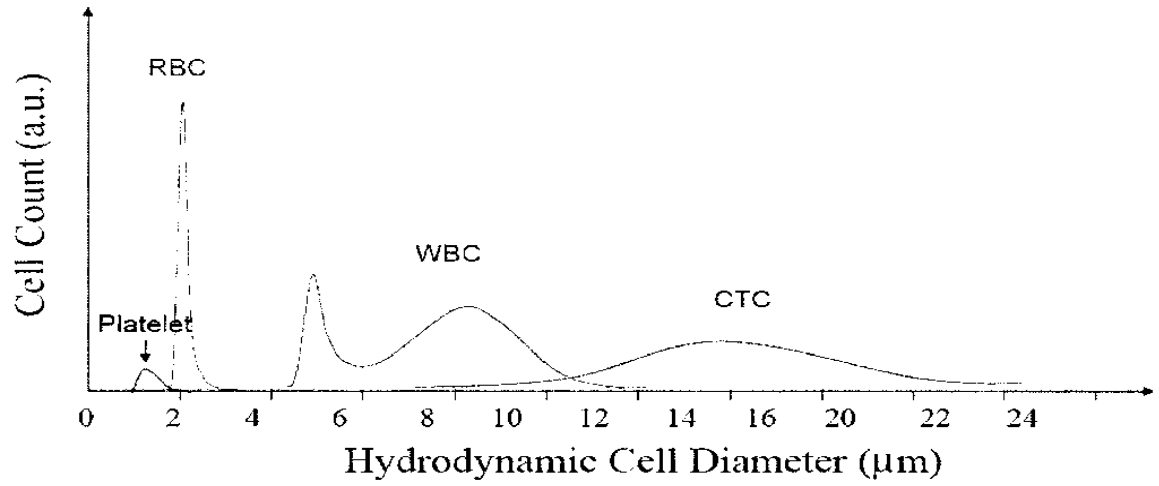


Figure 20B

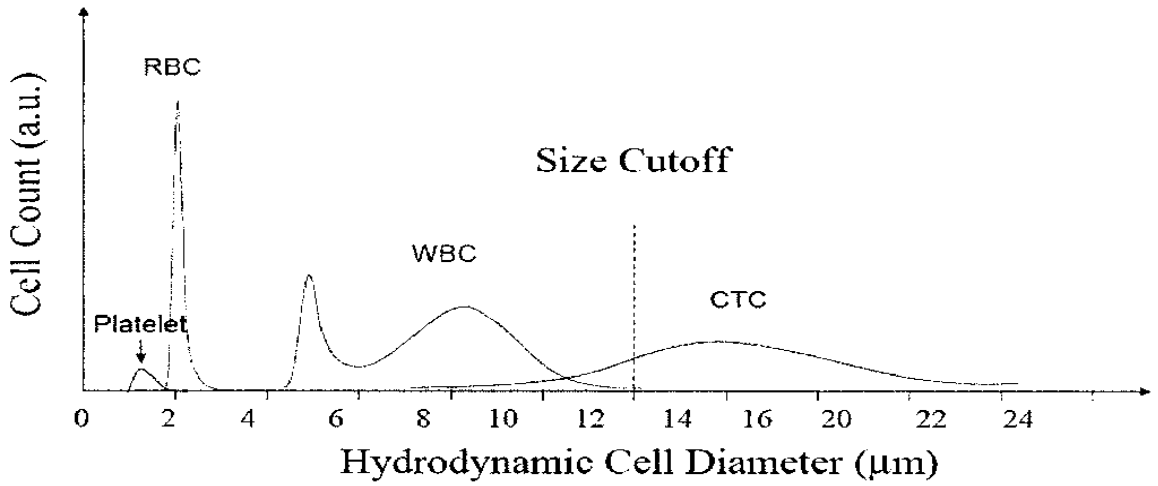


Figure 20C

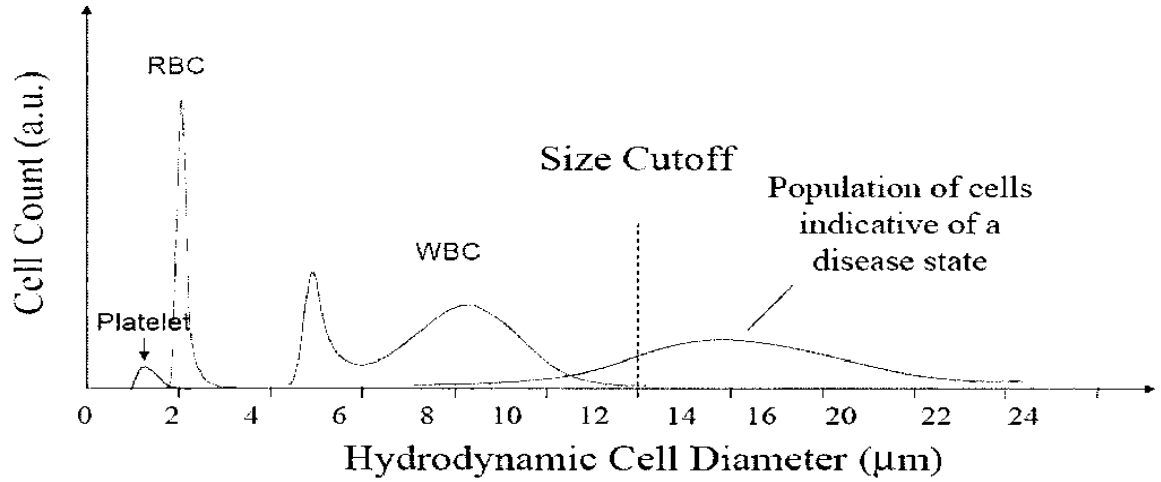


Figure 20D

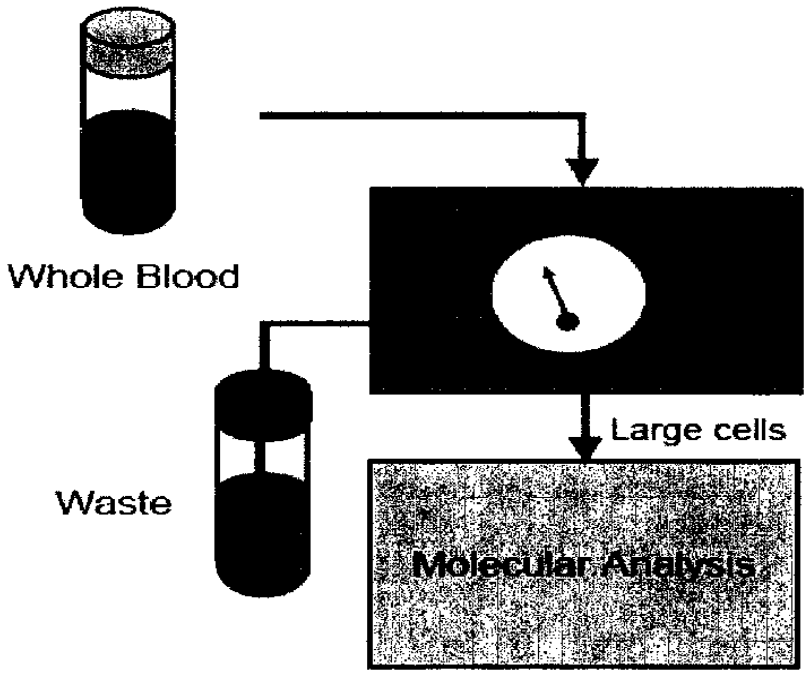


Figure 21

10x Genomics, Inc. Exhibit 2004
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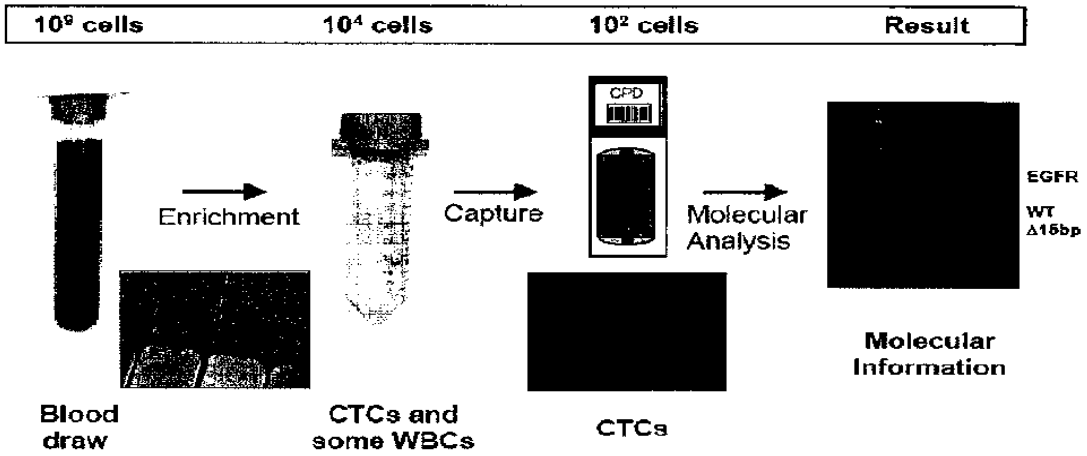


Figure 22

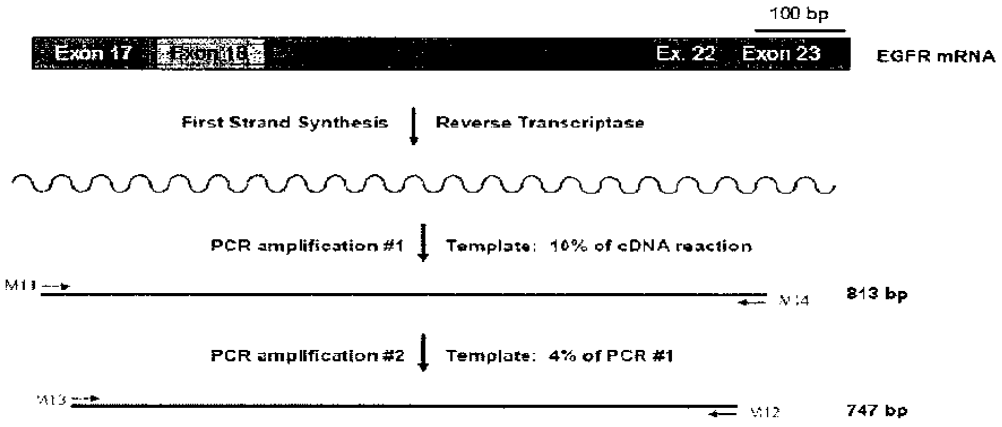


Figure 23

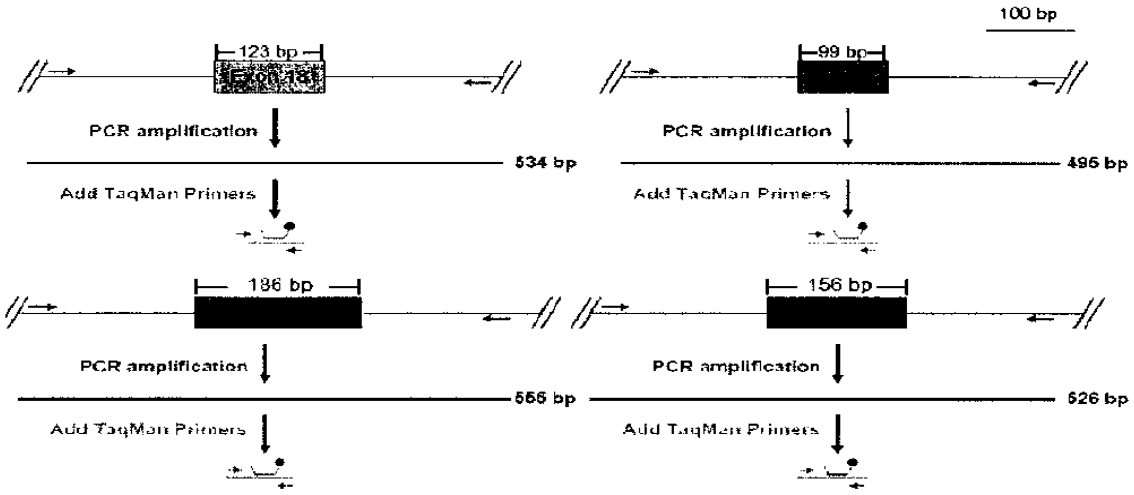

100 bp
123 bp
Exon 18
99 bp
PCR amplification
534 bp
PCR amplification
496 bp
Add TaqMan Primers
Add TaqMan Primers
186 bp
156 bp
PCR amplification
556 bp
PCR amplification
526 bp
Add TaqMan Primers
Add TaqMan Primers

Figure 24

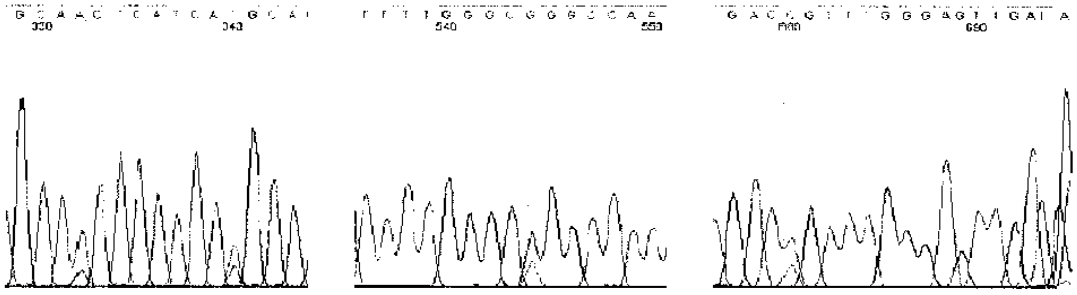

CAA → CAG (silent) at 2361
ACG → ATG (T790M) at 2369
CTG → CGG (L828R) at 2573
ACC → ACT (silent) at 2709

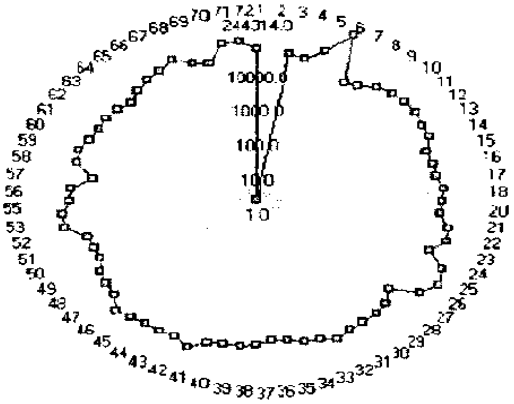
Figure 25



WBC cDNA	-	+	-	-	+	-	-	+	-	-	+	-
H1650 cDNA	-	-	+	-	-	+	-	-	+	-	-	+
Target mRNA	BCKD			CD45			EpCaM			EGFR		

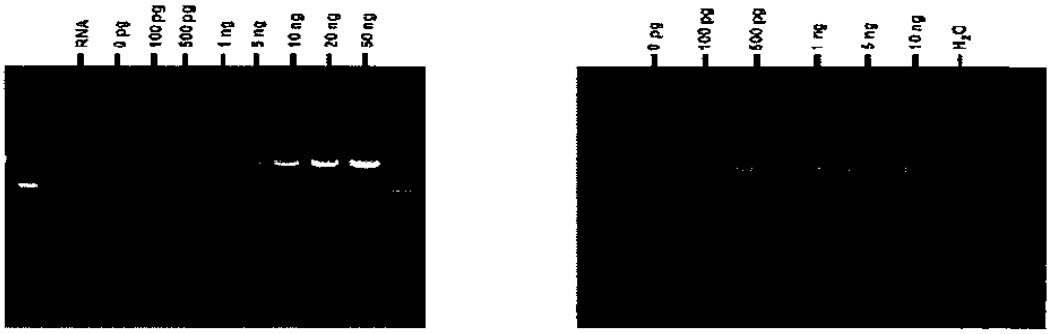
Size Standards

Figure 26A



	Tissue	Ct	[mRNA]
1	Renal Blood Vessel	24.02	25,916
2	Blood Mononuclear Cells (PBMC)	>40	0
3	Colon	24.28	21,968
4	Primary Lung Bronchus	24.49	19,224

Figure 26B



PCR #1 PCR #2 (Nested)

NIH-3T3 – 100 pg total RNA = ~20 cells
NCI-H1975 -- 100 pg total RNA = ~10 cells
COS-7 -- 100 pg total RNA = ~6 cells

Figure 27

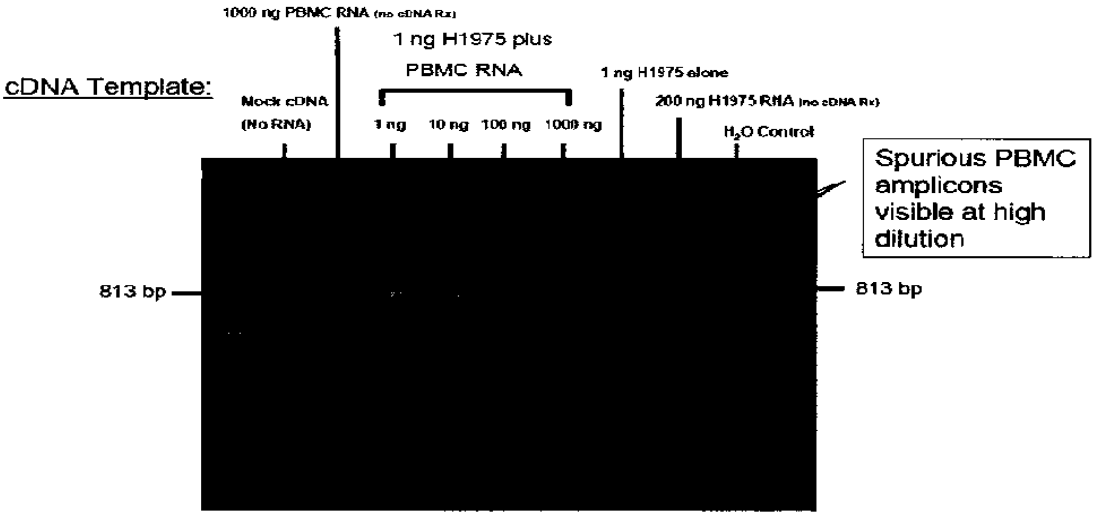


Figure 28A

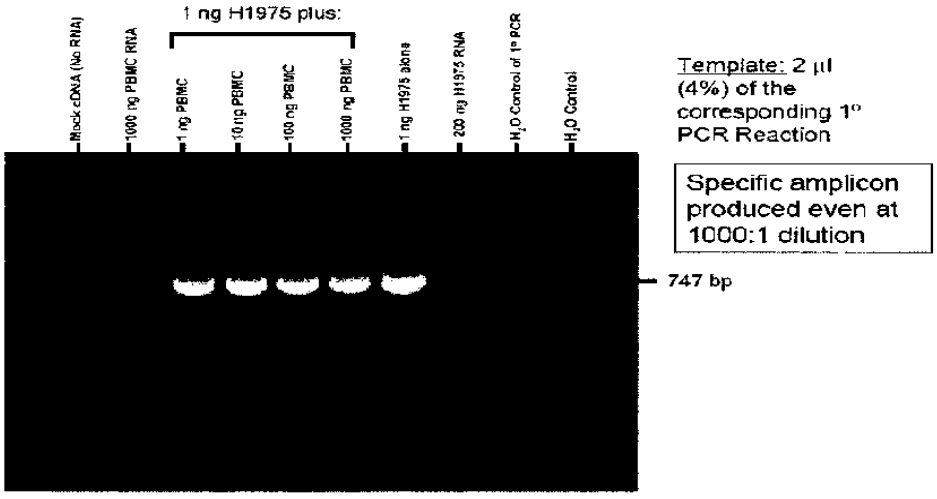


Figure 28B

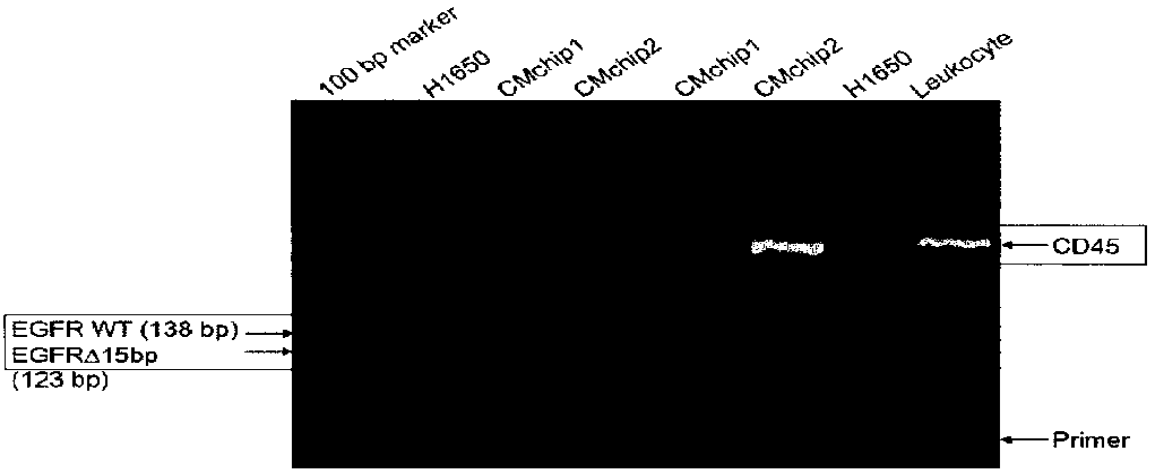

100 bp marker
H1650
CMchip1
CMchip2
CMchip1
CMchip2
H1650
Leukocyte
CD45
EGFR WT (138 bp)
EGFRΔ15bp
(123 bp)
Primer

Figure 29

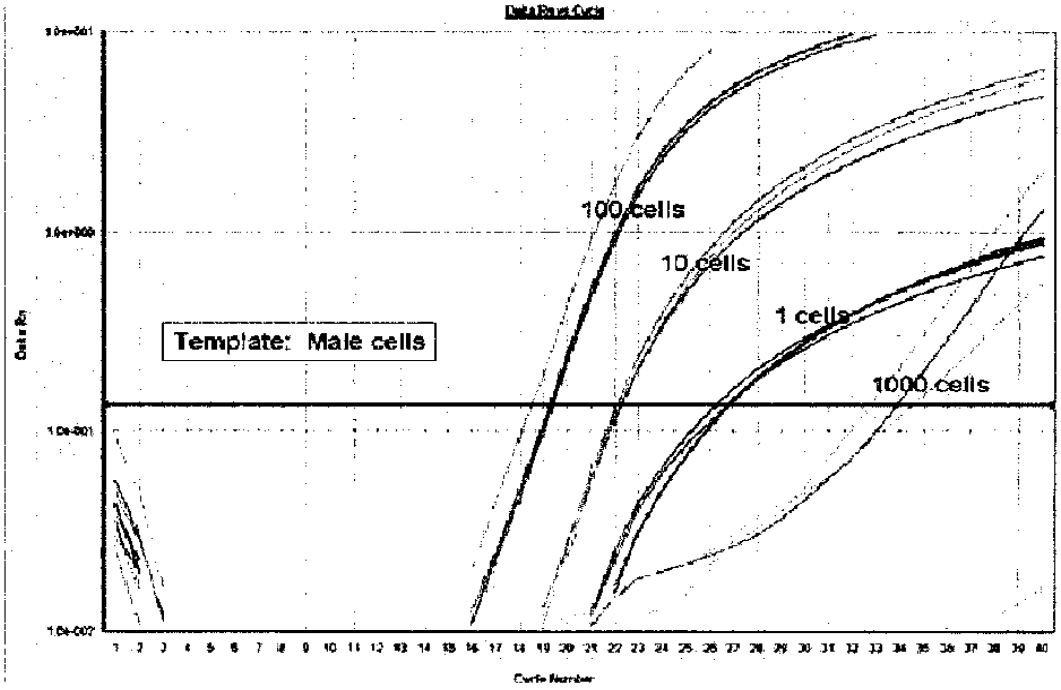


FIGURE 30

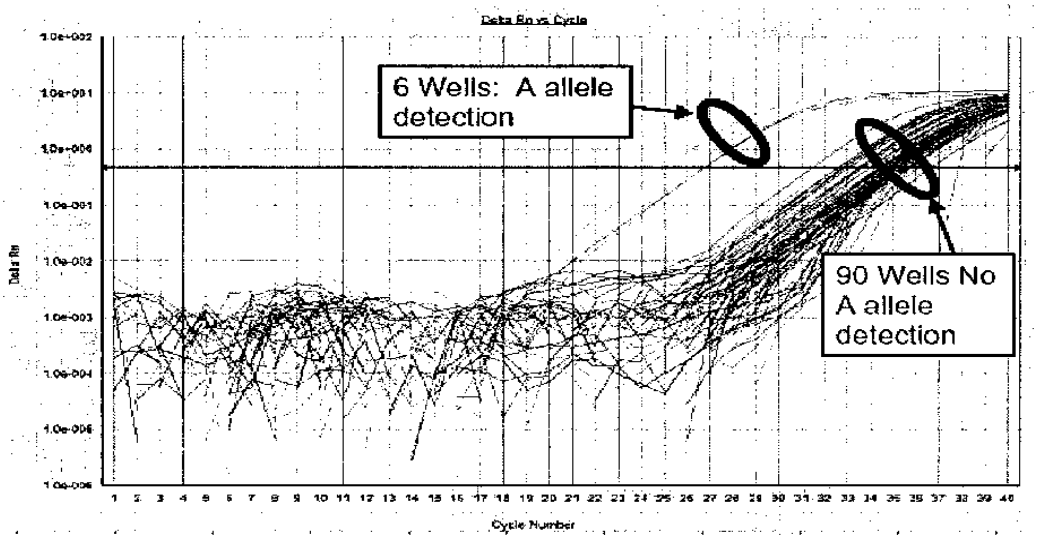

Delta Rn vs Cycle
6 Wells: A allele detection
90 Wells No A allele detection
Cycle Number

FIGURE 31

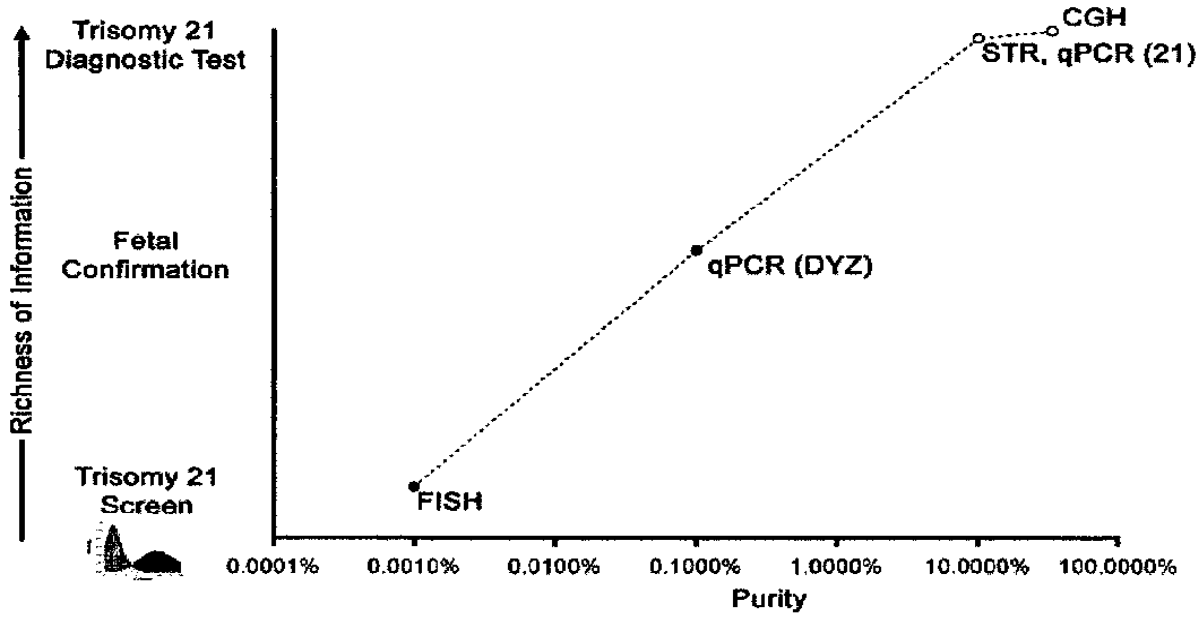

Trisomy 21
Diagnostic Test
Fetal
Confirmation
Trisomy 21
Screen
Richness of Information
CGH
STR, qPCR (21)
qPCR (DYZ)
FISH
0.0001%
0.0010%
0.0100%
0.1000%
1.0000%
10.0000%
100.0000%
Purity

FIGURE 32

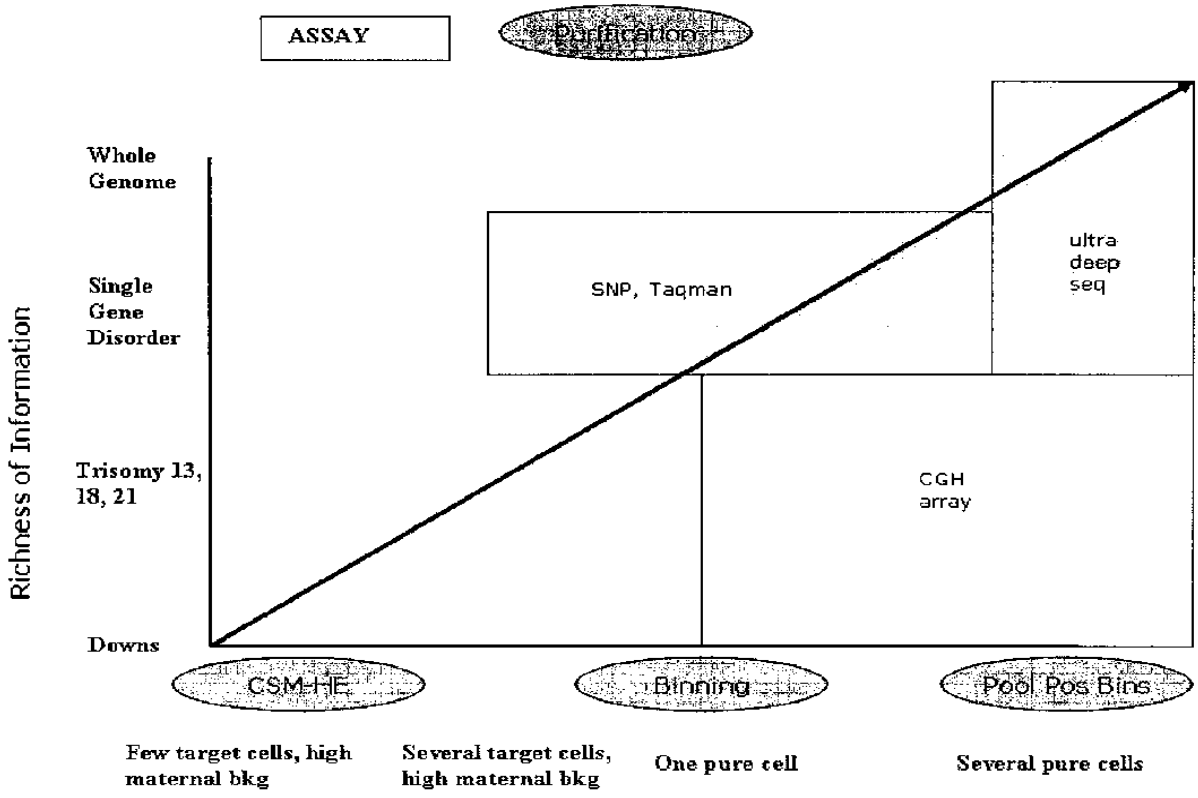

ASSAY
Purification
Whole Genome
Single Gene Disorder
Trisomy 13, 18, 21
Downs
Richness of Information
SNP, Taqman
ultra deep seq
CGH array
CSM-HE
Binning
Pool Pos Bins
Few target cells, high maternal bkg
Several target cells, high maternal bkg
One pure cell
Several pure cells

FIGURE 33

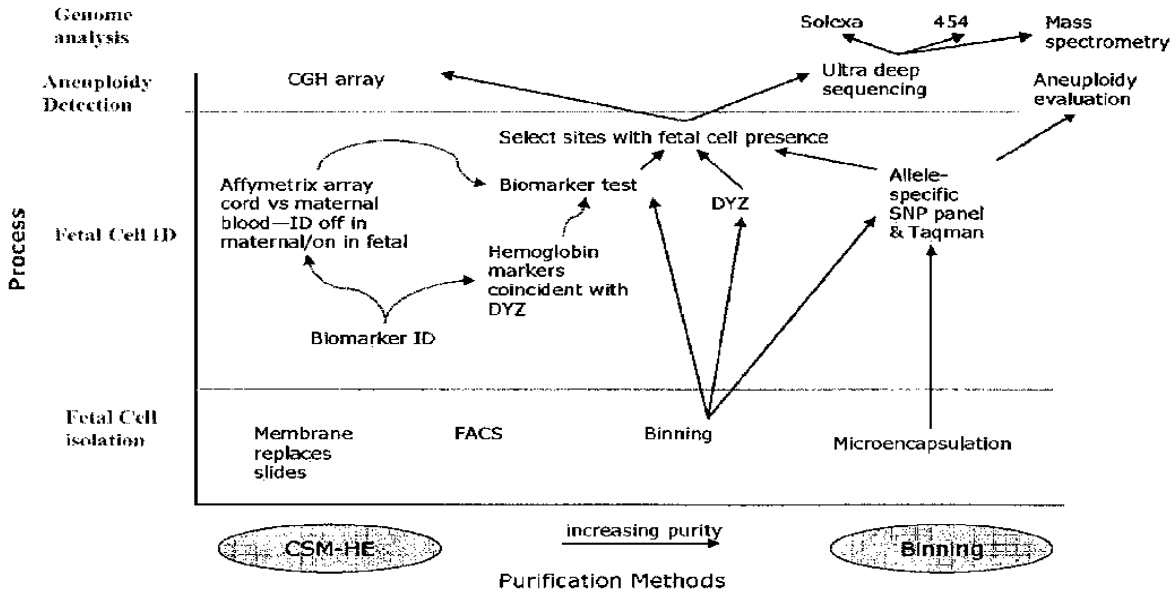

Genome analysis
Solexa
454
Mass spectrometry
Aneuploidy Detection
CGH array
Ultra deep sequencing
Aneuploidy evaluation
Select sites with fetal cell presence
Affymetrix array cord vs maternal blood—ID off in maternal/on in fetal
Biomarker test
DYZ
Allele-specific SNP panel & Taqman
Process
Fetal Cell ID
Hemoglobin markers coincident with DYZ
Biomarker ID
Fetal Cell isolation
Membrane replaces slides
FACS
Binning
Microencapsulation
CSM-HE
increasing purity
Binning
Purification Methods

FIGURE 34

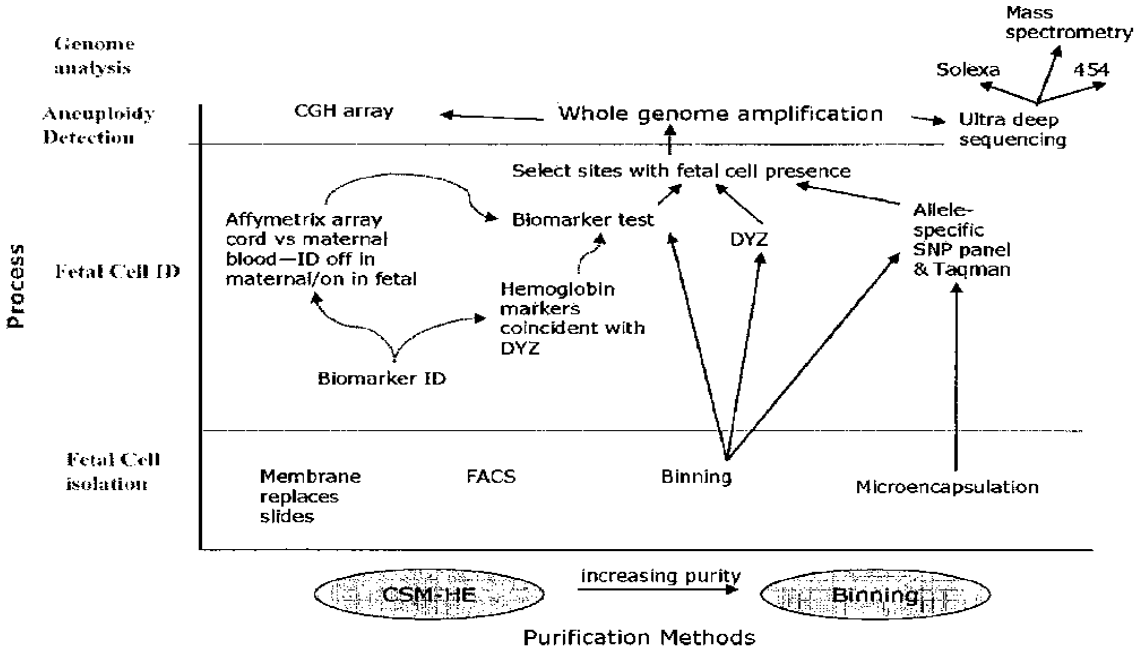

Genome analysis
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454
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CGH array
Whole genome amplification
Ultra deep sequencing
Select sites with fetal cell presence
Affymetrix array cord vs maternal blood—ID off in maternal/on in fetal
Biomarker test
DYZ
Allele-specific SNP panel & Taqman
Process
Fetal Cell ID
Hemoglobin markers coincident with DYZ
Biomarker ID
Fetal Cell isolation
Membrane replaces slides
FACS
Binning
Microencapsulation
increasing purity
CSM-HE
Binning
Purification Methods

FIGURE 35

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(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **LINNARSSON, Sten** [SE/SE]; C/o 9885 Towne Centre Drive, San Diego, CA 92121 (US).

(74) Agents: **HEBERT, Michael L.** et al.; Mcdermott Will & Emery LLP, 11682 El Camino Real, Suite 400, San Diego, CA 92130 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) Title: GENE EXPRESSION ANALYSIS IN SINGLE CELLS

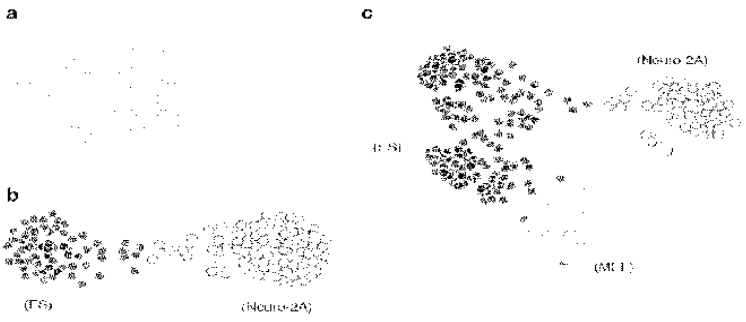


Figure 21

(57) Abstract: The present invention provides methods and compositions for the analysis of gene expression in single cells or in a plurality of single cells. The invention provides methods for preparing a cDNA library from individual cells by releasing mRNA from each single cell to provide a plurality of individual mRNA samples, synthesizing cDNA from the individual mRNA samples, tagging the individual cDNA, pooling the tagged cDNA samples and amplifying the pooled cDNA samples to generate a cDNA library. The invention also provides a cDNA library produced by the methods described herein. The invention farther provides methods for analyzing gene expression in a plurality of cells by preparing a cDNA library as described herein and sequencing the library.

WO 2010/117620 A2

GENE EXPRESSION ANALYSIS IN SINGLE CELLS

The application claims the benefit of priority to United States Provisional application serial No. 61/164,759, filed March 30, 2009, the entire contents of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to the analysis of gene expression in single cells. In particular, the invention relates to a method for preparing a cDNA library from a plurality of single cells, and to a cDNA library produced by this method. The cDNA libraries prepared by the method of the invention are suitable for analysis of gene expression by sequencing.

BACKGROUND OF THE INVENTION

The determination of the mRNA content of a cell or tissue (i.e. "gene expression profiling") provides a method for the functional analysis of normal and diseased tissues and organs. For example, gene expression profiling can be used in the study of embryogenesis; for the characterization of primary tumor samples; for the analysis of biopsies from diseased and normal tissue in, for example, psoriasis; for the comparative analysis of cell types from different species to delineate the evolution of development; as an assay system for diagnostics; as a quality control system in cell replacement therapy (i.e. to ensure that a culture of cells is sufficiently pure, and the cells are correctly differentiated); and as an *in vitro* tool to measure the effect of a transfected gene or siRNA on downstream targets in spite of less than 100% transfection efficiency.

Gene expression profiling is usually performed by isolating mRNA from tissue samples and subjecting this mRNA to microarray hybridization. However, such methods only allow previously known genes to be analyzed, and cannot be used to analyze alternative splicing, promoters and polyadenylation signals.

Therefore, direct sequencing of the all, or parts, of the mRNA content of a tissue is being increasingly used (Cloonan et al., Nat Methods 5(7):613-9 (2008)). However, current methods of analyzing the mRNA content of cells by direct sequencing rely on analyzing bulk mRNA obtained from tissue samples typically containing millions of cells. This means that much of the functional information present in single cells is lost or blurred when gene expression is analyzed in bulk mRNA. In addition, dynamic processes, such as the cell cycle,

cannot be observed in population averages. Similarly, distinct cell types in a complex tissue (e.g. the brain) can only be studied if cells are analyzed individually.

Gene expression in single cells has previously been analyzed using a variety of methods (see, for example, Brail et al., Mutat Res 406(2-4):45-54 (1999) ; Levsky et al., Science 297(5582):836-40 (2002); Bengtsson et al. Genome Res 15(10):1388-92 (2005); Esumi et al., Nat Genet 37(2):171-6 (2005). In particular, single cell gene expression in neural cells has been studied by microarray analysis (see Esumi et al. Neurosci Res 60(4):439-51 (2008)). However, these methods require that each single cell is analyzed individually and treated separately during the entire procedure, which is time-consuming and expensive. In addition, the preparation and amplification of samples from single cells independently potentially introduces cell-to-cell variation. Furthermore, as the cDNA of each cell must be amplified to an amount that can be reasonably handled for the subsequent analysis, there is potential amplification bias. For example, a single cell contains about 0.3 pg of mRNA, and at least a 300 ng is commonly needed for subsequent analysis by sequencing. Therefore, an amplification of at least a million-fold is required.

Additionally, microarrays have two major shortcomings: they are linked to known genes, and they have limited sensitivity and dynamic range. RNA sequencing (RNA-Seq) overcomes these problems by sequencing RNA directly (Ozsolak et al., Nature 461:814-818 (2009)) or after reverse-transcription to cDNA (Cloonan et al., Nat. Methods 5:613-619 (2008); Mortazavi et al., Nat. Methods 5:621-628 (2008); Wang et al., Nature 456:470-476 (2008)). Sequence reads are mapped to the genome to reveal sites of transcription, and quantitation is based simply on hit counts, with great sensitivity and nearly unlimited dynamic range.

Tissues are rarely homogeneous, however, and therefore any expression profile based on a tissue sample, biopsy or cell culture will confound the true expression profiles of its constituent cells. One way of getting around this problem would be to analyze single cells instead of cell populations, and indeed single-cell methods have been developed for both microarrays (Esumi et al., Neurosci. Res. 60:439-451 (2008) and Kurimoto et al., Nucleic Acids Res. 34:42 (2006)). These methods are suitable for the analysis of small numbers of single cells, and in particular may be used to study cells that are difficult to obtain in large numbers, such as oocytes and the cells of the early embryo. Cells may be isolated for example by laser capture microdissection, or by microcapillary, and marker genes may be used to locate cells of interest. However, single-cell transcriptomics must confront two great challenges. First, markers suitable for the prospective isolation of defined cell populations are not available for every cell

type, reflecting the fact that few cell types are clearly defined in molecular terms. Second, transcript abundances vary greatly from cell to cell. For example, (β-Actin (Actb) mRNA content varies more than three orders of magnitude between pancreatic islets cells (Bengtsson et al., Genome Res. 15:1388-1392 (2005)). Similar results have been reported, using a variety of detection methods, for RNA polymerase II (Raj et al., PLoS Biol 4:309 (2006)), GAPDH (Lagunavicius et al., RNA 15:765-771 (2009) and Warren et al., Proc. Natl. Acad. Sci. U.S.A. 103:17807-17812 (2006)), PU.1 (Warren et al., *supra*), and TBP, B2M, SDHA and EEIFG mRNAs (Taniguchi et al., Nat Methods 6:503-506 (2009)), and at present seems to be a common feature of the transcriptome.

Most of the variation may be intrinsic, caused by burst-like stochastic activation of transcription, where brief episodes of mRNA synthesis lasting a few minutes are separated by periods of transcriptional silence of similar duration (Chubb et al., Curr Biol 16:1018-1025 (2006)). Each burst would give rise to a dense population of mRNA in the nucleus, which is then exported to the cytoplasm and rapidly decays. As a consequence, a random sample of cells would show great variation in their content of particular mRNAs, ranging from those cells that have just undergone a burst, to those that have nearly completely degraded their mRNA; this has been directly observed for RNA polymerase II transcription *in situ* using a fluorescent probe targeting the 52-copy repeat in that gene (Raj et al., PLoS Biol 4:309 (2006)).

In summary, there are often no suitable cell-surface markers to use in isolating single cells for study, and even when there are, a small number of single cells is not sufficient to capture the range of natural variation in gene expression. The present invention aims to overcome, or reduce, these problems by providing a method of preparing cDNA libraries which can be used to analyze gene expression in a plurality of single cells.

SUMMARY OF THE INVENTION

The invention provides method for preparing a cDNA library from a plurality of single cells. In one aspect, the method includes the steps of releasing mRNA from each single cell to provide a plurality of individual mRNA samples, synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, pooling the tagged cDNA samples and amplifying the pooled cDNA samples to generate a cDNA library having double-stranded cDNA. The invention also provides a cDNA library produced by the methods described herein. The

invention further provides methods for analyzing gene expression in a plurality of cells by preparing a cDNA library as described herein and sequencing the library.

BRIEF DESCRIPTION OF THE DRAWINGS

The figures are intended to illustrate broad concepts of the invention by reference to representative examples for ease of discussion. They are not intended to limit the scope of the invention by showing one out of several alternate embodiments or by showing or omitting optional features of the invention.

Figure 1, panels A-F, show an overview of a method of analyzing gene expression in a plurality of single cells. (A) the tissue of interest is dissected; (B) a plurality of single cells are selected; (C) single cells are placed in separate wells of a 96-well plate and lysed; tagged reverse transcription is performed on each sample to produce cDNA; (D) cDNA samples are pooled and amplified; (E) sequencing is performed to obtain 100 million reads; and (F) identification of expressed genes and identification of cells from which they originated.

Figure 2 shows the synthesis of cDNA by template switching.

Figure 3 shows an example of a template switching oligonucleotide comprising a 5' amplification primer sequence (APS), a cell tag and a 3' sequence for template switching.

Figure 4 shows an example of a cDNA synthesis primer (CDS) comprising a 5' amplification primer sequence (APS), a cell tag and a 3' RNA complementary sequence (RCS).

Figure 5 shows the visualization of cDNA samples L001 and L002 following full-length cDNA amplification by PCR. Lane 1: 100 bp marker ladder; lanes 2-3: 25 cycles; lanes 4-5: 30 cycles; lanes 6-7: 35 cycles. Even lanes contain sample L001 and odd lanes contain sample L002.

Figure 6 shows a dilution series from a test PCR using sample L001 (lanes 3-10). Lanes 2 and 11 contain a 100 bp ladder as a size marker.

Figure 7, panels A and B, show the gel electrophoresis separation and isolation of cDNA libraries. Panel A shows a cDNA library following final amplification by PCR (16 cycles) (lanes 5 and 6). Panel B shows the 125-200 bp region has been excised. Lanes 3 and 8 contain 100 bp ladder as a size marker.

Figure 8 shows an example of a sequenced cDNA molecule from a tagged cDNA library. The primer sequences for SOLiD sequencing (P1 and P2) are underlined. The cell-specific tag is boxed. The 2-5 Gs from the template-switching mechanism are shaded by a grey box. Sequence from the TOPO cloning vector is shown in italics. The insert in this case is tubulin beta 2c.

Figure 9 shows a graphical representation of results from a quantitative real-time PCR comparing bulk cDNA (horizontal axis) *versus* 96-cell tagged cDNA (vertical axis). Each circle represents a PCR primer pair directed against the indicated genes. The units are arbitrary and are derived from the cycles-to-threshold value, C_T.

Figure 10, panels A-E, show an overview of the single-cell tagged reverse transcription (STRT) method and exemplary results. (A) Overview of the method, illustrating how single cells were tracked. Well-specific (and hence cell-specific) barcodes were incorporated during cDNA synthesis, resulting in a library where each molecule carried a barcode identifying the cell of origin. (B) Example of reads mapped to both strands of the 5 kb Pou5f1 locus, shown as a coverage plot. Reads were strand-specific and mapped mostly to exons. Non-mRNA background was minimal as judged by hits to introns on the forward strand (top lane) or to the reverse strand (bottom lane). (C) Comparison with standard RNA-Seq (Cloonan et al. Nat. Methods 5:613-619 (2008)) for the 6 kb Nanog locus. Since cDNA synthesis was primed from the poly(A) tail, reads were typically clustered at the 3' end (top lane). The extended 3' UTR of Nanog (light blue) was clearly detected by both methods. SQRL reads extended to 5' exons, but showed more intronic background. (D) The mitochondrial genome. As expected, transcription was nearly exclusively detected on the H strand (top), with only a few truncated transcripts (arrow) originating on the L strand. Protein-coding genes are indicated on the bottom. (E) Gene expression on chromosome 19. Coverage on the forward (top lane) and reverse (middle) strands was highly correlated with local gene density (bottom). Genes are shown as horizontal stacked bars. For clarity, the vertical scales in (B-E) are truncated at half maximal values.

Figure 11, panels A-F, show graphical representation of steps optionally used in the STRT method. Panel A shows a reverse transcription step. A tailed oligo-dT primer directs synthesis of a cDNA strand. When the end of the template RNA is reached, reverse transcriptase adds 3-4 Cs at 3' of cDNA strand (due to its terminal transferase activity). Panel B shows a template switching step. Barcoded helper oligo anneals transiently, and cDNA synthesis proceeds using the oligo as template. As a consequence, the 3' end of the cDNA will carry barcode (XXXX),

BtsCI recognition (Bts) and primer annealing (Pr) sequences. Panel C shows a single primer PCR step. The ends of the cDNA have identical sequences and are amplified using a single PCR primer, which helps suppress short amplicons. Panel D shows a fragmentation step. The amplified library is fragmented to 200 - 300 bp using controlled DNase digestion. Panel E shows an immobilization and end-repair step. Both 5' (with barcode (Br) and BtsCI site (Bts)) and 3' fragments are bound to beads, while internal fragments are washed away. Panel F shows fragment release and adapter ligation. 5' fragments are released by BtsCI digestion, leaving just the barcode (Bc) and insert (white area). 3' fragments remain stuck on the beads. Genome Analyzer paired-end compatible adapters (P1 and P2) are ligated. The library is sequenced from the P1 primer (and could optionally be sequenced also from P2). P1 reads begin with a 5 bp barcode, followed by 3-4 Gs, followed by the cDNA insert. P2 reads would yield only cDNA sequence.

Figure 12, panels A-D, show a graphical representation of an absence of motifs surrounding the template-switching site. All reads were examined in sample I 006 for the presence of any motif around the template-switching site (that is, around the 5' end of each read). Sequence logos are shown for the 20 bases of genomic sequence upstream and downstream of the first nucleotide (arrow) of each mapped read. As exemplified in A and B, in typical cases (92 of 96), no strong motif was detected, indicating an absence of significant mispriming events, which would have generated an upstream motif complementary to the primer. In four cases (C and D), there was a general preference for T-rich sequences particularly in the first 20 bases of the read. This occurred in wells with very small numbers of reads, indicating a failed reaction. However, in a single case the T-rich motif was observed despite a large number of reads.

Figure 13, panels A and B, show a graphical representation of hotspots for template switching. (A) The Actb locus is expressed from the lower (reverse) strand, right to left in the figure. The top two tracks show aggregate hits on the forward and reverse strands, respectively, demonstrating strand specificity and lack of background in introns. The middle (blue) track shows the exon/intron structure of the gene. The lower track shows individual hits from single cells. Each pixel row represents the hits from a single cell as black dots. There are 96 pixel rows altogether. (B) The same analysis was done for Sox2, a single-exon gene transcribed on the forward (upper) strand, showing the usual 3' bias. In both (A) and (B), hotspots were clearly visible, and were shared among cells, suggesting they represent structural sites on mRNA that favor termination of cDNA synthesis, RNA hydrolysis and/or template switching.

Figure 14, panels A and B, show a graphical representation of the new discovery rate. (A) shows the rate of discovery of distinct mapping reads as a function of the total number of reads. None of the samples were sequenced to saturation, and most libraries would likely contain at least 3 million distinct molecules, indicating that on average at least 30,000 distinct molecules per cell were successfully converted to amplifiable cDNA. The curves are wiggly due to inhomogeneities in the data, presumably caused by imperfections in the cluster PCR process that can generate local duplicates and thus less than random sampling. In (B) the rate of discovery of distinct annotated features is shown as a function of the number of mapped reads (for sample L006). Saturation was reached quickly, showing that most features present in the sample could be discovered at modest sampling depth.

Figure 15 shows a graphical representation for distinguishing expression of overlapping genes. Because of the strand-specificity of the template-switching mechanism, strandedness could be maintained throughout the protocol. This was especially important for genes with overlapping exons. Represented in the figure is an example of such a pair of genes, Cathepsin A (Ctsa) and Phospholipid transfer protein (Pltp), whose last exons overlap. Without strand information, the reads in the last four exons of Pltp could not be distinguished from the reads originating in the last exon of Ctsa. There are about 3,000 genes with similar overlapping 3' exons.

Figure 16 shows a graphical representation of length bias for transcripts. In order to detect any bias against short or long transcripts, the average expression level was calculated as a function of mRNA length (in 200 bp bins) for sample L019. Each bar shows the expression level of genes with transcripts shorter than the indicated length (thus the first bar contains transcripts 0 - 200 bp long). Over a wide range of mRNA length, there was no apparent difference in measured expression levels. The shortest transcripts (<200 bp) were presumably suppressed by the gel purification step where inserts >100 bp were selected. The apparent overexpression of genes in the 400 - 800 bp range can possibly be explained by a more efficient template switching in this range, where cDNA synthesis would often reach the 5' cap of mRNA. Alternatively, it can simply be due to the presence of a few very highly expressed genes in this range, including Dppa5 and Rps14.

Figure 17, panels A-E, show a graphical representation of the quantitative accuracy of the STRT method. (A) Distribution of gene expression levels, in transcripts per million (t.p.m.) showed predominantly low expression, in the 10 - 100 t.p.m. range. (B) Comparison of two cells sequence to a depth of approximately 500 000 reads/cell. In this case, genes below 100 t.p.m. could be accurately quantified. (C) Comparison of two cells sequenced to

approximately 100 000 reads/cell. In this case, sensitivity dropped to about 1000 t.p.m. (D) Probability of detection as a function of expression level. Each dot shows a gene, with a given average expression level (across all cells) and fraction of cells having non-zero expression of this gene. The distribution approach the theoretical limit of random sampling given the actual depth of sequencing used here (dashed line). (E) Comparison with quantitative real-time PCR for the four ES cells shown in (B) and (C), using select markers of pluripotency and differentiation. In general, the quantitative accuracy was good, with a single putative false positive (Eomes in ES Cell #4). However this was a rare event, and was not observed for this gene in any of the other 160 ES cells examined. Q-PCR levels were converted to t.p.m. by normalization of the Actb/1081 primer pair with ES cells #1 and #2. Actin was then independently measured using a different primer pair (Actb/1832) to confirm the accuracy of the normalization.

Figure 18 shows a graphical comparison between STRT, Q-PCR and microarray analyses. Genes expected to be expressed (Actb, Pou5f1, Zfp42, Sox2, Klf4, Nanog, Plk1, Zic3) or not expressed (Gata4, Brachyury, Eomes, Otx1, Cdx2, Gata5, Calb1, Gfap, Dppa3 and NeuroD1) in undifferentiated ES cells were analyzed by STRT, quantitative real-time PCR (Q-PCR) and Illumina microarray. There was good correlation between STRT and Q-PCR, and to a lesser extent with microarray data. In particular, Sox2 was undercalled on the microarray, while Otx1 and Dppa3 were apparent false positives. The microarray data is the mean of two hybridization reactions, Q-PCR was performed in duplicate and repeated once for confirmation, and the STRT data is the mean of 160 single ES cells.

Figure 19, panels A and B, show a graphical representation of gene expression distribution across cells. (A) shows the distribution of Actb expression levels across all cells using STRT. (B) shows Actb expression in pancreatic islet cells from Bengtsson et al. Genome Res 15(10):1388-92 (2005) for comparison.

Figure 20 shows a principal component analysis. In order to discover and cluster cell types based on expression data, the five independently prepared 96-cell samples were subjected to principal component analysis. The three types of cells (ES, Neuro-2A and MEF) clearly clustered separately, although MEFs did not form a very distinct cluster. Moreover, ES cells prepared independently clustered together, showing that the PCA did not simply pick up sample preparation differences. This demonstrates that single-cell expression data can be used to accurately classify cell types.

Figure 21, panels A-C, show a graph-based visualization ("cell map") of the expression pattern. (A) Cells, represented by graph nodes (circles) were laid out randomly, and edges were drawn from each cell to the five other cells it was most highly correlated with. (B) Force-directed layout was used to lay out the graph on a plane. In this layout, cells repelled each other uniformly, but were held together by edges acting as elastic springs. The resulting visual map was consistent with known cell identities (ES cells vs Neuro-2A cells), with some less deeply sequenced cells showing poor separation. (C) Adding more ES cells as well as fibroblasts (MEFs) expanded the map and demonstrated that independently prepared ES cells grouped together accurately.

Figure 22 shows the visualizing of gene expression on a cell map of **Figure 21**. Each map retains its layout from **Figure 21C**, but cells are shaded according to expression of the indicated gene. A logarithmic scale was used (upper right). Mitochondrial ribosomal RNA 2 (mt_Rnr2) was the highest-expressed gene of all. Housekeeping genes such as Actin (Actb) and ribosomal protein L4 (Rpl4) were detected in all cell types, but not in every single cell. The power of shotgun single cell expression profiling was revealed for low-expressed genes like K-ras (Kras), which was detected only in approximately half of all cells, but still clearly expressed in all cell types. Calbindin (Calb1) was absent, as expected and confirmed by Q-PCR. A set of well-known ES cell markers (Dppa5, Sox2, Sall4, Pou5f1, Nanog, Zfp42, Zic3 and Esrrb) were clearly expressed specifically in the ES cell cluster, whereas Klf4, Myc and Klf2 were more widely distributed. Dppa3 was not detected, as confirmed by Q-PCR (**Figure 18**)

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for the analysis of gene expression in single cells or in a plurality of single cells. In particular, the invention provides methods for preparing a cDNA library from a plurality of single cells. The methods are based on determining gene expression levels from a population of individual cells, which can be used to identify natural variations in gene expression on a cell by cell level. The methods can also be used to identify and characterize the cellular composition of a population of cells in the absence of suitable cell-surface markers. The methods described herein also provide the advantage of generating a cDNA library representative of RNA content in a cell population by using single cells, whereas cDNA libraries prepared by classical methods typically require total RNA isolated from a large population (see Example 1). Thus, a cDNA library produced using the methods of the invention provide at least equivalent representation of RNA content in a

population of cells by utilizing a smaller subpopulation of individual cells along with additional advantages as described herein.

Embodiments of the invention also provide sampling of a large number of single cells. Using similarity of expression patterns, a map of cells can be built showing how the cells relate. This map can be used to distinguish cell types *in silico*, by detecting clusters of closely related cells (see Example II). By sampling not just a few, but large numbers of single cells, similarity of expression patterns can be used to build a map of cells and how they are related. This method permits access to undiluted expression data from every distinct type of cell present in a population, without the need for prior purification of those cell types. In addition, where known markers are available, these can be used *in silico* to delineate cells of interest. The validity of this approach is shown in Example II, which analyzes a collection of cells sampled from three distinct cell types (mouse embryonic stem cells, embryonic fibroblasts and neuroblastoma cells) of distinct embryonic origins (pluripotent stem cells vs. mesodermal and ectodermal germ layers) and disease state (normal vs. transformed).

Embodiments of the invention provide a method of preparing a cDNA library from a plurality of single cells by releasing mRNA from each single cell to provide a plurality of individual samples, wherein the mRNA in each individual mRNA sample is from a single cell, synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell pooling the tagged cDNA samples and amplifying the pooled cDNA samples to generate a cDNA library comprising double-stranded cDNA. By utilizing the above method, it is feasible to prepare samples for sequencing from several hundred single cells in a short time and with a minimal amount of work. Traditional methods for preparing a fragment library from RNA for sequencing include gel excision steps that are laborious. In the absence of special equipment, it is not convenient to prepare more than a handful of samples in parallel. In some aspects of the methods described herein, a set of 96 cells is prepared as a single sample (after cDNA synthesis), which makes it feasible to prepare several hundred cells for sequencing. Additionally, technical variation is minimized because each set of 96 cells is prepared together (in a single tube).

In some aspects of the invention, each cDNA sample obtained from a single cell is tagged, which allows gene expression to be analyzed at the level of a single cell. This allows dynamic processes, such as the cell cycle, to be studied and distinct cell types in a complex tissue (e.g.

the brain) to be analyzed. In some aspects of the invention, the cDNA samples can be pooled prior to analysis. Pooling the samples simplifies handling of the samples from each single cell and reduces the time required to analyze gene expression in the single cells, which allows for high throughput analysis of gene expression. Pooling of the cDNA samples prior to amplification also provides the advantage that technical variation between samples is virtually eliminated. In addition, as the cDNA samples are pooled before amplification, less amplification is required to generate sufficient amounts of cDNA for subsequent analysis compared to amplifying and treating cDNA samples from each single cell separately. This reduces amplification bias, and also means that any bias will be similar across all the cells used to provide pooled cDNA samples. RNA purification, storage and handling are also not required, which helps to eliminate problems caused by the unstable nature of RNA.

As the cDNA libraries produced by the method of the invention are suitable for the analysis the gene expression profiles of single cells by direct sequencing, it is possible to use these libraries to study the expression of genes which were not previously known, and also to analyze alternative splicing, promoters and polyadenylation signals. Preparing the cDNA libraries as described herein, provides for a sensitive method for detecting a single or low copy RNA transcript. The sensitivity of the method is shown in **Figure 17D** and described in Example II. For example, genes expressed at 100 transcripts per million (t.p.m.) are detected about 50% of the time. However, as shown in **Figure 14A**, the samples were not saturated, so there is additional sensitivity that is achievable with deeper sequencing of the samples. Accordingly, the method for preparing the cDNA libraries as described herein detect a single or low copy RNA transcript at least 30% of the time, or alternatively at least 40% of the time, or at least 50% of the time, or alternatively at least 60% of the time, or alternatively at least 70 % of the time, or alternatively at least 80% of the time or alternatively at least 90% of the time, or alternatively at least 95% of the time.

Embodiments of the invention also provide a method for identifying a single cell type out of a sample and/or determining the transcriptome of a single cell by preparing a cDNA library as described herein, determining the expression levels of individual cells in a population, and mapping of the individual cells based on similarity of expression patterns. Mapping of individual cells can be done *in silico* by one of skill in the art and in particular utilizing the methods described herein, such as shown in Example II. The number of cells needed to determine the frequency of a given cell type in the plurality of cells will follow a binomial distribution. For example, a predetermined number of individual cells can be sampled so that

at least ten of the desired type are expected to be detected. Accordingly, if the frequency of the cell type in the sample is 10%, a cDNA library from approximately 100 cells will need to be prepared and analyzed as described herein.

The term "cDNA library" refers to a collection of cloned complementary DNA (cDNA) fragments, which together constitute some portion of the transcriptome of a single cell or a plurality of single cells. cDNA is produced from fully transcribed mRNA found in a cell and therefore contains only the expressed genes of a single cell or when pooled together the expressed genes from a plurality of single cells.

As used herein, a "plurality" refers to a population of cells and can include any number of cells desired to be analyzed. In some aspects of the invention, a plurality of cells includes at least 10 cells, or alternatively at least 25 cells, or alternatively at least 50 cells, or alternatively at least 100 cells, or alternatively at least 200 cells, or alternatively at least 500 cells, or alternatively at least 1000 cells, or alternatively 5,000 cells or alternatively 10,000 cells. In another aspect of the invention, a plurality of cells includes from 10 to 100 cells, or alternatively from 50 to 200 cells, alternatively from 100 to 500 cells, or alternatively from 100 to 1000, or alternatively from 1,000 to 5,000 cells.

The expression "amplification" or "amplifying" refers to a process by which extra or multiple copies of a particular polynucleotide are formed. Amplification includes methods such as PCR, ligation amplification (or ligase chain reaction, LCR) and amplification methods. These methods are known and widely practiced in the art. See, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Innis et al., "PCR protocols: a guide to method and applications" Academic Press, Incorporated (1990) (for PCR); and Wu et al. (1989) Genomics 4:560-569 (for LCR). In general, the PCR procedure describes a method of gene amplification which is comprised of (i) sequence-specific hybridization of primers to specific genes within a DNA sample (or library), (ii) subsequent amplification involving multiple rounds of annealing, elongation, and denaturation using a DNA polymerase, and (iii) screening the PCR products for a band of the correct size. The primers used are oligonucleotides of sufficient length and appropriate sequence to provide initiation of polymerization, i.e. each primer is specifically designed to be complementary to each strand of the genomic locus to be amplified.

Reagents and hardware for conducting amplification reaction are commercially available. Primers useful to amplify sequences from a particular gene region are preferably complementary to, and hybridize specifically to sequences in the target region or in its flanking

regions and can be prepared using the polynucleotide sequences provided herein. Nucleic acid sequences generated by amplification can be sequenced directly.

When hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides, the reaction is called "annealing" and those polynucleotides are described as "complementary". A double-stranded polynucleotide can be complementary or homologous to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the second. Complementarity or homology (the degree that one polynucleotide is complementary with another) is quantifiable in terms of the proportion of bases in opposing strands that are expected to form hydrogen bonding with each other, according to generally accepted base-pairing rules.

As used herein, a "single cell" refers to one cell. Single cells useful in the methods described herein can be obtained from a tissue of interest, or from a biopsy, blood sample, or cell culture. Additionally, cells from specific organs, tissues, tumors, neoplasms, or the like can be obtained and used in the methods described herein. Furthermore, in general, cells from any population can be used in the methods, such as a population of prokaryotic or eukaryotic single celled organisms including bacteria or yeast. In some aspects of the invention, the method of preparing the cDNA library can include the step of obtaining single cells. A single cell suspension can be obtained using standard methods known in the art including, for example, enzymatically using trypsin or papain to digest proteins connecting cells in tissue samples or releasing adherent cells in culture, or mechanically separating cells in a sample. Single cells can be placed in any suitable reaction vessel in which single cells can be treated individually. For example a 96-well plate, such that each single cell is placed in a single well.

Methods for manipulating single cells are known in the art and include fluorescence activated cell sorting (FACS), micromanipulation and the use of semi-automated cell pickers (e.g. the Quixell™ cell transfer system from Stoelting Co.). Individual cells can, for example, be individually selected based on features detectable by microscopic observation, such as location, morphology, or reporter gene expression.

In some aspects of the invention, mRNA can be released from the cells by lysing the cells. Lysis can be achieved by, for example, heating the cells, or by the use of detergents or other chemical methods, or by a combination of these. However, any suitable lysis method known in the art can be used. A mild lysis procedure can advantageously be used to prevent the release of nuclear chromatin, thereby avoiding genomic contamination of the cDNA library, and to

minimise degradation of mRNA. For example, heating the cells at 72°C for 2 minutes in the presence of Tween-20 is sufficient to lyse the cells while resulting in no detectable genomic contamination from nuclear chromatin. Alternatively, cells can be heated to 65°C for 10 minutes in water (Esumi et al., Neurosci Res 60(4):439-51 (2008)); or 70°C for 90 seconds in PCR buffer II (Applied Biosystems) supplemented with 0.5% NP-40 (Kurimoto et al., Nucleic Acids Res 34(5):e42 (2006)); or lysis can be achieved with a protease such as Proteinase K or by the use of chaotropic salts such as guanidine isothiocyanate (U.S. Publication No. 2007/0281313).

Synthesis of cDNA from mRNA in the methods described herein can be performed directly on cell lysates, such that a reaction mix for reverse transcription is added directly to cell lysates. Alternatively, mRNA can be purified after its release from cells. This can help to reduce mitochondrial and ribosomal contamination. mRNA purification can be achieved by any method known in the art, for example, by binding the mRNA to a solid phase. Commonly used purification methods include paramagnetic beads (e.g. Dynabeads). Alternatively, specific contaminants, such as ribosomal RNA can be selectively removed using affinity purification.

cDNA is typically synthesized from mRNA by reverse transcription. Methods for synthesizing cDNA from small amounts of mRNA, including from single cells, have previously been described (Kurimoto et al., Nucleic Acids Res 34(5):e42 (2006); Kurimoto et al., Nat Protoc 2(3):739-52 (2007); and Esumi et al., Neurosci Res 60(4):439-51 (2008)). In order to generate an amplifiable cDNA, these methods introduce a primer annealing sequence at both ends of each cDNA molecule in such a way that the cDNA library can be amplified using a single primer. The Kurimoto method uses a polymerase to add a 3' poly-A tail to the cDNA strand, which can then be amplified using a universal oligo-T primer. In contrast, the Esumi method uses a template switching method to introduce an arbitrary sequence at the 3' end of the cDNA, which is designed to be reverse complementary to the 3' tail of the cDNA synthesis primer. Again, the cDNA library can be amplified by a single PCR primer. Single-primer PCR exploits the PCR suppression effect to reduce the amplification of short contaminating amplicons and primer-dimers (Dai et al., J Biotechnol 128(3):435-43 (2007)). As the two ends of each amplicon are complementary, short amplicons will form stable hairpins, which are poor templates for PCR. This reduces the amount of truncated cDNA and improves the yield of longer cDNA molecules.

In some aspects of the invention, the synthesis of the first strand of the cDNA can be directed by a cDNA synthesis primer (CDS) that includes an RNA complementary sequence (RCS). In some aspects of the invention, the RCS is at least partially complementary to one or more mRNA in an individual mRNA sample. This allows the primer, which is typically an oligonucleotide, to hybridize to at least some mRNA in an individual mRNA sample to direct cDNA synthesis using the mRNA as a template. The RCS can comprise oligo (dT), or be gene family-specific, such as a sequence of nucleic acids present in all or a majority related genes, or can be composed of a random sequence, such as random hexamers. To avoid the CDS priming on itself and thus generating undesired side products, a non-self-complementary semi-random sequence can be used. For example, one letter of the genetic code can be excluded, or a more complex design can be used while restricting the CDS to be non-self-complementary.

The terms "oligonucleotide" and "polynucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and can perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this invention that comprises a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. Thus, the term polynucleotide sequence is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

A "primer" is a short polynucleotide, generally with a free 3'-OH group that binds to a target or template potentially present in a sample of interest by hybridizing with the target, and thereafter promoting polymerization of a polynucleotide complementary to the target. Primers

of the instant invention are comprised of nucleotides ranging from 17 to 30 nucleotides. In one aspect, the primer is at least 17 nucleotides, or alternatively, at least 18 nucleotides, or alternatively, at least 19 nucleotides, or alternatively, at least 20 nucleotides, or alternatively, at least 21 nucleotides, or alternatively, at least 22 nucleotides, or alternatively, at least 23 nucleotides, or alternatively, at least 24 nucleotides, or alternatively, at least 25 nucleotides, or alternatively, at least 26 nucleotides, or alternatively, at least 27 nucleotides, or alternatively, at least 28 nucleotides, or alternatively, at least 29 nucleotides, or alternatively, at least 30 nucleotides, or alternatively at least 50 nucleotides, or alternatively at least 75 nucleotides or alternatively at least 100 nucleotides.

The RCS can also be at least partially complementary to a portion of the first strand of cDNA, such that it is able to direct the synthesis of a second strand of cDNA using the first strand of the cDNA as a template. Thus, following first strand synthesis, an RNase enzyme (e.g. an enzyme having RNaseH activity) can be added after synthesis of the first strand of cDNA to degrade the RNA strand and to permit the CDS to anneal again on the first strand to direct the synthesis of a second strand of cDNA. For example, the RCS could comprise random hexamers, or a non-self complementary semi-random sequence (which minimizes self-annealing of the CDS).

A template-switching oligonucleotide (TSO) that includes a portion which is at least partially complementary to a portion of the 3' end of the first strand of cDNA can be added to each individual mRNA sample in the methods described herein. Such a template switching method is described in (Esumi et al., Neurosci Res 60(4):439-51 (2008)) and allows full length cDNA comprising the complete 5' end of the mRNA to be synthesized. As the terminal transferase activity of reverse transcriptase typically causes 2-5 cytosines to be incorporated at the 3' end of the first strand of cDNA synthesized from mRNA, the first strand of cDNA can include a plurality of cytosines, or cytosine analogues that base pair with guanosine, at its 3' end (see US 5,962,272). In one aspect of the invention, the first strand of cDNA can include a 3' portion comprising at least 2, at least 3, at least 4, at least 5 or 2, 3, 4, or 5 cytosines or cytosine analogues that base pair with guanosine. A non-limiting example of a cytosine analogue that base pairs with guanosine is 5-aminoallyl-2'-deoxycytidine.

In one aspect of the invention, the TSO can include a 3' portion comprising a plurality of guanosines or guanosine analogues that base pair with cytosine. Non-limiting examples of guanosines or guanosine analogues useful in the methods described herein include, but are not limited to, deoxyriboguanosine, riboguanosine, locked nucleic acid-guanosine, and peptide

nucleic acid-guanosine. The guanosines can be ribonucleosides or locked nucleic acid monomers.

A locked nucleic acid (LNA) is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation. Some of the advantages of using LNAs in the methods of the invention include increasing the thermal stability of duplexes, increased target specificity and resistance from exo- and endonucleases.

A peptide nucleic acid (PNA) is an artificially synthesized polymer similar to DNA or RNA, wherein the backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The backbone of a PNA is substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This provides two non-limiting advantages. First, the PNA backbone exhibits improved hybridization kinetics. Secondly, PNAs have larger changes in the melting temperature (Tm) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4° C drop in Tm for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9° C. This can provide for better sequence discrimination. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration.

A nucleic acid useful in the invention can contain a non-natural sugar moiety in the backbone. Exemplary sugar modifications include but are not limited to 2' modifications such as addition of halogen, alkyl, substituted alkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, SO_2CH_3, OSO_2, SO_3, CH_3, ONO_2, NO_2, N_3, NH_2, substituted silyl, and the like. Similar modifications can also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Nucleic acids, nucleoside analogs or nucleotide analogs having sugar modifications can be further modified to include a reversible blocking group, peptide linked label or both. In those embodiments where the above-described 2' modifications are present, the base can have a peptide linked label.

A nucleic acid used in the invention can also include native or non-native bases. In this regard a native deoxyribonucleic acid can have one or more bases selected from the group consisting of adenine, thymine, cytosine or guanine and a ribonucleic acid can have one or more bases selected from the group consisting of uracil, adenine, cytosine or guanine. Exemplary non-

native bases that can be included in a nucleic acid, whether having a native backbone or analog structure, include, without limitation, inosine, xathanine, hypoxathanine, isocytosine, isoguanine, 5-methylcytosine, 5-hydroxymethyl cytosine, 2-aminoadenine, 6-methyl adenine, 6-methyl guanine, 2-propyl guanine, 2-propyl adenine, 2-thiolJracil, 2-thiothymine, 2-thiocytosine, 15 -halouracil, 15 -halocytosine, 5-propynyl uracil, 5-propynyl cytosine, 6-azo uracil, 6-azo cytosine, 6-azo thymine, 5-uracil, 4-thiouracil, 8-halo adenine or guanine, 8-amino adenine or guanine, 8-thiol adenine or guanine, 8-thioalkyl adenine or guanine, 8-hydroxyl adenine or guanine, 5-halo substituted uracil or cytosine, 7-methylguanine, 7-methyladenine, 8-azaguanine, 8-azaadenine, 7-deazaguanine, 7-deazaadenine, 3-deazaguanine, 3-deazaadenine or the like. A particular embodiment can utilize isocytosine and isoguanine in a nucleic acid in order to reduce non-specific hybridization, as generally described in U.S. Pat. No. 5,681,702.

A non-native base used in a nucleic acid of the invention can have universal base pairing activity, wherein it is capable of base pairing with any other naturally occurring base. Exemplary bases having universal base pairing activity include 3-nitropyrrole and 5-nitroindole. Other bases that can be used include those that have base pairing activity with a subset of the naturally occurring bases such as inosine, which basepairs with cytosine, adenine or uracil.

In one aspect of the invention, the TSO can include a 3' portion including at least 2, at least 3, at least 4, at least 5, or 2, 3, 4, or 5, or 2-5 guanosines, or guanosine analogues that base pair with cytosine. The presence of a plurality of guanosines (or guanosine analogues that base pair with cytosine) allows the TSO to anneal transiently to the exposed cytosines at the 3' end of the first strand of cDNA. This causes the reverse transcriptase to switch template and continue to synthesis a strand complementary to the TSO. In one aspect of the invention, the 3' end of the TSO can be blocked, for example by a 3' phosphate group, to prevent the TSO from functioning as a primer during cDNA synthesis.

In one aspect of the invention, the mRNA is released from the cells by cell lysis. If the lysis is achieved partially by heating, then the CDS and/or the TSO can be added to each individual mRNA sample during cell lysis, as this will aid hybridization of the oligonucleotides. In some aspects, reverse transcriptase can be added after cell lysis to avoid denaturation of the enzyme.

In some aspects of the invention, a tag can be incorporated into the cDNA during its synthesis. For example, the CDS and/or the TSO can include a tag, such as a particular nucleotide

sequence, which can be at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15 or at least 20 nucleotides in length. For example, the tag can be a nucleotide sequence of 4-20 nucleotides in length, e.g. 4, 5, 6, 7, 8, 9, 10, 15 or 20 nucleotides in length. As the tag is present in the CDS and/or the TSO it will be incorporated into the cDNA during its synthesis and can therefore act as a "barcode" to identify the cDNA. Both the CDS and the TSO can include a tag. The CDS and the TSO can each include a different tag, such that the tagged cDNA sample comprises a combination of tags. Each cDNA sample generated by the above method can have a distinct tag, or a distinct combination of tags, such that once the tagged cDNA samples have been pooled, the tag can be used to identify which single cell from each cDNA sample originated. Thus, each cDNA sample can be linked to a single cell, even after the tagged cDNA samples have been pooled in the methods described herein.

Before the tagged cDNA samples are pooled, synthesis of cDNA can be stopped, for example by removing or inactivating the reverse transcriptase. This prevents cDNA synthesis by reverse transcription from continuing in the pooled samples. The tagged cDNA samples can optionally be purified before amplification, ether before or after they are pooled.

The pooled cDNA samples can be amplified by polymerase chain reaction (PCR) including emulsion PCR and single primer PCR in the methods described herein. For example, the cDNA samples can be amplified by single primer PCR. The CDS can comprise a 5' amplification primer sequence (APS), which subsequently allows the first strand of cDNA to be amplified by PCR using a primer that is complementary to the 5' APS. The TSO can also comprise a 5' APS, which can be at least 70% identical, at least 80% identical, at least 90% identical, at least 95% identical, or 70%, 80%, 90% or 100% identical to the 5' APS in the CDS. This means that the pooled cDNA samples can be amplified by PCR using a single primer (i.e. by single primer PCR), which exploits the PCR suppression effect to reduce the amplification of short contaminating amplicons and primer-dimers (Dai et al., J Biotechnol 128(3):435-43 (2007)). As the two ends of each amplicon are complementary, short amplicons will form stable hairpins, which are poor templates for PCR. This reduces the amount of truncated cDNA and improves the yield of longer cDNA molecules. The 5' APS can be designed to facilitate downstream processing of the cDNA library. For example, if the cDNA library is to be analyzed by a particular sequencing method, e.g. Applied Biosystems' SOLiD sequencing technology, or Illumina's Genome Analyzer, the 5' APS can be designed to be identical to the primers used in these sequencing methods. For example, the 5' APS can be

identical to the SOLiD P1 primer, and/or a SOLiD P2 sequence inserted in the CDS, so that the P1 and P2 sequences required for SOLiD sequencing are integral to the amplified library.

Another exemplary method for amplifying pooled cDNA includes PCR. PCR is a reaction in which replicate copies are made of a target polynucleotide using a pair of primers or a set of primers consisting of an upstream and a downstream primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme. Methods for PCR are well known in the art, and taught, for example in MacPherson et al. (1991) PCR 1 : A Practical Approach (IRL Press at Oxford University Press). All processes of producing replicate copies of a polynucleotide, such as PCR or gene cloning, are collectively referred to herein as replication. A primer can also be used as a probe in hybridization reactions, such as Southern or Northern blot analyses.

For emulsion PCR, an emulsion PCR reaction is created by vigorously shaking or stirring a "water in oil" mix to generate millions of micron-sized aqueous compartments. The DNA library is mixed in a limiting dilution either with the beads prior to emulsification or directly into the emulsion mix. The combination of compartment size and limiting dilution of beads and target molecules is used to generate compartments containing, on average, just one DNA molecule and bead (at the optimal dilution many compartments will have beads without any target) To facilitate amplification efficiency, both an upstream (low concentration, matches primer sequence on bead) and downstream PCR primers (high concentration) are included in the reaction mix. Depending on the size of the aqueous compartments generated during the emulsification step, up to $3x10^9$ individual PCR reactions per μl can be conducted simultaneously in the same tube. Essentially each little compartment in the emulsion forms a micro PCR reactor. The average size of a compartment in an emulsion ranges from sub-micron in diameter to over a 100 microns, depending on the emulsification conditions.

"Identity," "homology" or "similarity" are used interchangeably and refer to the sequence similarity between two nucleic acid molecules. Identity can be determined by comparing a position in each sequence which can be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of identity between sequences is a function of the number of matching or identical positions shared by the sequences. An unrelated or non-homologous sequence shares less than 40% identity, or alternatively less than 25% identity, with one of the sequences of the present invention.

A polynucleotide has a certain percentage (for example, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent sequence identity or homology can be determined using software programs known in the art, for example those described in Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., (1993). Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the National Center for Biotechnology Information.

The method of preparing a cDNA library described herein can further comprise processing the cDNA library to obtain a library suitable for sequencing. As used herein, a library is suitable for sequencing when the complexity, size, purity or the like of a cDNA library is suitable for the desired screening method. In particular, the cDNA library can be processed to make the sample suitable for any high-throughout screening methods, such as Applied Biosystems' SOLiD sequencing technology, or Illumina's Genome Analyzer. As such, the cDNA library can be processed by fragmenting the cDNA library (e.g. with DNase) to obtain a short-fragment 5'-end library. Adapters can be added to the cDNA, e.g. at one or both ends to facilitate sequencing of the library. The cDNA library can be further amplified, e.g. by PCR, to obtain a sufficient quantity of cDNA for sequencing.

Embodiments of the invention provide a cDNA library produced by any of the methods described herein. This cDNA library can be sequenced to provide an analysis of gene expression in single cells or in a plurality of single cells.

Embodiments of the invention also provide a method for analyzing gene expression in a plurality of single cells, the method comprising the steps of preparing a cDNA library using the method described herein and sequencing the cDNA library. A "gene" refers to a polynucleotide containing at least one open reading frame (ORF) that is capable of encoding a particular polypeptide or protein after being transcribed and translated. Any of the polynucleotide sequences described herein can be used to identify larger fragments or full-length coding sequences of the gene with which they are associated. Methods of isolating larger fragment sequences are known to those of skill in the art.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression can include splicing of the mRNA in an eukaryotic cell.

The cDNA library can be sequenced by any suitable screening method. In particular, the cDNA library can be sequenced using a high-throughout screening method, such as Applied Biosystems' SOLiD sequencing technology, or Illumina's Genome Analyzer. In one aspect of the invention, the cDNA library can be shotgun sequenced. The number of reads can be at least 10,000, at least 1 million, at least 10 million, at least 100 million, or at least 1000 million. In another aspect, the number of reads can be from 10,000 to 100,000, or alternatively from 100,000 to 1 million, or alternatively from 1 million to 10 million, or alternatively from 10 million to 100 million, or alternatively from 100 million to 1000 million. A "read" is a length of continuous nucleic acid sequence obtained by a sequencing reaction.

"Shotgun sequencing" refers to a method used to sequence very large amount of DNA (such as the entire genome). In this method, the DNA to be sequenced is first shredded into smaller fragments which can be sequenced individually. The sequences of these fragments are then reassembled into their original order based on their overlapping sequences, thus yielding a complete sequence. "Shredding" of the DNA can be done using a number of difference techniques including restriction enzyme digestion or mechanical shearing. Overlapping sequences are typically aligned by a computer suitably programmed. Methods and programs for shotgun sequencing a cDNA library are well know in the art.

An embodiment of the method of the invention is summarized in **Figure 1**. Cells are obtained from a tissue of interest and a single-cell suspension is obtained. A single cell is placed in one well of a 96-well plate in Cell Capture Mix. The cells are lysed and reverse transcription reaction mix is added directly to the lysates without additional purification. This results in the synthesis of cDNA from cellular mRNA and incorporation of a tag into the cDNA. The tagged cDNA samples are pooled and amplified and then sequenced to produce 100 million reads. This allows identification of genes that are expressed in each single cell.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also provided within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE 1

Single-cell tagged reverse transcription (STRT)

An embodiment of the method of the invention may be called "single-cell tagged reverse transcription" (STRT) and is described in detail below.

Cell Collection and Lysis

A 96-well plate containing Cell Capture Mix was made by aliquoting 5 µl/well from the Cell Capture Master Plate (see Table 1 below) into an AbGene Thermo-Fast plate.

Reagent	**For one well**	**For one plate**	**Final concentration**
STRT-T30-BIO (100 µM)	0.25 µL	27.5 µL	400 nM
Reagent	**For one well**	**For one plate**	**Final concentration**
STRT buffer (5x)	12.5 µL	1375 µL	1x
STRT-FW-n (5 µM)	5 µL	(5 µL/well)	400 nM
Water	44.75	4.9 mL	
Total	**62.5 uL**		

Table 1 Making a STRT Cell Capture Master Plate.

27.5 µl. STRT-T30- BIO (100 µM) was mixed with 1375 µL STRT 5x buffer and 4.9 mL Rnase/Dnase-free water. 57.5 µL of this solution was aliquoted to each well of a 96-well plate and 5 µL/well of STRT-FW-n (from 5 µM stock plate) was added, i.e. a different oligo in each well.

The sequence of STRT-T30-BIO (which is a CDS) is:

5'-BIO-AAGCAGTGGTATCAACGCAGAGT$_{30}$VN-3',

and the sequence of STRT-FW-n (which is a TSO) is:

5'-AAGCAGTGGTATCAACGCAGAGTGGATGCTXXXXXrGrGrG-3'(X=cell tag)

BtsCI->2/0

n is 1-96 and each oligonucleotide has a distinct cell tag, such that a different oligonucleotide is added to each well containing a single cell.

Mouse embryonic stem cells (R1) were grown without feeder cells, trypsinized, cleared through cell strainer and resuspended in 1x PBS. Cells were then picked by FACS into the

Capture Plate, with a single cell being placed in each well. The Capture Plate was transferred to a PCR thermocycler and incubated at 72°C for 2 minutes, and then cooled to 4°C for 5 minutes to allow annealing to occur. The detergent in STRT buffer helps reduce adsorption of mRNA and cDNA to the walls of the reaction tube during subsequent steps, and also improves lysis of the cells. The heating step causes the cell to lyse completely and release its RNA. When the temperature is reduced, the oligo(dT) primer anneals.

Reverse transcription

5 µL/well of RT mix (see Table 2 below) was added and the plate was incubated at 42°C for 45 minutes, without heated lid.

Reagent	**For 96 reactions**	**For one reaction**	**Final conc.**
STRT buffer (5x)	110 µL	1 µL	1x
DTT (20 mM)	110 µL	1 µL	2 mM
dNTP (10 mM)	110 µL	1 µL	1 mM
Dnase/Rnase-free water	209 µL	1 µL	
Superscript II RT (200 U/µL)	11 µL	1 µL of 20 U/µL (dilute in 1x RT buffer)	2.5 U/µL
Total volume	**550 µL**	**5 µL**	

Table 2 Composition of RT mix

When the RT mix is added, the reverse transcriptase enzyme (Superscript II RT) synthesizes a first strand and the tagged template-switching oligo introduces an upstream primer sequence.

Figure 2 shows the synthesis of cDNA by template switching. The 5' end of the cDNA (which corresponds to the 3' end of the mRNA) can be controlled by adding a tail (which is oligo-dT in this case) to the cDNA synthesis primer (CDS). The 3' end of the cDNA can be controlled using the template-switching oligo (TSO). When reverse transcriptase reaches the 5' end of the mRNA template, it preferentially adds 2-5 cytosines. The template-switching oligo, which has 2-5 guanosines, anneals transiently, and reverse transcriptase then switches template and synthesizes the complementary strand. By this mechanism, both ends of the cDNA can be arbitrarily controlled.

The structure of a typical TSO is shown in **Figure 3**. In this particular TSO, the 3' template switching sequence includes three riboguanines (rG). The cell-tag is shown as "XXXXX" and

can in general have any length or nucleotide composition. Arbitrary sequence can be inserted at the 5' end of the TSO, after the 5' APS, or after the cell-tag, but not at the 3' end.

The structure of a typical CDS is shown in **Figure 4**. The RCS is oligo-dT with an anchor nucleotide (V = A,C,G degenerate). The cell tag "XXXXX" can have any length or nucleotide composition. Additional arbitrary sequences can be inserted at the 5' end, after the 5' APS, or after the cell-tag.

Purification of cDNA

50 µl PBI (Qiaquick PCR Purification Kit) was added to each well to inactivate reverse transcriptase. The PBI inactivates reverse transcriptase and cDNA from all the wells was then pooled. Adding PBI before pooling prevents cDNA synthesis from proceeding once the cDNA samples have been pooled. The pooled cDNA was loaded on a single Qiaquick column and the purified cDNA was eluted in 30 µl EB buffer into a Beckman Polyallomer tube. The purification step removes primers (<40 bp) as well as proteins and other debris.

Full length cDNA amplification

The cDNA was amplified by PCR by adding the reagents shown in Table 3.

Reagent	**For one tube:**	**Final conc.**
Rnase/Dnase-free water	54 µL	
Advantage2 PCR buffer (10x)	10 µL	1x
dNTP (10 mM)	2 µL	200 µM
STRT-PCR (10 µM)	2 µL	200 µM
Advantage2 DNA Polymerase mix (50x)	2 µL	1x
Total volume	**100 µL**	

Table 3. Reagents used for full-length cDNA amplification.

The sequence of STRT-PCR is:
5'-BIO-AAGCAGTGGTATCAACGCAGAGT-3'

PCR was performed using a heated lid as follows: 1 min @ 95°C, 25 cycles of [5 s @ 95°C, 5 s @ 65°C, 6 min @ 68°C], 4°C forever.

30 µL of the reaction was transferred to a new PCR tube, labeled "Optimization". The remaining 70 µL was stored at 4°C until later. 10 µL from the Optimization tube was removed and the rest of the sample was run for three more cycles. This was repeated to obtain aliquots

from 25, 28 and 31 cycles. A diagnostic 2% agarose gel was used to determine the optimal cycle number (which is the cycle just before saturation of the PCR), as well as to visualize the size range of the product (see **Figure 5**). Typically, the optimal number of cycles was around 28. The remaining 70 µL of reaction was run to reach the optimal number of cycles (in addition to the 25 cycles already run).

The PCR product was purified using a Qiaquick column (PCR purification kit) and eluted in 50 µL EB into a Beckman polyallomer tube. The expected concentration at this stage was about 20-40 ng/µL (1-2 µg total yield).

DNase treatment

The sample was treated with DNaseI in the presence of Mn^{2+} to generate double-strand breaks and reduce the size. First, the following components were mixed in the order shown in Table 4.

Reagent	**Volume**	**Final concentration**
cDNA template	50 µl	8 – 16 ng/µL
Water	42.8 µl	
10 x DNase I buffer	11.6 µl	
100 mM $MnCl_2$ (*)	11.6 µl	10 mM
Total volume	**116 µl**	

(*) It is crucial to add $MnCl_2$ last to the reaction, as the BSA present in the buffer will precipitate otherwise.

Table 4. Composition of reaction mix for DNase treatment.

Diluted DNaseI (0.01 units/µl) was prepared just before use as follows: 40 µL 10x DNase I buffer, 318 µL water, 40 µL 100 mM $MnCl_2$ and 2 µL DNaseI (2 U/µL).

4 µl of this diluted DNase I was added to the reaction mix described in Table 4, and was incubated at RT for exactly 10 minutes. The reaction was then stopped by adding 600 µL PBI. The sample was purified on a Qiaquick column and eluted in 30 µL EB.

Bead capture and end repair/nick translation

The fragments were next bound to beads to capture 5' and 3' ends, and then treated with TaqExpress to repair frayed ends and nicks. 30 µL Dynabeads MyOne C1 Streptavidin were washed twice in 2x B&W (Dynal), then added to the Dnase-treated sample, incubated for 10 minutes, and then washed 3x in 1x B&W. About 10% of the sample was bound to the beads (i.e. about 30 – 60 ng), since internal fragments were not biotinylated.

The beads were washed once in 1x TaqExpress buffer and resuspended in the reaction mix shown in Table 5:

Reagent	Volume	Final concentration
10x TaqExpress buffer (blue, with $MgCl_2$)	4 µL	1x
dNTP (10 mM)	0.8 µL	200 µM
Water	33 µl	
TaqExpress (5 U/µL)	2 µL	0.25 U/µL
Total volume	**40 µl**	

Table 5. Composition of the reaction mix used for end repair/nick translation.

The reaction was incubated at 37°C for 30 minutes, and then washed three times in 1x NEB4 buffer.

Fragment release and RDV/FDV adapter ligation

The fragments were released by BtsCI digestion, and simultaneously ligated to the FDV and RDV adapters. The beads were then resuspended in the reaction mix shown in Table 6.

Reagent	Volume	Final concentration
10x NEB4 buffer	4 µL	1x
ATP (10 mM)	4 µL	1 mM
Adapter STRT-RDV-A (10 µM)	4 µL	1 µM
Adapter STRT-FDV (10 µM)	4 µL	1 µM
Water	26 µL	
T4 DNA Ligase (5 U/µL; Invitrogen)	2 µL	0.25 U/µL
BtsCI (20 U/µL)	2 µL	1 U/µL
Total volume	**40 µl**	

Table 6. Reaction mix for resuspension of the beads.

The sequence of STRT-FDV, made by annealing STRT-ADP1U and STRT-ADP1L, was:

5'----- CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATCT-3'
3'-PHO-GGTGATGCGGAGGCGAAAGGAGAGATACCCGTCAGCCACTA-PHO-5'

The sequence of STRT-RDV-A, made by annealing STRT-ADP2U-T and STRT-ADP2L was:

5'----- AACTGCCCCGGGTTCCTCATTCTCTT-3'
3'-PHO-TTGACGGGGCCCAAGGAGTAAGAGA-PHO-5'

The beads were incubated for 30 min at 37°C. The reaction was stopped by adding 200 µL PBL while the beads were held on the magnet. The supernatant was loaded on a Qiaquick

column, purified and eluted in 30 µl EB in a Beckman polyallomer tube. The concentration of the cDNA was about 1 – 2 ng/µL.

Library PCR amplification

Eight reactions were set up using 4, 2, 1, 1/2, 1/4, 1/8, 1/16 and 1/32 µL aliquots of the adapted library, each in 4 µL. Each library was amplified using the PCR reaction mix shown in Table 7.

Reagent	**For one tube:**	**Final conc.**
cDNA library (dilution series)	4 µL	(20 pg/µL)
TaqExpress blue buffer (10x)	5 µL	1x
dNTP (10 mN)	1 µL	200 µM
SOLID-P1 (10 µM)	2 µL	400 nM
SOLID-P2 (10 µM)	2 µL	400 nM
Reagent	**For one tube:**	**Final conc.**
Rnase/Dnase-free water	35.7 µL	0.25 U/µL
TaqExpress polymerase (25 U/µL)	0.3 µL	0.15 U/µL
Total volume	**50 µl**	

Table 7. PCR reaction mix for cDNA library amplification.

The sequence of SOLID-P1 was:

5'-CCACTACGCCTCCGCTTTCCTCTCTATG-3'

The sequence of SOLID-P2 was:

5'-CTGCCCCGGGTTCCTCATTCTCT-3'

PCR was run with heated lid: 5 min @ 94°C, 18 cycles of [15 s @ 94°C, 15 s @ 68°C], 5 min @ 70°C.

All eight reactions were loaded on a 2% E-gel, 10 µL + 10 µL water to determine which reaction was just shy of saturated (see **Figure 6**).

A fresh PCR reaction was then performed using the optimal number of cycles and starting material. For example, if 1/4 µL was optimal at 18 cycles, then 14 cycles were performed.

The PCR product was loaded on a 2% E-gel, 125 – 200 bp region was excised from the gel and purified by Qiagen Gel Extraction Kit (see **Figure 7**). The purified cDNA was eluted in 50 µL EB.

The cDNA library was now prepared for SOLiD sequencing, and could go directly into emulsion PCR.

To verify cDNA library quality, an aliquot was cloned using Invitrogen TOPO TA cloning kit, and sequenced by Sanger sequencing. **Figure 8** shows a typical result demonstrating the presence of primer sequences for SOLiD (P1 and P2; underlined), the cell-specific tag (boxed), and the 2-5 Gs (shaded in a gray box) from the template-switching mechanism. From 22 Sanger sequences, 7 were not mappable to anything in GenBank. All except one of these were misligations of the SOLiD adapters, which can be re-designed to stop this happening. In separate experiments, we found no misligated adapters after blocking their blunt ends with 3' phosphate. Alternatively, the non-ligating 3' ends could be blocked using dideoxy nucleotides or by designing a protruding strand incompatible with the ligating ends of the adapters.

Of the remaining 15 sequences, one was a ribosomal RNA (45S), which is not polyadenylated. It probably occurred due to internal mispriming during first-strand synthesis. The remaining 14 reads were all from polyadenylated mRNA, in the correct orientation and with correct cell tags.

To summarize this dataset, 15 of 22 reads were mappable and 14 of these 15 were correct transcript tags. All the transcripts seen in the Sanger sequence dataset are listed below:

Gene	Length (of mRNA)
Ribosomal protein L35	452
B2_Mm2	~200
Tubulin beta 2c	1 561
B2_Mm1	~195
RIKEN 1110008L16 gene	3 127
Sod2	661
Chchd2	910
mt-Cox2	947
Hnrnpab	2 545
Ribosomal protein L24	558
Ribosomal protein S18	524
RIKEN 2700060E02	941
B2_Mm1	~195
Ribosomal protein S28	356

As expected, the list was dominated by highly expressed genes like ribosomal proteins. Several long transcripts were present in this sample, indicating that there was no strong bias (if any) towards short mRNAs.

Interestingly, three copies of B2 repeats (of subfamilies Mm1 and Mm2) were observed. These are SINE-family repeats expressed from a pol III promoter (not pol II as most mRNAs), but with strong polyadenylation signals. They have been shown to be expressed at extremely high levels in ES cells, together comprising more than 10% of all mRNA. Even more interestingly, they peak just before S-phase in dividing cells, and are thus an early indication that it will be possible to characterize the cell cycle in unsynchronized primary cells using this method.

Quality control by quantitative real-time PCR

To verify that the libraries were representative of the mRNA content of the original ES cell population, quantitative real-time PCR was performed against a set of markers for pluripotency, as well as markers for differentiated tissues. A cDNA library prepared according to classical methods from 1 µg total RNA (~100,000 cells) was compared with the library prepared from 96 single cells using the STRT protocol.

Well-known markers of pluripotency, such as Sox2, Oct4 and Nanog were detected at similar levels in both samples, whereas markers of germ layer differentiation such as Brachyury, Gata4 and Eomes were detected only at very low levels in both samples (see **Figure 9**). The quantitative correlation was good (Pearson correlation coefficient 0.84), with the exception of Plk1, which was not detected in single cells in this experiment.

Reagents used

Reagent	**Source**
2x B&W	10 mM Tris HCl pH 7.5, 1 mM EDTA, 2 M NaCl
10% Tween-20 stock	Make 10% Tween-20, filter at 0.45 µm
Dynabeads MyOne Streptavidin C1	Invitrogen
5x STRT buffer	100 mM Tris-HCl pH 8, 375 mM KCl, 30 mM $MgCl_2$, 0.1% Tween-20
DNase/RNase-free water	Ambion
20 mM DTT	
10 mM and 1 mM dNTP	NEB / In Vitro
Superscript II RT	Invitrogen
10x Advantage2 PCR buffer	Clontech
50x Advantage2 polymerase mix	Clontech
10x DNase I buffer	0.5 M Tris pH 7.5, 0.5 mg/ml BSA
100 mM $MnCl_2$	Sigma 1M stock
Thermo-Fast 96-well plate	AbGene
Thermo-Strip tube strips	AbGene
1.5 mL polyallomer tubes	Beckman-Coulter
2% E-gel with SYBR Safe	Invitrogen

Reagent	Source
Qiaquick PCR Purification Kit	Qiagen
Qiaquick Gel Extraction kit	Qiagen
BtsCI restriction endonuclease	NEB
T4 DNA Ligase	Invitrogen
10 mM ATP	
10x NEB4 buffer	NEB
5 U/μl TaqExpress polymerase	Genetix, UK
10x TaqExpress buffer (blue)	Genetix, UK

Table 8. List of reagents used in the method described above.

EXAMPLE II

Characterization of single-cell transcriptional landscape by highly multiplex RNA-Seq

Understanding of the development and maintenance of tissues has been greatly aided by large-scale gene expression analysis. However, tissues are invariably complex, consisting of multiple cell types in a diversity of molecular states. As a result, expression analysis of a tissue confounds the true expression patterns of its constituent cell types. Described herein is a novel strategy, termed shotgun single-cell expression profiling, was used to access such complex samples. It is a simple and highly multiplexed method used to generate hundreds of single-cell RNA-Seq expression profiles. Cells are then clustered based on their expression profiles, forming a two-dimensional cell map onto which expression data can be projected. The resulting cell map integrates three levels of organization: the whole population of cells, the functionally distinct subpopulations it contains, and the single cells themselves—all without need for known markers to classify cell types. The feasibility of the strategy is demonstrated by analyzing the complete transcriptomes of 436 single cells of three distinct types. This strategy enables the unbiased discovery and analysis of naturally occurring cell types during development, adult physiology and disease.

METHODS

Cell culture

ES R1 cells were cultured as previously described (Moliner et al., Stem Cells Dev. 17:233-243 (2008)). MEFs and Neuro-2A cells were grown in DMEM with 10% FBS, 1x

penicillin/streptomycin, 1x Glutamax and 0.05 mM 2-mercaptoethanol. All culture reagents were from Gibco.

Quantitative real-time PCR (Q-PCR)

RNA was isolated using Trizol (Invitrogen) and 1 µg total RNA was reverse transcribed with Superscript III (Invitrogen) and oligo-(dT) primer. SYBR Green Master Mix (Applied Biosystems) and a cDNA amount corresponding to 5 ng RNA was mixed with 4 pmol primers (Eurofins MWG Operon, Germany) in a total volume of 10 µL, and analyzed on a 7900HT real-time thermocycler (Applied Biosystems). A dilution series of the template was used to determine primer efficiency.

Single-cell tagged reverse transcription (STRT)

Cells were dissociated enzymatically using TrypLE Express (Invitrogen), washed and resuspended in phosphate-buffered saline (PBS). A single cell was collected into each well of a 96-well capture plate (AbGene Thermo-Fast 96 cat. No. 0600) by fluorescence-activated cell sorting (FACS), and the plate was immediately frozen on dry ice. The FACS was used only to collect single cells and to reject dead cells and debris based on light scattering; no fluorescent reporter was used, and hence the collected cells would represent a random sample of the population.

The cell capture plate contained a single cell per well in 5 µL of *STRT* buffer (20 mM Tris-HCl pH 8.0, 75 mM KCl, 6 mM $MgCl^2$, 0.02% Tween-20) with 400 nM STRT-T30-BIO (5'-biotin-AAGCAGTGGTATCAACGCAGAGT$_{30}$VN-3'; this and all other oligos were from Eurofins MWG Operon) and 400 nM STRT-FW-n (5'-AAGCAGTGGTATCAACGCAGAGTGGATGCTXXXXXrGrGrG-3', where "rG" denotes a ribonucleotide guanine and "XXXXX" was a barcode). Each well of the capture plate contained a different template-switching helper oligo (STRT-FW-n) with a distinct barcode. For example, well A01 received STRT-FW-1 with sequence 5'-AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGAArGrGrG-3' having barcode sequence CAGAA. All 96 barcodes and helper oligo sequences are given in Table 9.

Barcode	Oligo name	Sequence (GGG) = ribonucleotides
CAGAA	STRT-FW-1	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGAA(GGG)
CATAC	STRT-FW-2	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATAC(GGG)
CAAAG	STRT-FW-3	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAAAG(GGG)
CACAT	STRT-FW-4	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACAT(GGG)
CATCA	STRT-FW-5	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATCA(GGG)

Barcode	Oligo name	Sequence (GGG) = ribonucleotides
CAGCC	STRT-FW-6	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGCC(GGG)
CACCG	STRT-FW-7	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACCG(GGG)
CAACT	STRT-FW-8	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAACT(GGG)
CAAGA	STRT-FW-9	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAAGA(GGG)
CACGC	STRT-FW-10	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACGC(GGG)
CATGT	STRT-FW-11	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATGT(GGG)
CACTA	STRT-FW-12	AAGCAGTGGTATCAACGCAGAGTGGATGCTCACTA(GGG)
CAATC	STRT-FW-13	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAATC(GGG)
CATTG	STRT-FW-14	AAGCAGTGGTATCAACGCAGAGTGGATGCTCATTG(GGG)
CAGTT	STRT-FW-15	AAGCAGTGGTATCAACGCAGAGTGGATGCTCAGTT(GGG)
CCTAA	STRT-FW-16	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTAA(GGG)
CCGAC	STRT-FW-17	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGAC(GGG)
CCAAT	STRT-FW-18	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCAAT(GGG)
CCGCA	STRT-FW-19	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGCA(GGG)
CCTCC	STRT-FW-20	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTCC(GGG)
CCACG	STRT-FW-21	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCACG(GGG)
CCAGC	STRT-FW-22	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCAGC(GGG)
CCTGG	STRT-FW-23	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTGG(GGG)
CCGGT	STRT-FW-24	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGGT(GGG)
CCATA	STRT-FW-25	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCATA(GGG)
CCGTG	STRT-FW-26	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCGTG(GGG)
CCTTT	STRT-FW-27	AAGCAGTGGTATCAACGCAGAGTGGATGCTCCTTT(GGG)
CGAAA	STRT-FW-28	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGAAA(GGG)
CGCAC	STRT-FW-29	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCAC(GGG)
CGGAG	STRT-FW-30	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGAG(GGG)
CGTAT	STRT-FW-31	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTAT(GGG)
CGCCA	STRT-FW-32	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCCA(GGG)
CGACC	STRT-FW-33	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGACC(GGG)
CGTCG	STRT-FW-34	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTCG(GGG)
CGGCT	STRT-FW-35	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGCT(GGG)
CGGGA	STRT-FW-36	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGGA(GGG)
CGTGC	STRT-FW-37	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTGC(GGG)
CGAGG	STRT-FW-38	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGAGG(GGG)
CGCGT	STRT-FW-39	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCGT(GGG)
CGTTA	STRT-FW-40	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGTTA(GGG)
CGGTC	STRT-FW-41	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGGTC(GGG)
CGCTG	STRT-FW-42	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGCTG(GGG)
CGATT	STRT-FW-43	AAGCAGTGGTATCAACGCAGAGTGGATGCTCGATT(GGG)
CTCAA	STRT-FW-44	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTCAA(GGG)
CTAAC	STRT-FW-45	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTAAC(GGG)
CTTAG	STRT-FW-46	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTAG(GGG)
CTGAT	STRT-FW-47	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGAT(GGG)
CTACA	STRT-FW-48	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTACA(GGG)
CTGCG	STRT-FW-49	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGCG(GGG)
CTTCT	STRT-FW-50	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTCT(GGG)
CTTGA	STRT-FW-51	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTGA(GGG)
CTGGC	STRT-FW-52	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGGC(GGG)
CTCGG	STRT-FW-53	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTCGG(GGG)
CTAGT	STRT-FW-54	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTAGT(GGG)

Barcode	Oligo name	Sequence (GGG) = ribonucleotides
CTGTA	STRT-FW-55	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTGTA(GGG)
CTTTC	STRT-FW-56	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTTTC(GGG)
CTATG	STRT-FW-57	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTATG(GGG)
CTCTT	STRT-FW-58	AAGCAGTGGTATCAACGCAGAGTGGATGCTCTCTT(GGG)
GACAA	STRT-FW-59	AAGCAGTGGTATCAACGCAGAGTGGATGCTGACAA(GGG)
GAAAC	STRT-FW-60	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAAAC(GGG)
GATAG	STRT-FW-61	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATAG(GGG)
GAGAT	STRT-FW-62	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGAT(GGG)
GAACA	STRT-FW-63	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAACA(GGG)
GAGCG	STRT-FW-64	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGCG(GGG)
GATCT	STRT-FW-65	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATCT(GGG)
GATGA	STRT-FW-66	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATGA(GGG)
GAGGC	STRT-FW-67	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGGC(GGG)
GACGG	STRT-FW-68	AAGCAGTGGTATCAACGCAGAGTGGATGCTGACGG(GGG)
GAAGT	STRT-FW-69	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAAGT(GGG)
GAGTA	STRT-FW-70	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAGTA(GGG)
GATTC	STRT-FW-71	AAGCAGTGGTATCAACGCAGAGTGGATGCTGATTC(GGG)
GAATG	STRT-FW-72	AAGCAGTGGTATCAACGCAGAGTGGATGCTGAATG(GGG)
GACTT	STRT-FW-73	AAGCAGTGGTATCAACGCAGAGTGGATGCTGACTT(GGG)
GCAAA	STRT-FW-74	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCAAA(GGG)
GCCAC	STRT-FW-75	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCCAC(GGG)
GCGAG	STRT-FW-76	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGAG(GGG)
GCTAT	STRT-FW-77	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTAT(GGG)
GCACC	STRT-FW-78	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCACC(GGG)
GCTCG	STRT-FW-79	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTCG(GGG)
GCGCT	STRT-FW-80	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGCT(GGG)
GCGGA	STRT-FW-81	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGGA(GGG)
GCTGC	STRT-FW-82	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTGC(GGG)
GCAGG	STRT-FW-83	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCAGG(GGG)
GCCGT	STRT-FW-84	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCCGT(GGG)
GCTTA	STRT-FW-85	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCTTA(GGG)
GCGTC	STRT-FW-86	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCGTC(GGG)
GCCTG	STRT-FW-87	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCCTG(GGG)
GCATT	STRT-FW-88	AAGCAGTGGTATCAACGCAGAGTGGATGCTGCATT(GGG)
GGTAA	STRT-FW-89	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGTAA(GGG)
GGCAG	STRT-FW-90	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGCAG(GGG)
GGAAT	STRT-FW-91	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGAAT(GGG)
GGTCC	STRT-FW-92	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGTCC(GGG)
GGACG	STRT-FW-93	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGACG(GGG)
GGCCT	STRT-FW-94	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGCCT(GGG)
GGCGA	STRT-FW-95	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGCGA(GGG)
GGAGC	STRT-FW-96	AAGCAGTGGTATCAACGCAGAGTGGATGCTGGAGC(GGG)

Table 9. Barcodes and helper oligonucleotide sequences.

The cell capture plate was thawed and then heated to lyse the cells (20°C for 5 minutes, 72°C for 4 minutes, 10°C for 5 minutes in a thermocycler). 5 µL reverse transcription mix (4 mM DTT, 2 mM dNTP, 5 U/µL Superscript II in *STRT* buffer) was added to each well and the plate

was incubated (10°C for 10 minutes, 42°C for 45 minutes) to complete reverse transcription and template switching.

To purify the cDNA and remove unreacted primers, 50 µL PB (Qiaquick PCR Purification Kit, Qiagen) was added to each well. All 96 reactions were pooled and purified over a single Qiaquick column. The cDNA was eluted in 30 µL EB in a 1.5 mL polyallomer tube (Beckman).

The whole 96-cell cDNA sample was amplified in a single tube in 100 µL of 200 µM dNTP, 200 µM STRT-PCR primer (5'-biotin-AAGCAGTGGTATCAACGCAGAGT-3'; Eurofins MWG Operon), 1x Advantage2 DNA Polymerase Mix (Clontech) in 1x Advantage2 PCR buffer (Clontech) with 1 min at 94°C followed by 25 cycles of 15 s at 95°C, 30 s at 65°C, 3 min at 68°C, with heated lid. An aliquot was visualized on a 1.2% agarose E-gel (Invitrogen) and the sample was amplified an additional 1-5 cycles if necessary. The product was purified (Qiaquick PCR Purification Kit, Qiagen) and quantified by fluorimetry (Qubit, Invitrogen). Typical yields were 0.5 – 1 µg total. Aliquots were taken at this stage for microarray analysis and Q-PCR.

Sample preparation for high-throughput sequencing

Amplified cDNA was fragmented by DNase I in the presence of Mn^{2+}, which causes a preference for double-strand breaks. 50 µL cDNA was fragmented in DNase I buffer supplemented with 10 mM $MnCl_2$ and DNase I diluted to 0.0003 U/µL in a total volume of 120 µL for exactly six minutes room temperature. The reaction was stopped by the addition of 600 µL PB (Qiaquick PCR Purification Kit, Qiagen), purified and eluted in 30 µL EB into a polyallomer tube (Beckman).

3' and 5' fragments were immobilized on 30 µL streptavidin-coated paramagnetic beads (Dynabeads MyOne C1, Invitrogen), then resuspended in 30 µL TaqExpress buffer (Genetix, UK). Ends were repaired and single A overhangs generated by incubating the beads in 40 µL of 200 µM dNTP, 0.25 U/µL TaqExpress (Genetix, UK) in TaqExpress buffer at 37°C for 30 minutes, followed by three washes in NEBuffer 4 (New England Biolabs).

5' fragments containing barcodes and cDNA inserts were released from the beads by BtsCI digestion, and adapters were simultaneously ligated to generate a sample suitable for sequencing on the Illumina Genome Analyzer. The beads were resuspended in 40 µL of 1 mM ATP, 1 µM SOLEXA-ADP1 adapter

(5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCT-3' and 3'-PHO-TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAG GCTA-PHO-5'), 1 µM SOLEXA-ADP2 adapter (5'-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT-3' and 3'-PHO-GTTCGTCTTCTGCCGTATGCTCGAGAAGGCTAG-PHO-5'), 0.25 U/µL T4 DNA ligase (Invitrogen), 1 U/µL BtsCI (New England Biolabs) in 1x NEBuffer 4, and incubated 30 minutes at 37°C. The beads were removed and the supernatant was purified using AmPure (Agencourt) and eluted in 40 µL EB (Qiagen).

The sample was loaded on a 2% SizeSelect E-gel and the range 200 – 300 bp was collected. A 4 µL aliquot was amplified in 50 µL total volume containing 200 µM dNTP, 400 nM each primer (5'- AATGATACGGCGACCACCGA-3' and 5'-CAAGCAGAAGACGGCATACGAG-3') and 0.15 U/µL Phusion polymerase in Phusion HF buffer (New England Biolabs) with 30 s at 98°C, 14-18 cycles of [10 s at 98°C, 30 s at 65°C, 30 s at 72°C] followed by 5 min at 70°C. Test amplifications were used to determine the minimal number of cycles needed. The amplified sample was purified by Qiaquick PCR Purification followed by a 2% SizeSelect E-gel, again collecting the region 200 – 300 bp. The concentration was measured by Qubit (Invitrogen) and was typically 5 ng/µL. Aliquots were cloned (TOPO, Invitrogen) and sequenced by Sanger sequencing to verify sample quality and determine the average fragment length. Based on this information, the molar concentration could be accurately determined and was generally above 10 nM. Cluster formation and sequencing-by-synthesis was performed on a Genome Analyzer *IIx* according to the manufacturer's protocols (Illumina, Inc., San Diego, USA) at a commercial service provider (Fasteris SA, Geneva, Switzerland).

Mapping, quantification and visualization

Raw reads were sorted by barcode (first five bases) and trimmed to remove up to five 5' Gs introduced by template-switching, and 3' As that sometimes occurred when a read extended into the poly(A) tail. Only exact barcodes were allowed, and the barcodes were designed so that no single error would convert one valid barcode into another. The reads were then mapped to the mouse genome using Bowtie (Langmead et al., Genome Biol. 10:R25 (2009)) with the default settings. Unmapped reads were discarded. Then, for each annotated feature in the NCBI 37.1 assembly, all mapping reads were counted to generate a raw count. That is, all reads that mapped to any of the exons of a gene were assigned to that gene; isoforms were not

distinguished. Finally, the raw reads for each cell were normalized to transcripts per million (t.p.m.). Wells with fewer than 1000 mapped reads were omitted from further analysis; presumably these included cases where the FACS instrument had failed to hit the reagent droplet while cells were picked.

To visualize cells in a two-dimensional landscape, all pairwise similarities were first computed. The Bray-Curtis distance was used as a similarity metric because it tended to handle the noise in low-expressed genes well. Standard correlation yielded similar results, but with a few more misplaced cells (data not shown). A similarity graph was then built by letting nodes represent cells, and connecting each cell to its five most similar cells (for clarity, cells with fewer than 10,000 reads were omitted, as they were liable to generate spurious edges). Thus every node (cell) had five outgoing edges and varying numbers of incoming edges. Force-directed layout was then used to project the graph to two dimensions, revealing the internal structure based on cell-cell similarities. The GraphPlot function of the Mathematica program (Wolfram Research Inc., USA) was used with the "SpringElectricalEmbedding" option.

RESULTS

Data from 436 single cells collected from three different mouse cell types: embryonic stem cells (ES R1, Wood et al., Nature 365: 87-89 (1993)), a neuroblastoma tumor cell line (Neuro-2A, Olmsted et al., Proc. Natl. Acad. Sci U.S.A. 65:129-136 (1970)) and embryonic fibroblasts (MEF) are reported. In brief, each sample was prepared by picking single cells by fluorescence-activated cell sorting (FACS) into the wells of a 96-well PCR plate preloaded with lysis buffer; heating the plate to complete lysis, then adding reverse transcription reagents to generate a first strand cDNA. To incorporate a well-specific (and hence cell-specific) barcode, the reverse transcriptase template-switching mechanism (Schmidt et al., Nucleic Acids Res. 27:e31 (1999)) was used whereby a helper oligo directs the incorporation of a specific sequence at the 3' end of the cDNA molecule (**Figure 10A**). A different helper oligo was used in each well, with distinct five-base barcodes and universal primer sequence. After cDNA synthesis, the 96 reactions were pooled, purified and amplified by single-primer PCR in a single tube. Cell-to-cell amplification bias was thus reduced, and the number of PCR cycles could be kept low since amplification started from 96-fold more material. The amplified samples were then adapted for sequencing using standard methods. The procedure was named 'STRT' (single-cell tagged reverse transcription). For details, see the Methods section and **Figure 11**.

5 – 12 million raw reads per sequencing lane were typically obtained on an Illumina Genome Analyzer IIx, and each sample was analyzed on up to eight lanes (but typically one or two). Reads lacking a proper barcode, mostly caused by errors in sample preparation such as misligated adaptors, were removed. Of the remaining 79±11% (mean±s.d.) reads, about three-quarters (75±12%) could be placed on the mouse genome allowing for up to two sequencing errors, resulting in hits to 14,718±3,006 distinct features (including mRNA, mitochondrial RNA and expressed repeats). These results are summarized in Table 10

Library	*Species*	*Cell source*	*Lanes*	*Length (bp)*	*Raw reads*	*Barcoded*	*Mapped*	*Features*	*Cum. features*
L006	Mouse	ES R1	8	1x36 7x50	46,130,389	38,284,613 (83%)	21,356,066 (56%)	13,168	13,168
L013	Mouse	ES R1	1	36	6,482,712	5,328,081 (82%)	3,199,050 (60%)	19,328	21,285
L019A	Mouse	ES R1	1	36	4,302,649	2,588,536 (60%)	2,344,394 (91%)	12,229	
L019B	Mouse	ES R1 (*)	1	36	5,134,171	3,323,243 (65%)	2,562,559 (77%)	14,794 (**)	23,152
L023	Mouse	Neuro2A	1	50	10,062,086	8,588,048 (86%)	5,963,143 (69%)	11,464	23,735
L026A	Mouse	MEF	1	36	4,278,888	3,729,229 (87%)	2,862,951 (77%)	12,924	
L026B	Mouse	MEF (*)	1	36	3,912,144	3,398,519 (87%)	2,599,074 (76%)	13,832 (**)	24,288

(*) these samples were independently amplified from corresponding 'A' libraries, but were not quality-controlled by Sanger sequencing af preparation

(**) cumulative with the previous sample (i.e. A and B together)

Table 10. Library summaries. Percentages refer to percent of the preceding column unless otherwise noted. "Features" includes mRNA, mitochondrial RNA and expressed retrotransposon families. "Cumulative features" tracks the cumulative number of features across all lib

Hits spanned some transcripts (**Figure 10B**), but were more commonly located in a region approximately 200 – 1500 bp from the 3' end of each gene, as illustrated in **Figure 10C**. This was expected because an oligo(dT) primer was used to generate cDNA from the 3' end and the mRNA was likely partially degraded during cell lysis by high-temperature hydrolysis in the presence of Mg^{2+}. The background from e.g. genomic DNA contamination, judged by hits to unannotated sequence, was minimal ($<10^{-3}$ reads per million per kilobase), as clearly seen in **Figure 10B** and **Figure 10C**. Note the paucity of hits to the reverse strand and to introns for both Pou5f1 and Nanog; other loci were similar.

Scrutinizing the mapped data, no evidence of mispriming or other undesired side-reactions was found. Control experiments showed that both RNA, reverse transcriptase and template switching oligo were individually required to yield product (data not shown). The vast majority of mapped reads had a properly oriented barcode, indicating that they were primed from the oligo-dT primer and correctly template-switched. No evidence of a motif complementary to any of the primers near read mapping sites or indeed of any other motif was found, except for a weak general T-bias in rare cases (**Figure 12**). On the other hand, there were frequent hotspots of template switching (**Figure 13**), indicating structural constraints in RNA affecting cDNA synthesis and/or template-switching at particular sites. Hotspots were consistent between cells, and should therefore not affect quantitative comparisons.

To characterize sample complexity, and to determine the depth of sequencing required to sample most of the available complexity, the 'new discovery' rate as function of read depth was studied. In other words, the number of new, distinct molecules that were discovered as more sequences were added was determined. It should be noted that, at most, one amplifiable clone was generated from each polyadenylated RNA molecule and this clone was then amplified and sequenced from its 5' end. Therefore reads mapping to distinct locations must have been generated from distinct mRNA molecules. On the other hand, reads mapping to the same location may have been coincidentally generated from two mRNA molecules, or may represent copies of the sample initial clone. The number of distinctly mapping reads was therefore a lower bound on the true sample complexity. As shown in **Figure 14A**, none of the samples reported here were sequenced to saturation, even at 21 million mapped reads, but the yield of new molecules tended to slow down after about 5-10 million reads. Projecting the curves to infinite read depth, most samples would seem to contain at least three million distinct molecules, or about 30,000 per cell. Assuming $10^5 – 10^6$ mRNA molecules per cell, this would suggest the method successfully converted 3-30% of the mRNA to mappable reads, as a (very)

conservative lower bound. The true proportion was likely significantly higher, as many coincident reads (discarded in this analysis) would be generated from template switching hot spots, as mentioned above.

In contrast, the rate of discovery of distinct features rapidly diminished, and 86% of all distinct feature were detected in the first 14% reads (**Figure 14B**). This suggests that the method successfully recovered most expressed features present in the samples, even at relatively low read depth.

Strand information is often required to properly assign reads to transcriptional units, since genes frequently overlap on opposite strands. For example, more than 3000 human genes overlap in this manner (Yelin et al., Nat. Biotechnol. 21:379-386 (2003)). Because the template-switching mechanism used to introduce a barcode occurs directionally, strandedness could be preserved throughout the protocol. To confirm this, the mitochondrial genome was examined, which is expressed as a single long transcript from one strand (the H strand), and is subsequently cleaved to excise tRNA transcripts located between protein-coding genes. Only the protein-coding genes are then polyadenylated. A single protein-coding transcript, ND6, is generated from the L strand, but it is very weakly expressed and irregularly polyadenylated (Slomovic et al., Mol. Cell. Biol. 25:6427-6435 (2005)). As shown in **Figure 10D**, very strong strand-specificity (>99% of reads on the H strand) was observed and no significant expression of tRNA genes was detected, which confirms that the method was poly(A)-specific. The small number of hits on the L strand occurred mainly near the L strand promoter, which may be explained by the polyadenylation of aborted L-strand transcripts (Slomovic et al., *supra*). Similarly, the apparent expression of ND6 on the wrong strand is likely explained by the natural polyadenylation of ND5 downstream of its open reading frame (Slomovic et al., *supra*). The strand specificity allowed us to unambiguously assign reads to expressed transcripts, even in cases where two overlapping genes were co-expressed (**Figure 15**).

On the larger scale of the nuclear chromosomes, hits were approximately equally distributed on the forward and reverse strands. Read density correlated strongly with gene density as shown for chromosome 19 in **Figure 10E**, again indicating that most reads originated specifically from expressed transcripts and were accurately mapped to the genome.

In order to generate a quantitative measure of gene expression, the number of hits to each annotated feature, normalized to transcripts per million (t.p.m.) were counted. Assuming 10^5 to 10^6 transcripts per cell, 1 to 10 t.p.m. corresponds to a single mRNA molecule per cell.

Transcript length (as in the RPKM measure (Mortazavi et al., Nat. Methods 5:621-628 (2008))) was not used to normalize because a single amplifiable 3'-end molecule was generated for each input mRNA molecule, irrespective of its length. An advantage of this approach was the lack of bias against short transcripts (which must be sampled more deeply to generate a detectable RPKM value) or long transcripts (which might otherwise be suppressed during PCR). Indeed, and in contrast to standard RNA-Seq (Oshlack et al., Biol. Direct 4:14 (2009)), no length-dependent bias for transcripts longer than 800 nucleotides was observed (**Figure 16**). Transcripts shorter than about 200 nucleotides were undercalled, likely because only samples above 100 bp were gel-selected. Additionally, transcripts around 600 nucleotides were slightly overrepresented, possibly because of the higher efficiency of template-switching at the 5'-cap of mRNA (Schmidt et al., Nucleic Acids Res. 27:e31 (1999)) or due to the presence of a few very highly expressed genes in this range (e.g. Dppa5 and Rps14).

Expression levels spanned four orders of magnitude in single cells (approximately 1 – 10,000 t.p.m.), with most genes expressed at low levels (<100 t.p.m.: **Figure 17A**). Given the relatively shallow depth of sequencing used here, genes expressed below 10 t.p.m. were generally undetectable solely due to the sampling limit. Since single-cell cDNA was pooled before amplification, the yields of different cells could not be subsequently normalized. As a consequence, cells were unequally sampled and the limit of detection varied. For example, compare two cells sampled at 500,000 reads (**Figure 17B**) and 100,000 reads (**Figure 17C**). In the former case, the apparent detection limit was around 10 t.p.m., whereas in the latter case genes below 100 t.p.m. were generally not detected. However, in both cases, genes above the detection limit were reproducibly quantified in single cells (the coefficient of variation was 46% at 500,000 reads; and 72% at 100,000 reads). Extending this analysis to all cells and genes showed that sensitivity approached the theoretical limit imposed by sampling depth (**Figure 17D**); the difference can be explained by losses in reverse transcription, template switching and sample handling. Measured expression levels were generally accurate, as determined by comparison with Q-PCR (**Figure 17E**), and microarray hybridization (**Figure 18**). In agreement with published reports based on Q-PCR (Bengtsson et al., Genome Res. 15:1388-1392 (2005)), Actb mRNA abundance showed an approximately log-normal distribution across cells (**Figure 19**). RNA polymerase II (large subunit) was expressed at 25±123 t.p.m. in ES cells, comparable to the 27 RPKM found by RNA-Seq (Cloonan et al., Nat. Methods 5:613-619 (2008)) and to the 33±79 t.p.m. found in CHO cells by direct detection *in situ* (assuming 300,000 transcripts per cell) (Raj et al., PLoS Biol 4:309 (2006)).

The cell-cell relationships on a two-dimensional map were visualized, such that more closely related cells would be located near each other. In this way, cell types based solely on expression data were able to be detected and distinguished, without relying on pre-existing markers. A conventional principal component analysis (PCA) revealed three distinct groups of cells, as expected (**Figure 20**). However, a more complete separation into distinct cell-type clusters was achieved using a graph-based method (see **Methods**). Briefly, a graph with nodes representing cells, and edges representing cell-cell similarity of expression pattern was constructed (**Figure 21A**). Force-directed layout to project the graph to two dimensions was used. In this test case using only two cell types (ES and Neuro2A cells), a near-perfect separation was achieved (**Figure 21B**). A larger map incorporating MEFs and additional ES cells showed good separation (**Figure 21C**); demonstrating that the single-cell expression profiles contained enough information to distinguish cell types *de novo*. Both PCA and graph-based analysis clearly distinguished the cell types tested here, but the graph-based method generated more homogenous, well-separated clusters. Both methods accurately grouped independently prepared ES cells together as distinct from the other cell types, showing that clusters did not represent sample preparation artifacts.

Gene expression data was projected onto the map, which provided an easy way to quickly grasp gene expression patterns in both single cells and in the clusters representing cell types (**Figure 22**). A set of well-known ES cell markers (Dppa5, Sox2, Sall4, Pou5f1, Nanog, Zfp42, Zic3, Esrrb) were clearly specifically expressed in ES cells, although their expression levels varied widely from cell to cell (note the logarithmic color scale). A few genes important for pluripotency (Klf4, Myc and Klf2) were more broadly expressed. The power of large-scale single-cell analysis was evident in the fact that while not every cell expressed every marker, patterns of gene activity were highly consistent at the cluster level. For example, even a highly expressed cytoskeletal gene like Actb was not always detected, but its expression in each of the three major clusters was obvious. Correspondingly, lower expressed transcription factors characteristic of ES cells went undetected in some individual ES cells, but the overall pattern of expression in the ES cell cluster was unambiguous and consistent with their identity as ES cells. In general, as the average expression levels decreased from 45 000 t.p.m. (mt_Rnr2) to 1700 (Actb), 850 (Rpl4), 73 (Kras) and 0 t.p.m. (Calb1), the number of expressing cells also decreased, reflecting the stochastic nature of gene expression as well as the sensitivity limits of the method.

The cell map representation demonstrated that (1) individual cells showed highly variable expression patterns, yet their overall pattern of expression was sufficient to group cells of one type together as a cluster; (2) once a cluster of cells was formed, representing a distinct cell type, patterns of gene expression (at the cluster level) were unambiguous. Thus, shotgun single-cell expression profiling is an efficient strategy to access single-cell expression data in heterogeneous populations of cells.

DISCUSSION

Described herein is a reliable and accurate method to obtain RNA-Seq transcription profiles from hundreds of single cells, and shown that single-cell expression profiles can be used to form cell type-specific clusters. This allows analysis of cell type-specific patterns of gene expression both at the single-cell level and the population level, without the need for known markers or even a prior knowledge that a certain cell type exists. This general strategy can be extended to study all kinds of mixed samples. For example, it could be applied to monitor the emergence of specific cell types during organogenesis, without the need to purify those cell types using cell surface markers. Similarly, it could be used to study small populations of stem cells embedded in adult tissues, such as the stem cells that maintain intestinal crypts. The method could also be applied to disease, including the characterization of heterogeneous tumor cell samples or the rare circulating cancer cells that can contribute to metastasis.

What unites all these disparate scientific lines of inquiry is the need to unmix heterogeneous populations of cells. Currently, unmixing is primarily achieved either by physically isolating cells based on known cell surface markers, or by genetically labeling the desired cells so that they can be isolated based on e.g. GFP expression. However, the use of previously known markers precludes the discovery of new cell types, and always risks resulting in mixed data if the markers were not truly specific. In contrast, the methods described herein have shown that cells of distinct types can be unmixed purely *in silico*, provided that large numbers of single-cell expression profiles are generated.

Importantly, then, a very high-throughput, scalable method for single-cell expression profiling is required. Therefore, a method was developed to prepare a barcoded single-cell cDNA sample from 96 cells in a single incubation step. As a consequence, 96 cells could be pooled and treated as a single sample throughout the procedure, which greatly increased throughput and reduced cost. It can also reduce amplification bias, since all 96 cells were amplified in a single closed tube. The entire procedure takes two days to perform, from 96 living cells to

finished samples loaded on the Genome Analyzer. The cost, including all reagents and consumables to generate 10-15 million 36 bp reads using commercial services, was approximately $3500 (that is, about $35/cell).

The date generated herein was on a large number of single cells, each analyzed at a relatively shallow depth of coverage. This allowed the generation of data on far more single cells than have ever been reported in a single study (no single-cell transcriptome experiments with more than a dozen cells have been published), and to produce a cell map with high resolution. In fact, provided that each cell is sampled deeply enough to cluster correctly, it would often make more sense to analyze a larger number of cells than to analyze each cell more deeply. The more cells are added, the more accurate will be the aggregate data obtained from each distinct cell type (cluster), and the better the resolution in "cell type space". For example, many of the ES cells here were sampled at less than 100,000 reads/cell, but altogether 160 ES cells were identified in the cell map, comprising over 1.5 million reads. Sampling a large number of cells will be especially important when the approach is applied to complex tissues, where some types of cells can be present only in a small minority. In addition, as sequencing costs continue to decrease, the tradeoff between number of cells and number of reads will become less pressing.

The use of very large-scale single-cell transcriptional profiling to build a detailed map of naturally occurring cell types is envisioned, which would give unprecedented access to the genetic machinery active in each type of cell at each stage of development. The same strategy can be used to dissect the mutational heterogeneity of neoplasms at the single-cell level.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains. Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. A method of preparing a cDNA library from a plurality of single cells, the method comprising the steps of:

(i) releasing mRNA from each single cell to provide a plurality of individual mRNA samples, wherein the mRNA in each individual mRNA sample is from a single cell;

(ii) synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell;

(iii) pooling the tagged cDNA samples; and

(iv) amplifying the pooled cDNA samples to generate a cDNA library comprising double-stranded cDNA.

2. The method according to claim 1, wherein in step (ii) the tag is incorporated into the cDNA during its synthesis.

3. The method according to claim 1 or 2, wherein synthesis of the first strand of cDNA in step (ii) is directed by a cDNA synthesis primer (CDS) that includes an RNA complementary sequence (RCS) that is at least partially complementary to one or more mRNA in an individual mRNA sample.

4. The method according to claim 3, wherein the RCS is at least partially complementary to a portion of the first strand of cDNA, such that it is able to direct the synthesis of a second strand of cDNA using the first strand of cDNA as a template.

5. The method according to claim 3, wherein a template-switching oligonucleotide (TSO) is added to each individual mRNA sample, wherein said TSO comprises a portion which is at least partially complementary to a portion at the 3' end of the first strand of cDNA.

6. The method according to claim 1, wherein the first strand of cDNA includes a 3' portion comprising a plurality of cytosines or cytosine analogues that base pair with guanosine.

7. The method according to claim 6, wherein the TSO includes a 3' portion comprising a plurality of guanosines or guanosine analogues that base pair with cytosine.

8. The method according to claim 7, wherein the guanosines are ribonucleosides or locked nucleic acid monomers.

9. The method according to claim 5, wherein the CDS or the TSO includes a tag.

10. The method according to claim 5, wherein both the CDS and the TSO include a tag.

11. The method according to claim 10, wherein the CDS and the TSO each include a different tag, such that the tagged cDNA sample comprises a combination of tags.

12. The method according to claim 9, wherein the tag is a nucleotide sequence of 4-20 nucleotides in length.

13. The method according to claim 1, wherein each cDNA sample has a distinct tag or combination of tags.

14. The method according to claim 3, wherein the CDS comprises a 5' amplification primer sequence (APS) and a 3' RCS.

15. The method according to claim 14, wherein the 3' RCS comprises oligo(dT), a gene family-specific sequence, a random sequence or a non-self-complementary semi-random sequence.

16. The method according to claim 14, wherein the TSO includes a 5' APS.

17. The method according to claim 16, wherein the CDS and the 5' APS of the TSO is at least 80% identical to the 5' APS of the CDS.

18. The method according to claim 16, wherein the CDS and the 5' APS of the TSO is 100% identical to the 5' APS of the CDS.

19. The method according to claim 1, wherein the cells are lysed to release mRNA.

20. The method according to claim 1, wherein the mRNA is purified following step (i).

21. The method according to claim 1, wherein the synthesis of cDNA from mRNA is stopped before the tagged cDNA samples are pooled.

22. The method according to claim 1, wherein the tagged cDNA samples are purified before amplification of the cDNA.

23. The method according to claim 1, wherein in step (iv) the pooled cDNA samples are amplified by PCR.

24. The method according to claim 23, wherein the pooled cDNA samples are amplified by emulsion PCR.

25. The method according to claim 23 or 24, wherein the pooled cDNA samples are amplified by single primer PCR.

26. The method according to claim 1, wherein the method further comprises processing the cDNA library to obtain a library suitable for sequencing.

27. The method according to claim 26, wherein the processing comprises fragmenting the cDNA library.

28. The method according to claim 26 or 27, wherein the processing includes the step of adding an adapter to the cDNA.

29. The method according to claim 26, wherein the cDNA library is amplified.

30. A cDNA library produced by the method of claim 1 or 26.

31. A method for analysing gene expression in a plurality of single cells, the method comprising the steps of:

(i) preparing a cDNA library according to the method of claim 1 or 26; and

(ii) sequencing the cDNA library.

32. The method according to claim 31, wherein sequencing is by shotgun sequencing.

33. The method according to claim 32, wherein the cDNA library is sequenced to obtain at least 10,000, at least 1 million, at least 10 million, at least 100 million, or at least 1 billion reads, wherein a read is a length of continuous nucleic acid obtained by a sequencing reaction.

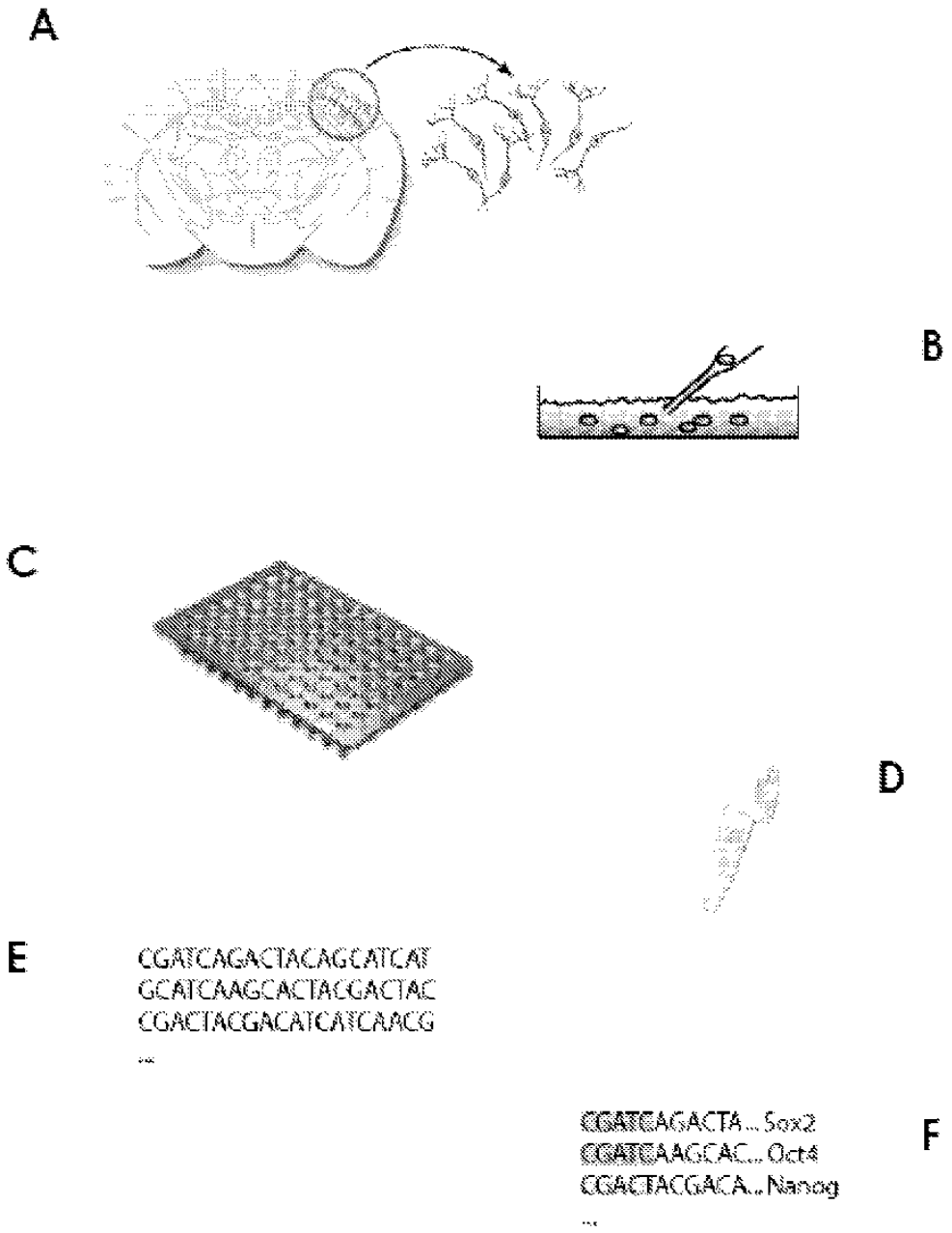


Figure 1

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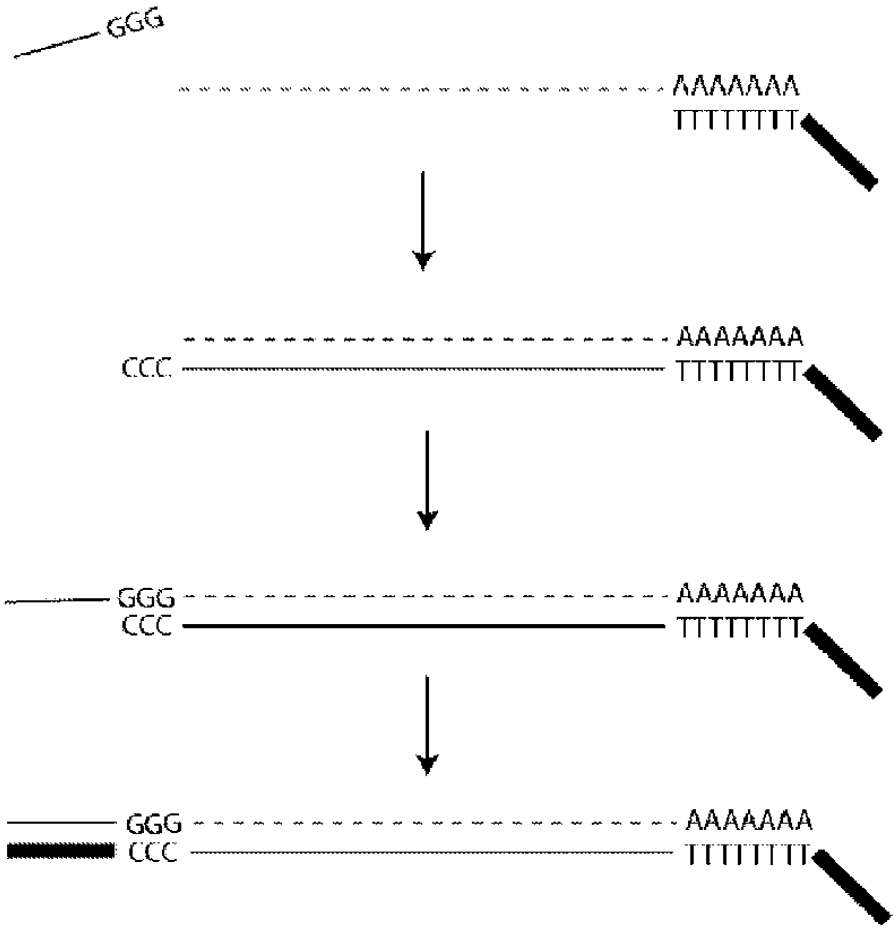


Figure 2

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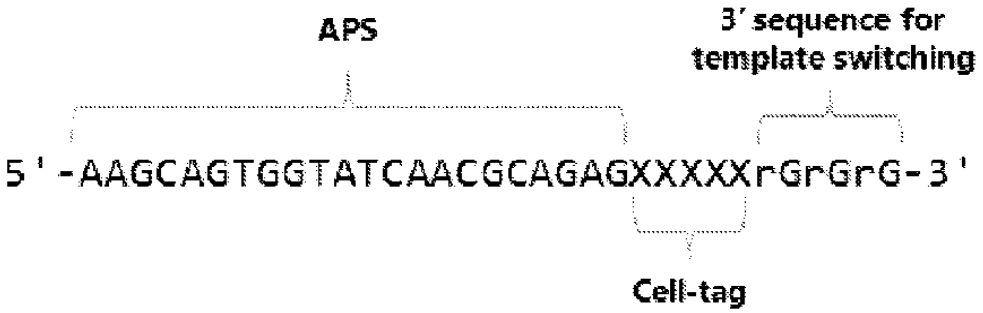


Figure 3

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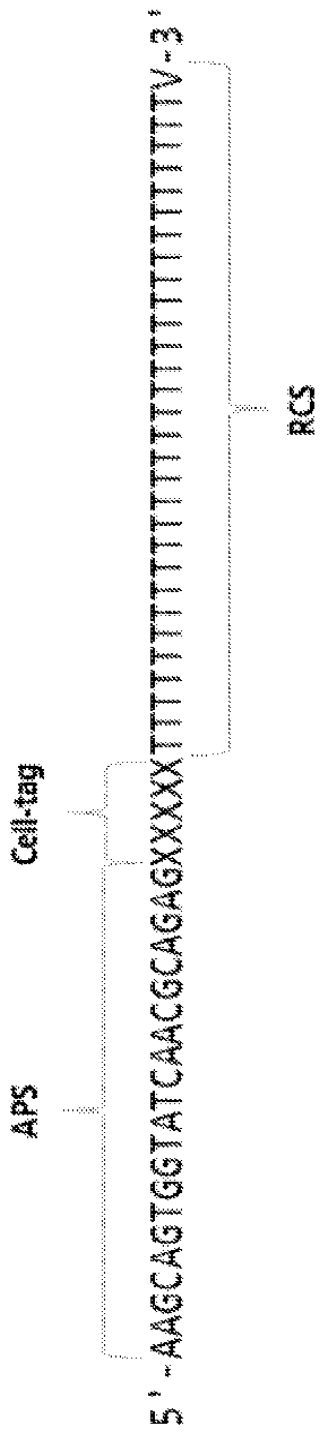


Figure 4

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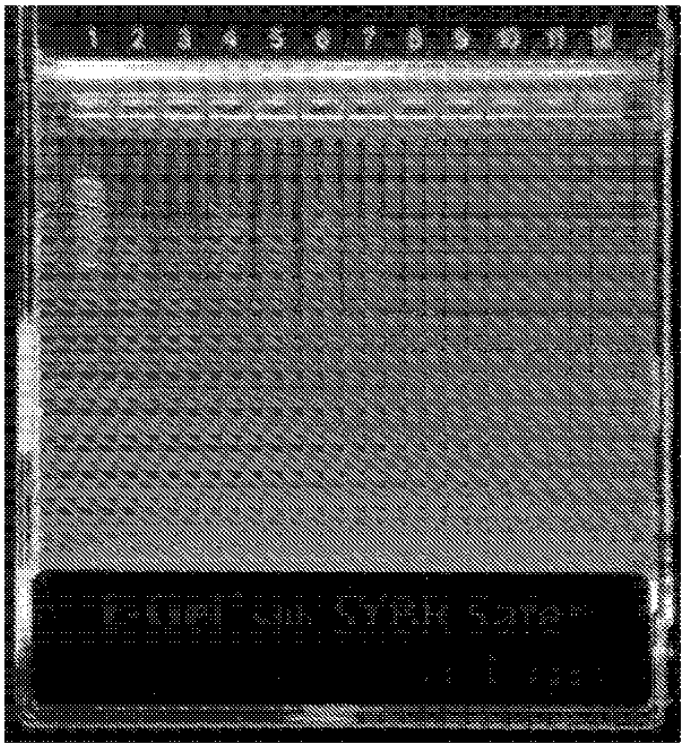


Figure 5

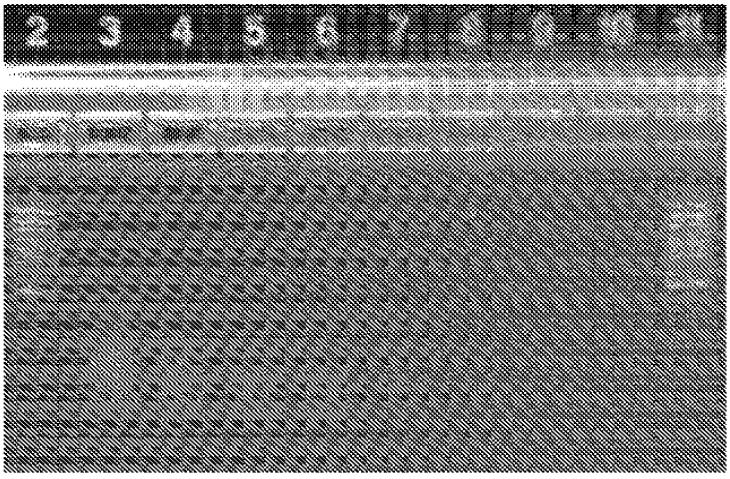


Figure 6

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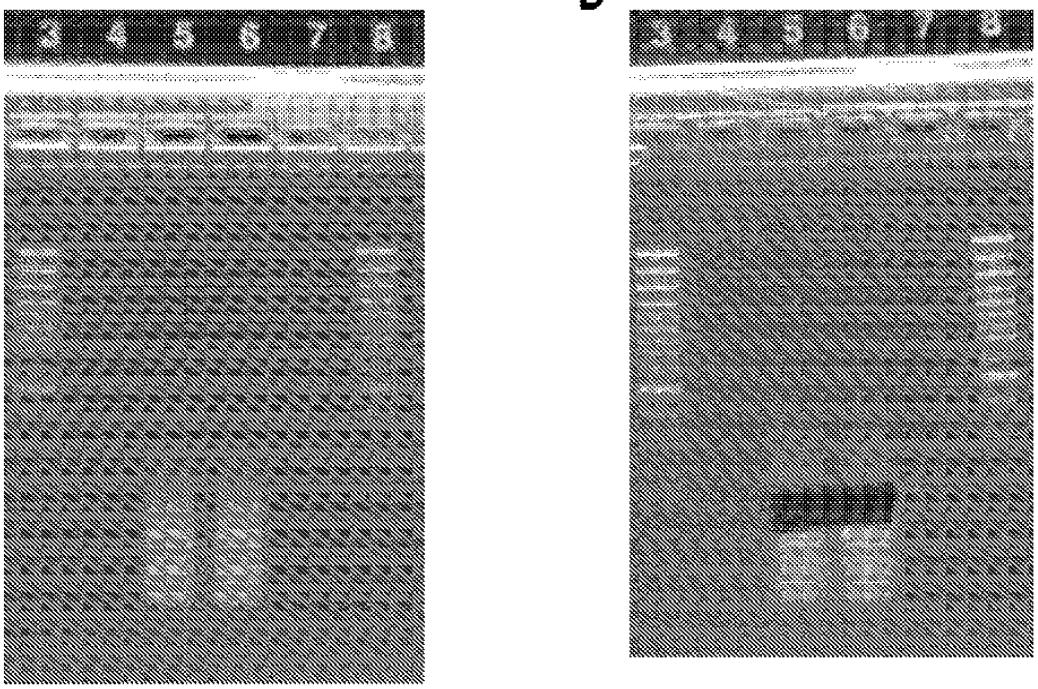


Figure 7

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Figure 8

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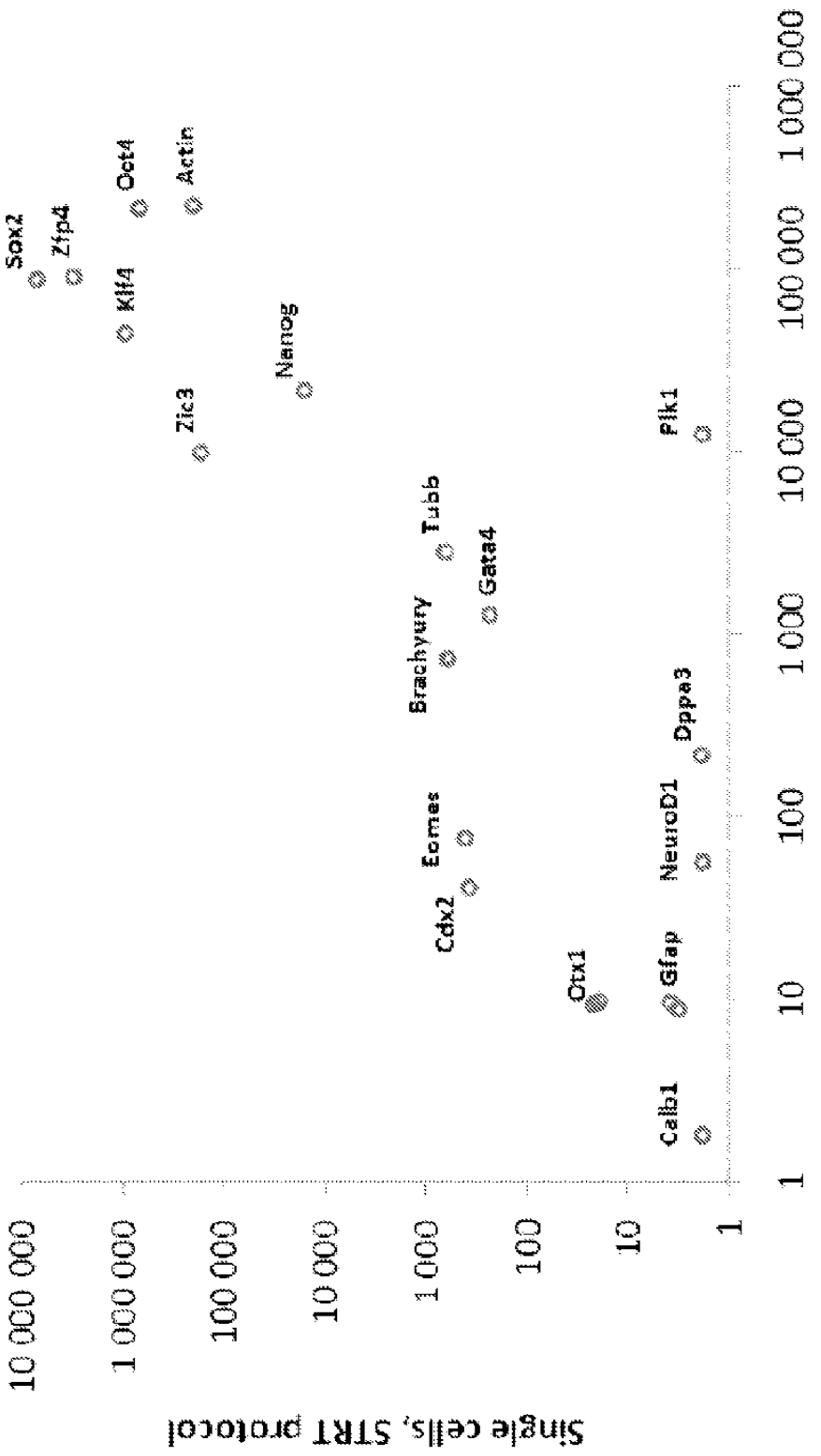


Figure 9

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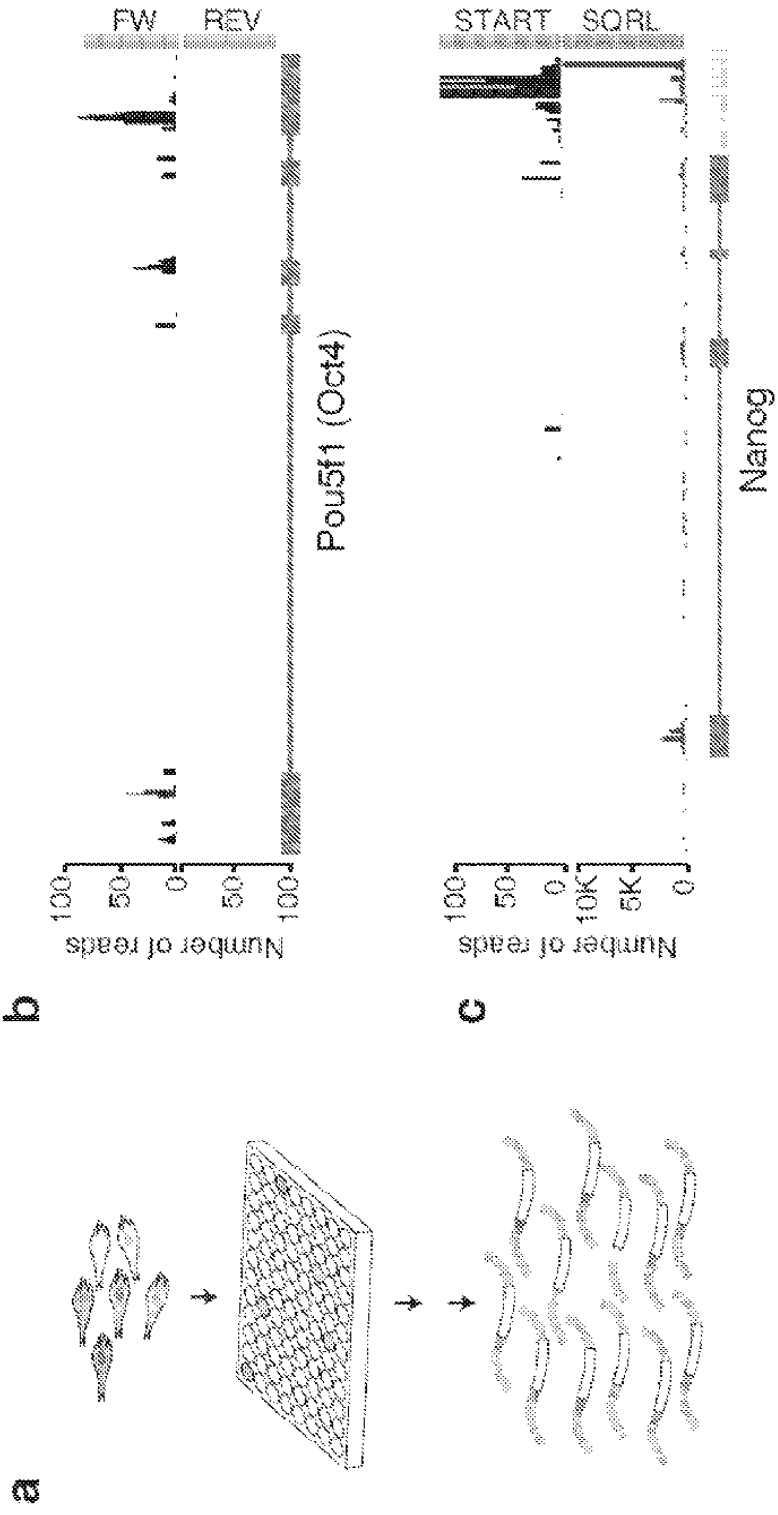


Figure 10A-C

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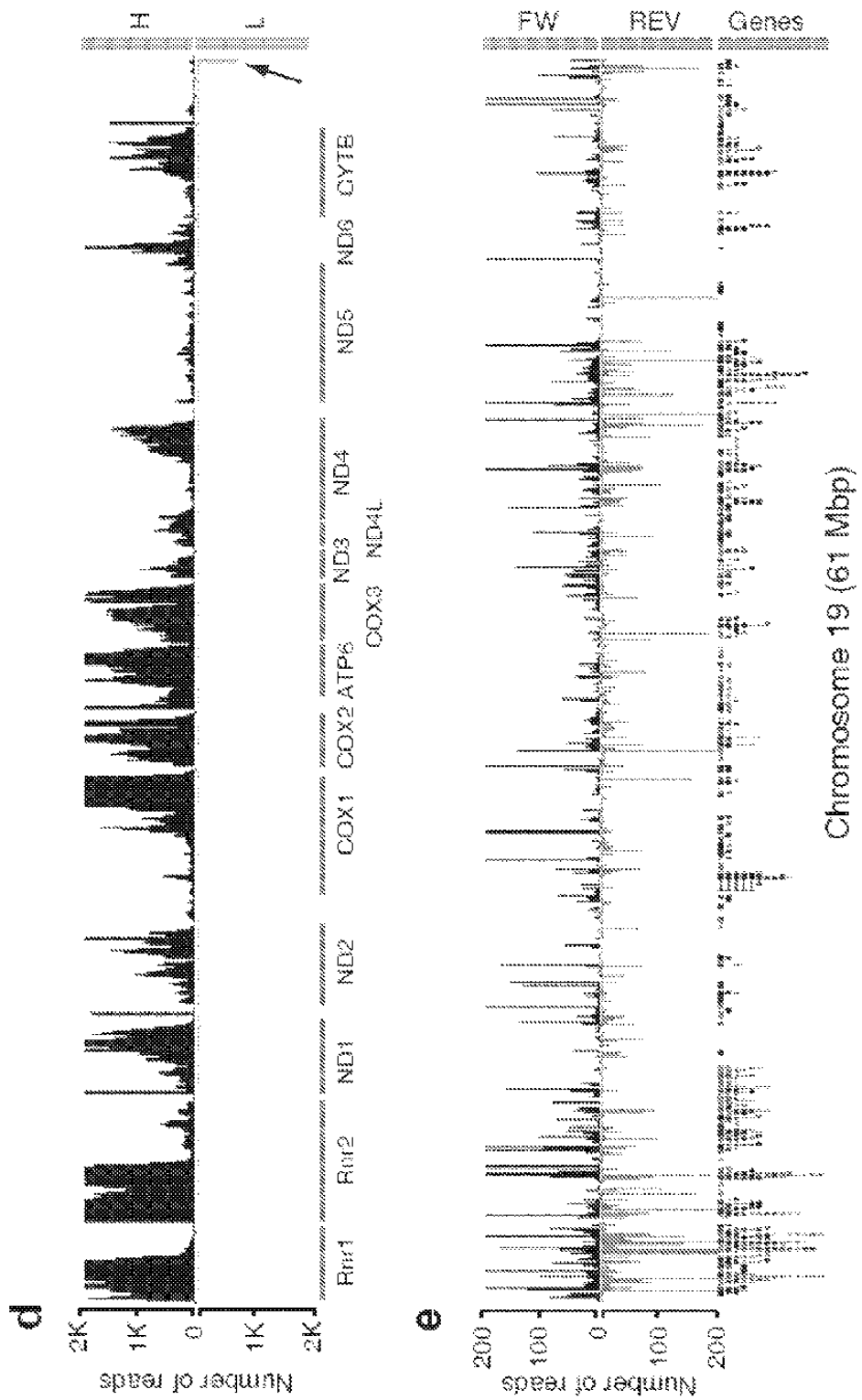


Figure 10D-E

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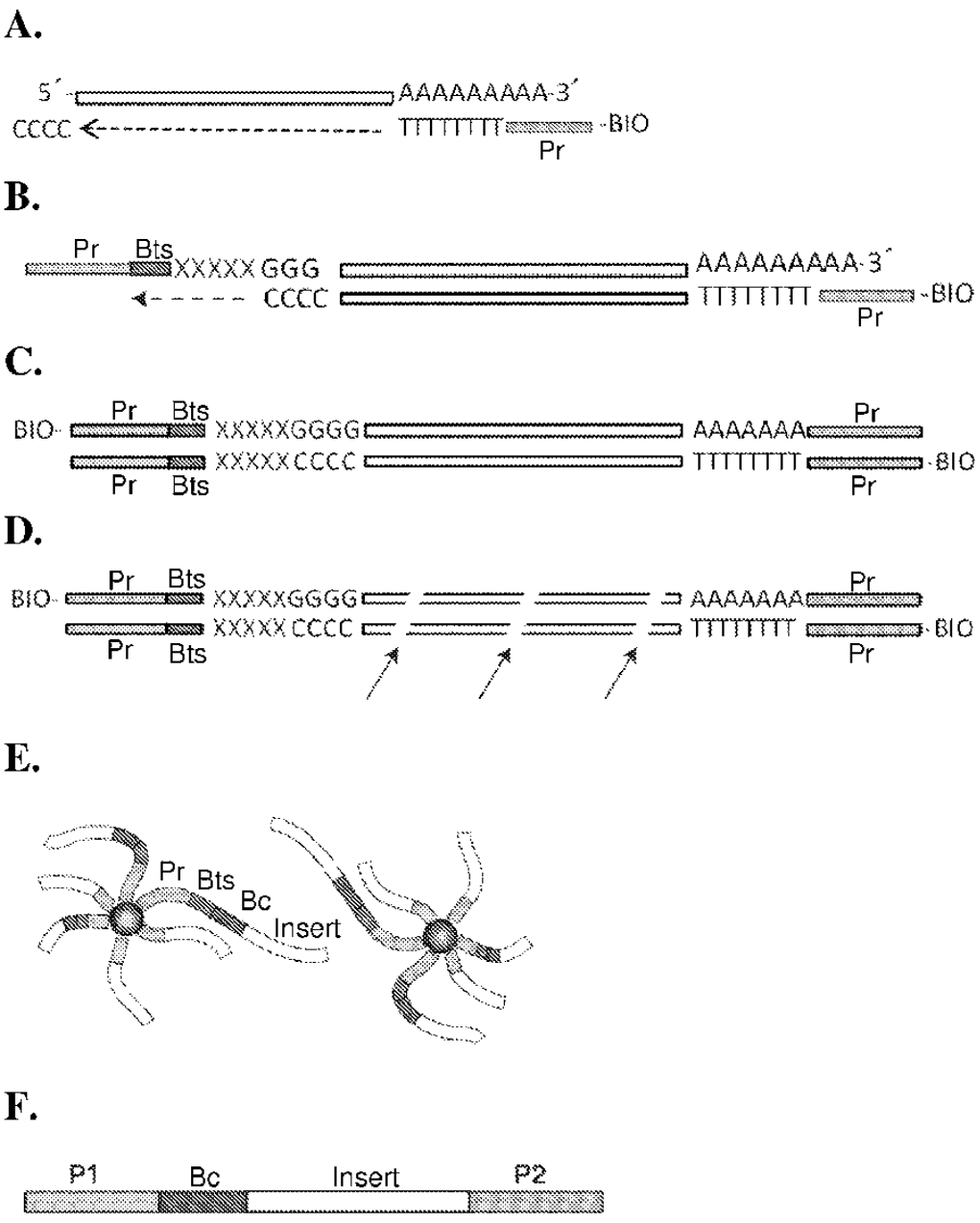


Figure 11

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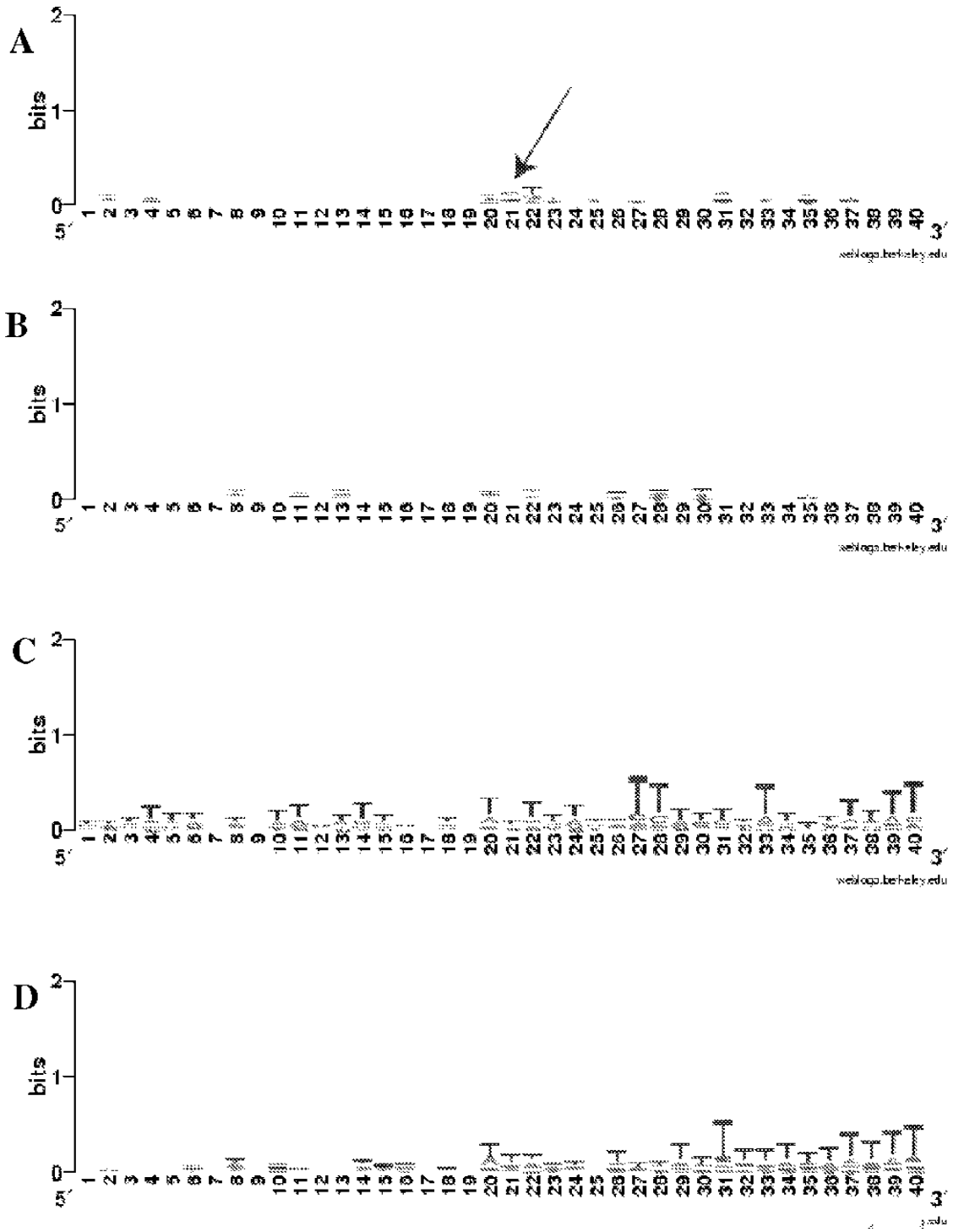


Figure 12

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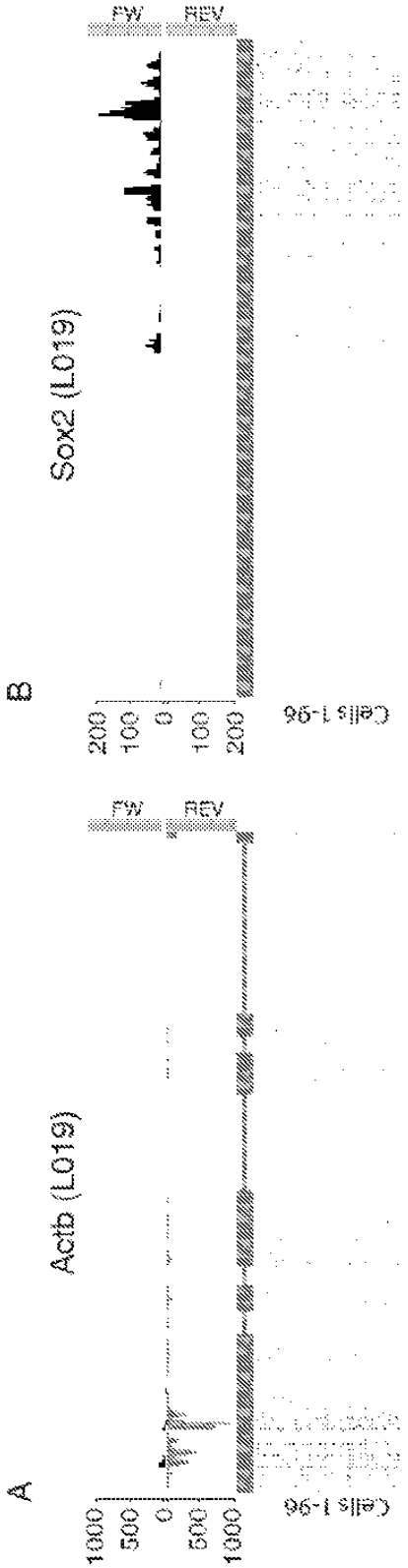


Figure 13

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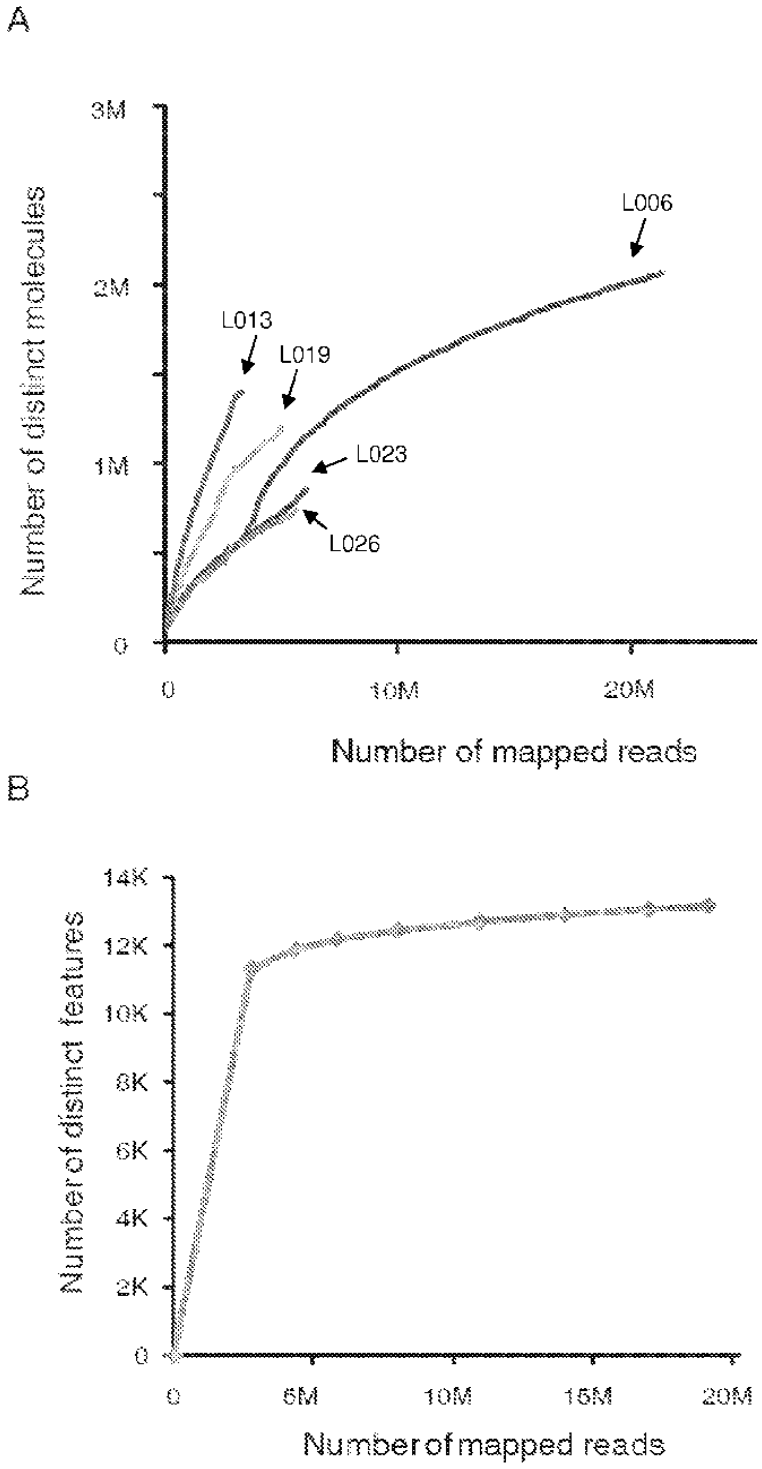


Figure 14

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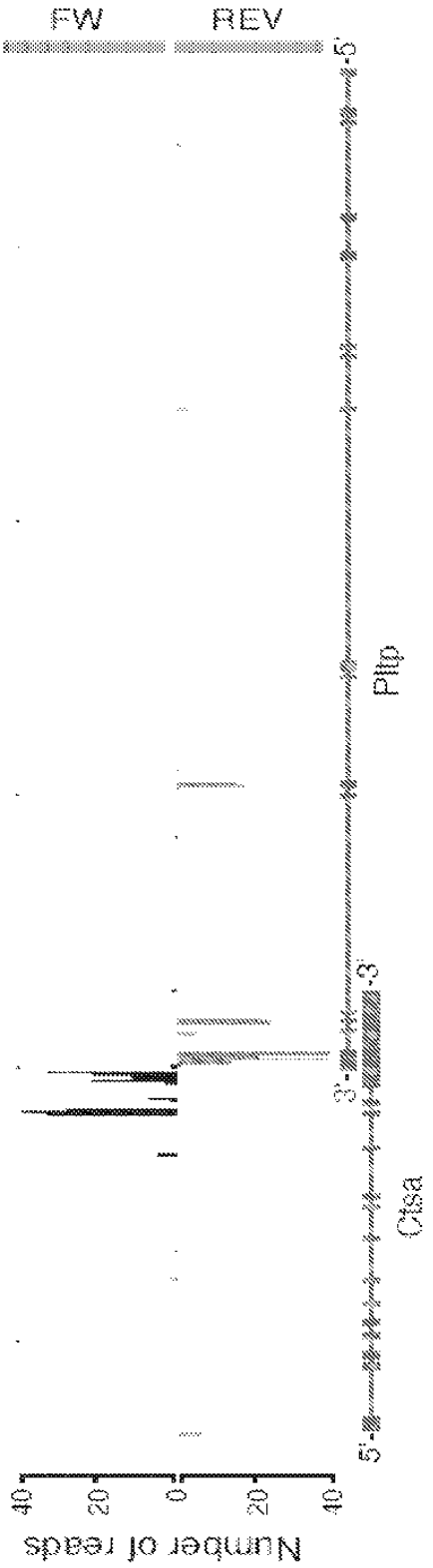


Figure 15

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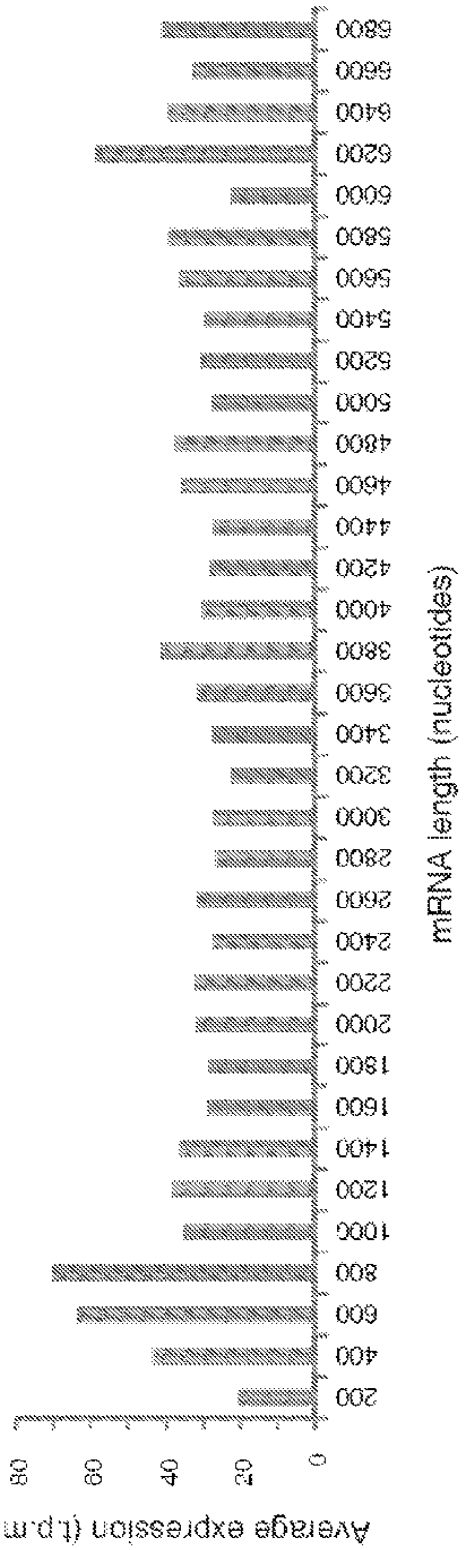


Figure 16

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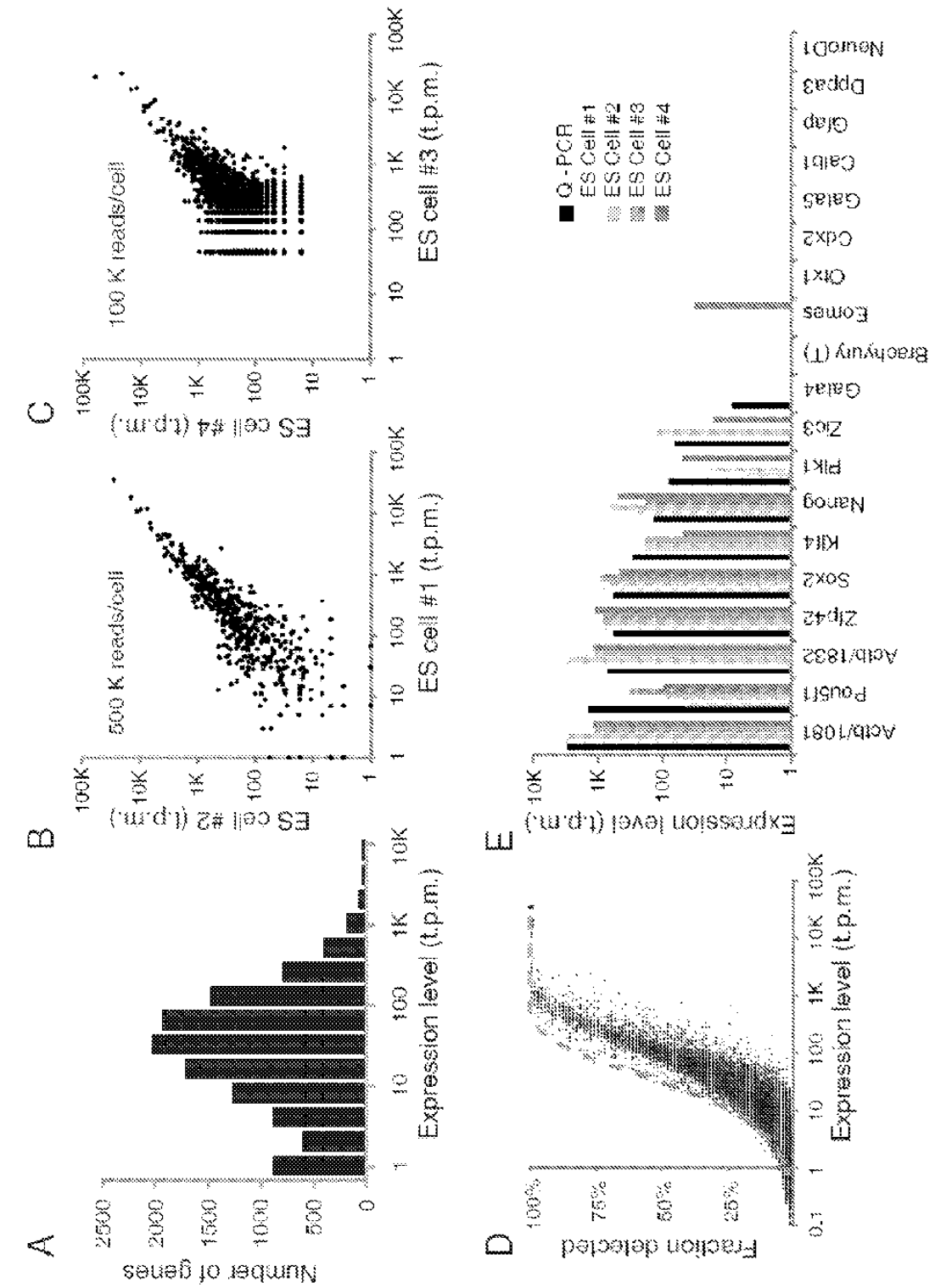


Figure 17

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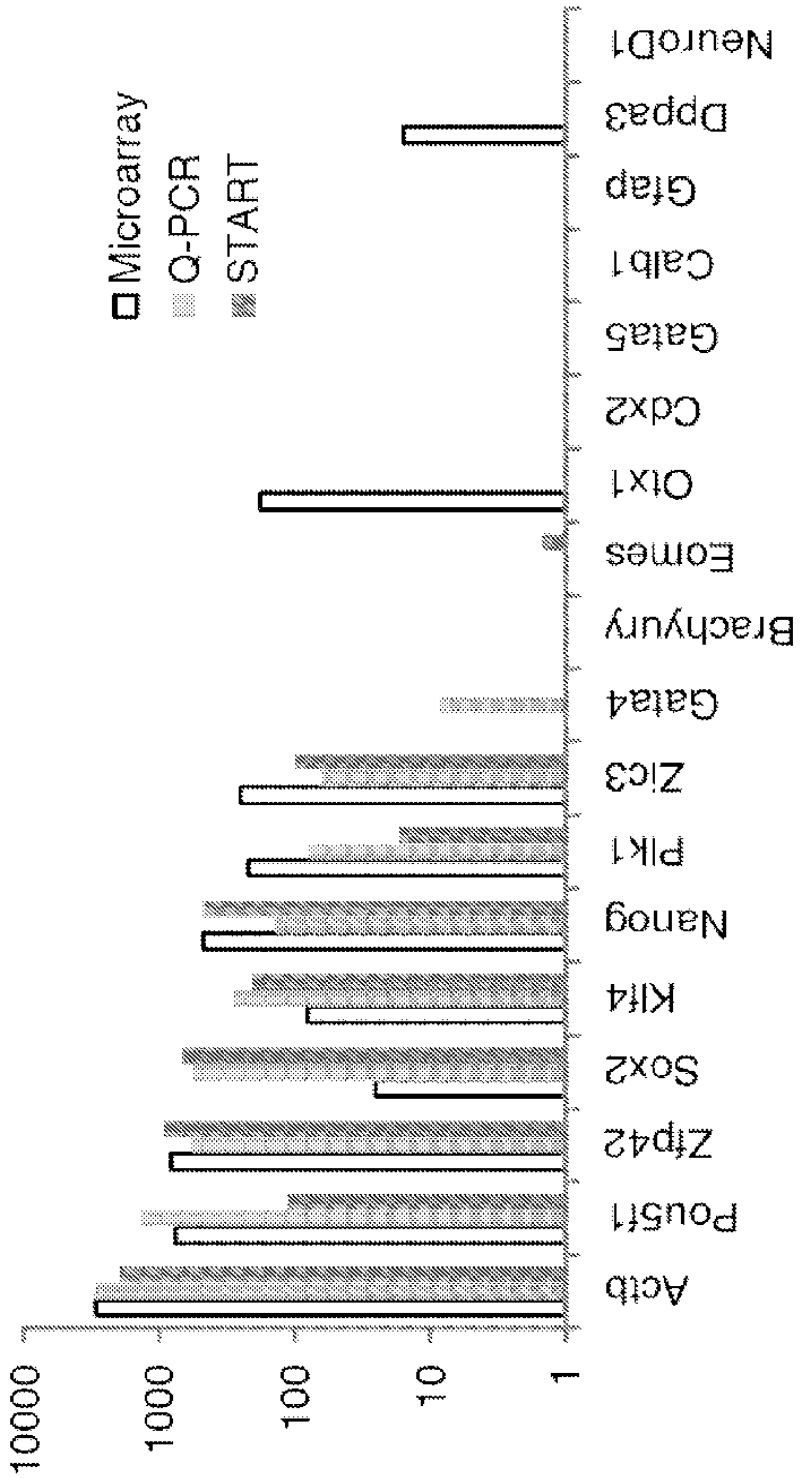


Figure 18

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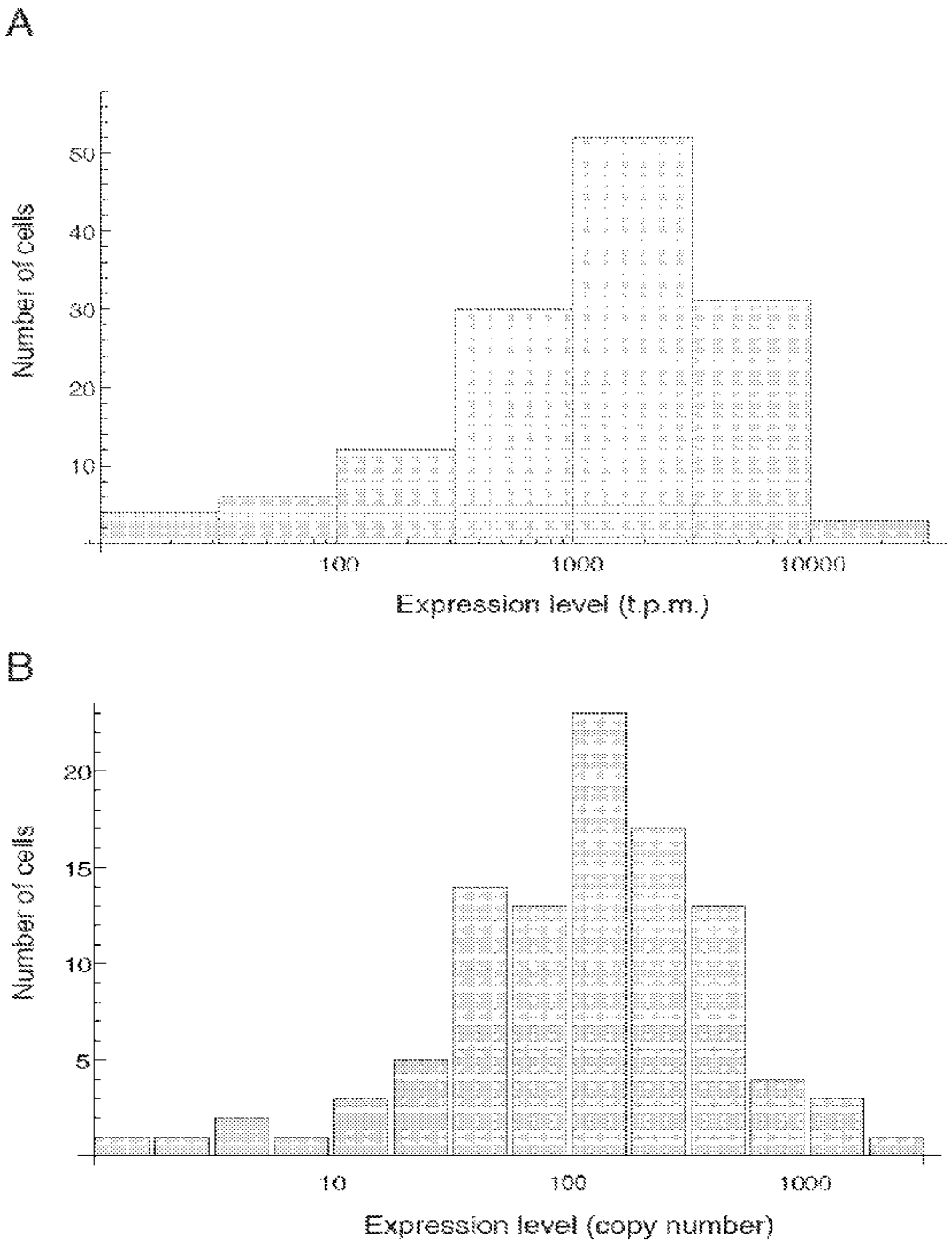


Figure 19

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 01031

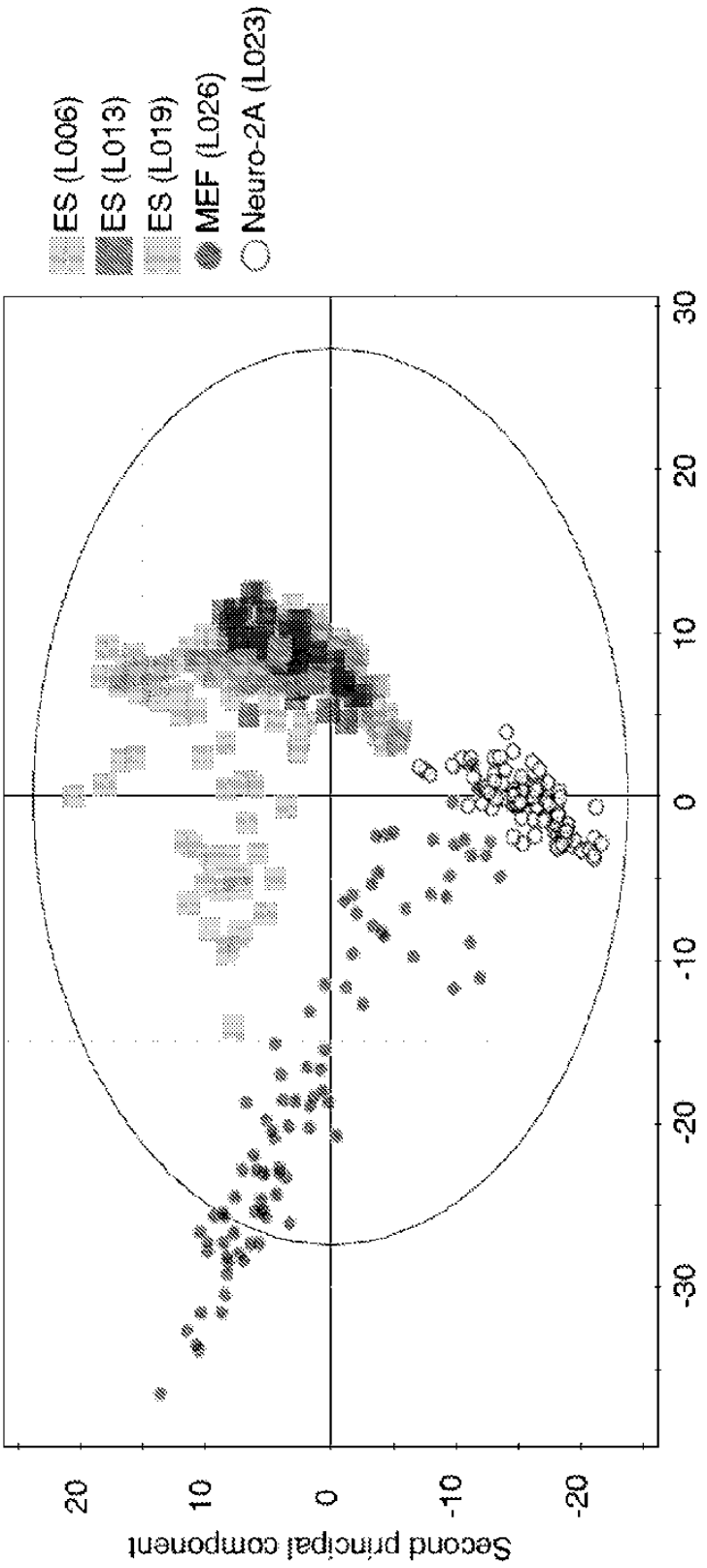


Figure 20

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 01032

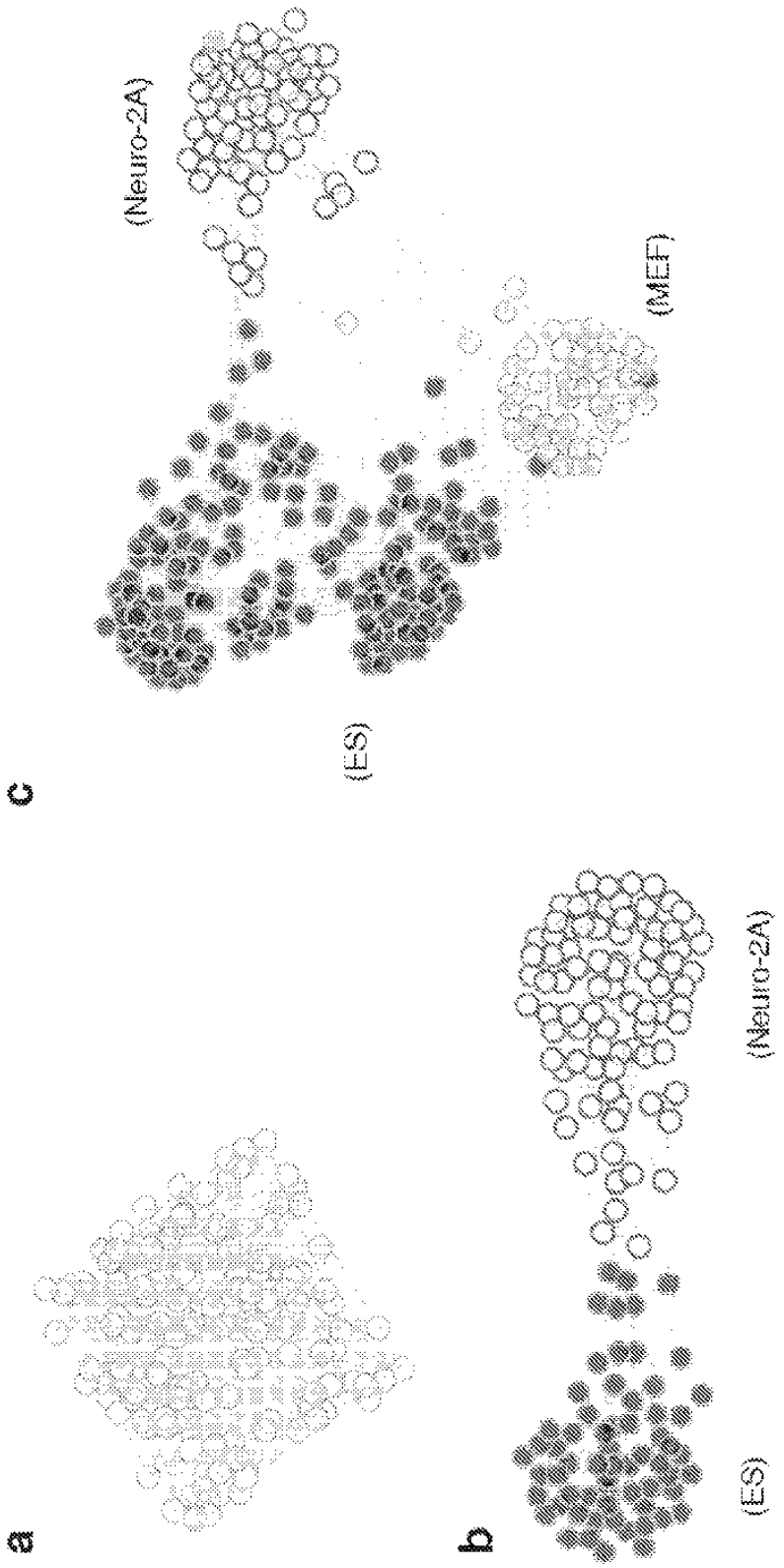


Figure 21

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 01033

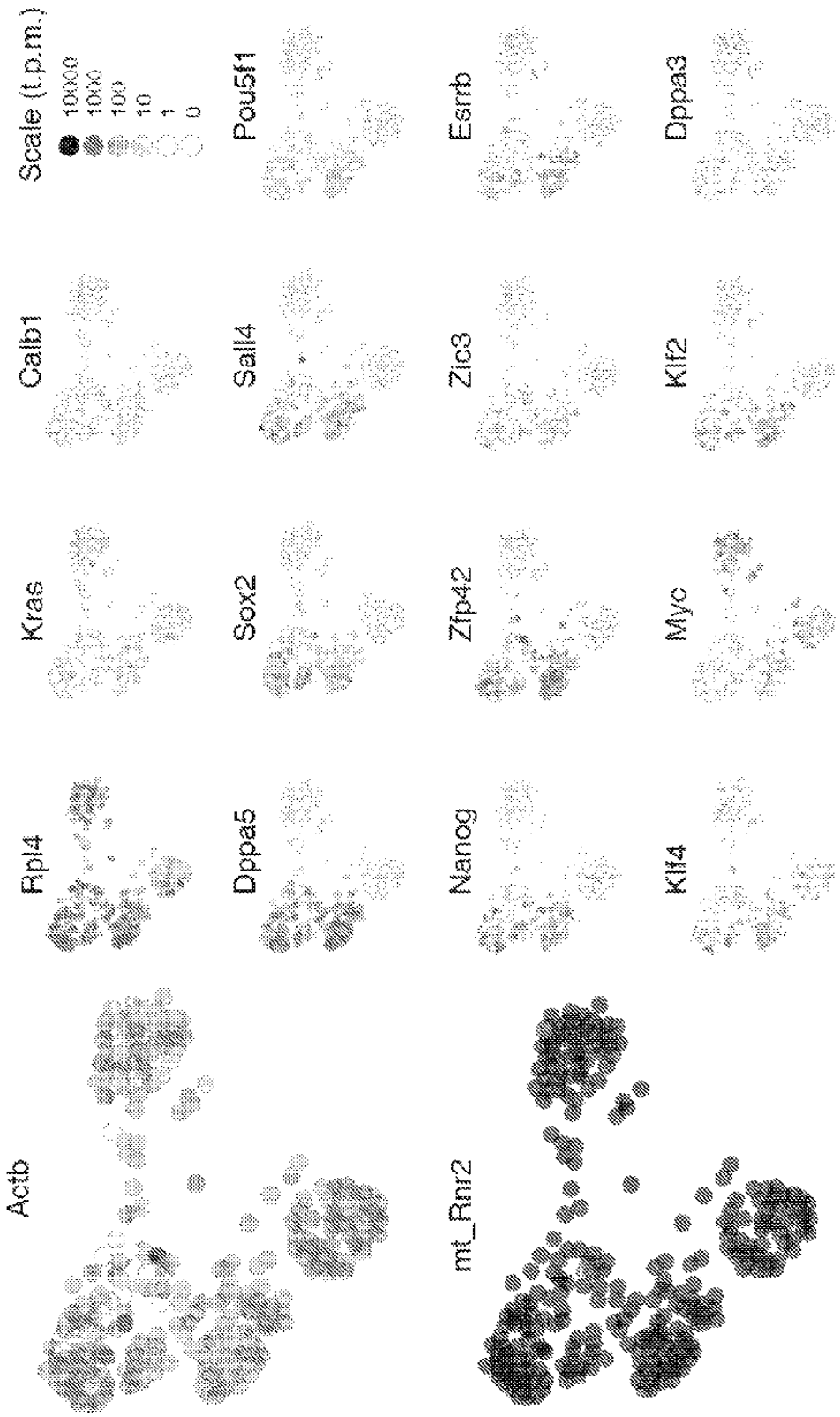


Figure 22

10x Genomics, Inc. Exhibit 2004
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 01034

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Conf. No.: **9697**
Sidney BRENNER	Art Unit: **1634**
Application No.: **14/792,094**	Examiner: **Narayan Kameshwar Bhat**
Filed: **July 6, 2015**	Docket No.: **104371-5016-US05**
For: **Compositions and Methods for Intramolecular Nucleic Acid Rearrangement**	*Certificate of Transmission (37 C.F.R. § 1.8)* *I hereby certify that this correspondence is being transmitted to the U.S. Patent and Trademark Office via the Office electronic filing system (EFS-Web) to the USPTO on August 30, 2017.* / Sheila R. Gloss / *Sheila R. Gloss*

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Alexandria, VA 22313-1450

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Commissioner:

This Information Disclosure Statement is hereby submitted in accordance with 37 C.F.R. 1.98 and pursuant to Applicant's continuing duty under 37 C.F.R. 1.56 to bring any information which may be material to patentability of this application to the Examiner's attention.

Applicant makes no representation that a search has been conducted by the Applicant, or that there is not possibly more relevant art. 37 C.F.R. § 1.97(g). Applicant also makes no representation that the information submitted herewith is in fact material to patentability. The filing of this Information Disclosure Statement shall not be construed as an admission against interest in any manner. 37 C.F.R. § 1.97(h).

The Examiner's attention is directed to the reference(s) cited on the accompanying substitute for forms 1449A/PTO and PTO/SB/08. Except for U.S. Patents and U.S. Patent Application Publications, *see* 37 C.F.R. § 1.98(a)(2)(i), copies of the cited references are enclosed, unless otherwise noted below.

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IPR2023-00876
Page 01035

This Information Disclosure Statement is being filed within three months of the filing date of a national application other than a continued prosecution application, within three months of the date of entry of a national stage, before the mailing of a first Office action on the merits, or before the mailing of a first Office action after the filing of a request for continued examination. 37 C.F.R. § 1.97(b). No fee is required.

The Commissioner is hereby authorized to charge any underpayment of the following fees associated with this communication, including any necessary fees for the submission of an information disclosure statement under 37 CFR § 1.17(p) or for the extension of time, or credit any overpayment to Deposit Account No. 50-0310.

Respectfully submitted,

Date: August 30, 2017

By: /Gargi Talukder/
Gargi Talukder, Reg. No. 61,368
Filed Under 37 C.F.R. § 1.34

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Fax: 415.442.1001

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00876
Page 01036

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(Substitute PTO/SB/8A)

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet	1	of	1

Complete if Known	
Application Number	14/792,094
Filing Date	July 6, 2015
First Named Inventor	Sidney BRENNER
Art Unit	1634
Examiner Name	Narayan Kameshwar Bhat
Attorney Docket Number	104371-5016-US05

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	U.S. Patent Document Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	A1	US 2006/0177833A1	08-10-2006	Brenner	
	A2	US 7,329,493 B2	02-12-2008	Chou et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[2] Number[3] Kind Code[4] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[5]
	A3	WO 2007147079 A2	12-21-2007	Living Microsystems, Inc. et al.		
	A4	WO 2010117620 A3	10-14-2010	Illumina, Inc.		

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	A5	"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Basic MID Set" Genome Sequencer FLX System, Technnical Bulletin 004-2009, (Apr 1 2009) pp 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09004 Using MultiplexIdentifierAdaptorsForTheGSFLXTitaniumSeriesChemistry-BasicMIDSet.pdf	
	A6	"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Extended MID Set" Genome Sequencer FLX System, Technnical Bulletin 005-2009, (Apr 1 2009) pp 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09005 UsingMultiplexIdentifierAdaptorsForTheGSFLXTitaniumChemistry-ExtendedMIDSet.pdf	
	A7	AHERN, "Biochemical, Reagents Kits Offer Scientists Good Return on Investment" The Scientist (1995) 9(15):1-7	
	A8	REPP et al. "Genotyping by Multiplex Polymerase Chain Reaction for Detection of Endemic Hepatitis B Virus Transmission" J Clinical Microbiology (1993) 31:1095-1102	
	A9	WISEMAN, R.W. et al. "Major histocompatibility complex genotyping with massively parallel pyrosequencing" Nature Medicine (Oct 11 2009) 15(11):1322-1326	
	A10	Xiao, et al, "Determination of haplotypes from single DNA molecules: a method for single-molecule barcoding", Hum Mutat., 28:913-21, 2007.	

Examiner Signature		Date Considered	

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DB2/ 31885247.1

Electronic Patent Application Fee Transmittal

Application Number:	14792094
Filing Date:	06-Jul-2015
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	Gargi Talukder/Sheila Gloss
Attorney Docket Number:	104371-5016-US05

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
RCE- 2ND AND SUBSEQUENT REQUEST	2820	1	850	850
		Total in USD ($)		**850**

Electronic Acknowledgement Receipt	
EFS ID:	30232442
Application Number:	14792094
International Application Number:	
Confirmation Number:	9697
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US05
Receipt Date:	30-AUG-2017
Filing Date:	06-JUL-2015
Time Stamp:	19:53:45
Application Type:	Utility under 35 USC 111(a)

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Payment Type	DA
Payment was successfully received in RAM	$850
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1	Request for Continued Examination (RCE)	104371-5016-US05_RCE.pdf	1349886 1acc27577aa3c6e9195166a46acdd800f7d2982	no	3
Warnings:					
Information:					
2		104371-5016-US05_Response.pdf	180562 470384200d4172a31a65aa8d585e006dfe5566d5	yes	6
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Response After Final Action		1	1	
	Claims		2	3	
	Applicant Arguments/Remarks Made in an Amendment		4	6	
Warnings:					
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3	Foreign Reference	WO2007147079-PAMPH-618.pdf	6657853 31e9a83321056ca6ce9e5679aa65560f62779a2	no	99
Warnings:					
Information:					
4	Foreign Reference	WO2010117620-PAMPH-142.pdf	7973504 5aac1ce727109a9f8320ddaa6d025612037c70c7	no	71
Warnings:					
Information:					
5	Non Patent Literature	BasicMIDSet.pdf	614106 30f6a99ffeca354ed45046f40f6648644eaca99c13	no	11

Warnings:					
Information:					
6	Non Patent Literature	UsingMIDAdapters_TechnicalBulletin.pdf	500692 8f52d<<<9adaace92e9382aaa33ed4aa259d08	no	7
Warnings:					
Information:					
7	Non Patent Literature	AHERN.pdf	538716 e2020218a32e32aaae9cf1af1df399d10650ce65	no	7
Warnings:					
Information:					
8	Non Patent Literature	REPP.pdf	789531 fe12aa6a8e9878a5e917118565cdffa9efa6a5d	no	9
Warnings:					
Information:					
9	Non Patent Literature	WISEMAN_RW.pdf	1284288 4172e8a20aa2054a3f0d0acd5cd33e8ce6e62f30	no	14
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10	Non Patent Literature	XIAO.pdf	884830 01e5da1e6510be44ad749a21ac42afe95958e87	no	9
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PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/792,094	Filing Date 07/06/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	140
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 = *		X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 = *		X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	140

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)		
	08/30/2017	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	* 9	Minus	** 20	= 0	X $40 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $210 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)		
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

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This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Substitute for form 1449A/PTO (Substitute PTO/SB/8A)				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	**14/792,094**
				Filing Date	**July 6, 2015**
				First Named Inventor	**Sidney BRENNER**
(use as many sheets as necessary)				Art Unit	**1634**
				Examiner Name	**Narayan Kameshwar Bhat**
Sheet	1	of	1	Attorney Docket Number	**104371-5016-US05**

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	U.S. Patent Document Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	A1	US 2006/0177833A1	08-10-2006	Brenner	
	A2	US 7,329,493 B2	02-12-2008	Chou et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[2] Number[4] Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
	A3	WO 2007147079 A2	12-21-2007	Living Microsystems, Inc. et al.		
	A4	WO 2010117620 A3	10-14-2010	Illumina, Inc.		

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[6]
	A5	"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Basic MID Set" Genome Sequencer FLX System, Technnical Bulletin 004-2009, (Apr 1 2009) pp 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09004 Using MultiplexIdentifierAdaptorsForTheGSFLXTitaniumSeriesChemistry-BasicMIDSet.pdf	
	A6	"Using Multiplex Identifier (MID) Adaptors for the GS FLX Titanium Chemistry Extended MID Set" Genome Sequencer FLX System, Technnical Bulletin 005-2009, (Apr 1 2009) pp 1-7. URL:http://454.com/downloads/my454/documentation/technical-bulletins/TCB-09005 UsingMultiplexIdentifierAdaptorsForTheGSFLXTitaniumChemistry-ExtendedMIDSet.pdf	
	A7	AHERN, "Biochemical, Reagents Kits Offer Scientists Good Return on Investment" The Scientist (1995) 9(15):1-7	
	A8	REPP et al. "Genotyping by Multiplex Polymerase Chain Reaction for Detection of Endemic Hepatitis B Virus Transmission" J Clinical Microbiology (1993) 31:1095-1102	
	A9	WISEMAN, R.W. et al. "Major histocompatibility complex genotyping with massively parallel pyrosequencing" Nature Medicine (Oct 11 2009) 15(11):1322-1326	
	A10	Xiao, et al, "Determination of haplotypes from single DNA molecules: a method for single-molecule barcoding", Hum Mutat., 28:913-21, 2007.	

Examiner Signature	/NARAYAN BHAT/	Date Considered	09/13/2017

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☐ A 3D digital atlas of C. elegans and its application to **single-cell** analyses.

1. Long F, Peng H, Liu X, Kim SK, Myers E.
Nat Methods. 2009 Sep;6(9):667-72. doi: 10.1038/nmeth.1366. Epub 2009 Aug 16.
PMID: 19684595 **Free PMC Article**

☐ Graded information extraction by neural-network dynamics with multihysteretic neurons.

2. Tsuboshita Y, Okamoto H.
Neural Netw. 2009 Sep;22(7):922-30. doi: 10.1016/j.neunet.2009.07.005. Epub 2009 Jul 16.
PMID: 19632088

☐ A toolbox for the fast information analysis of multiple-site LFP, EEG and spike train recordings.

3. Magri C, Whittingstall K, Singh V, Logothetis NK, Panzeri S.
BMC Neurosci. 2009 Jul 16;10:81. doi: 10.1186/1471-2202-10-81.
PMID: 19607698 **Free PMC Article**

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4. Dupuy F, Casas J, Bagnères AG, Lazzari CR.
J Neurosci Methods. 2009 Oct 15;183(2):195-201. doi: 10.1016/j.jneumeth.2009.06.031. Epub 2009 Jul 5.
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☐ DNA-**barcode** directed capture and electrochemical metabolic analysis of **single** mammalian **cells** on a microelectrode array.

5. Douglas ES, Hsiao SC, Onoe H, Bertozzi CR, Francis MB, Mathies RA.
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☐ **Automatic** summarization of changes in biological image sequences using algorithmic information theory.

6. Cohen AR, Bjornsson CS, Temple S, Banker G, Roysam B.
IEEE Trans Pattern Anal Mach Intell. 2009 Aug;31(8):1386-403. doi: 10.1109/TPAMI.2008.162.
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☐ Chemogenomic approaches to elucidation of gene function and genetic pathways.

7. Pierce SE, Davis RW, Nislow C, Giaever G.
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PMID: 19521822

☐ Feature extraction from spike trains with Bayesian binning: 'latency is where the signal starts'.

8. Endres D, Oram M.
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IPR2023-00876
Page 01046

9. Species identification through DNA "barcodes".
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Genet Test Mol Biomarkers. 2009 Jun;13(3):421-6. doi: 10.1089/gtmb.2008.0144.
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10. Issuing of blood components dispensed in syringes and bar code-based pretransfusion check at the bedside for pediatric patients.
Ohsaka A, Abe K, Nakamura Y, Ohsawa T, Sugita S.
Transfusion. 2009 Jul;49(7):1423-30. doi: 10.1111/j.1537-2995.2009.02164.x. Epub 2009 Apr 3.
PMID: 19374727

11. MetaboliteDetector: comprehensive analysis tool for targeted and nontargeted GC/MS based metabolome analysis.
Hiller K, Hangebrauk J, Jäger C, Spura J, Schreiber K, Schomburg D.
Anal Chem. 2009 May 1;81(9):3429-39. doi: 10.1021/ac802689c.
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Genomics, gene expression and DNA arrays
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... Now that **nucleic** acid arrays have been constructed for many different organisms14,2629 and ... strain corresponds to a specific location on an array that contains **oligonucleotide** probes that ... first is a PCR-based approach that has been used to make **single-cell cDNA** libraries7678 ...
Cited by 2399 Related articles All 24 versions Cite Save

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Cited by 328 Related articles All 7 versions Cite Save

Single-cell gene expression profiling
JM Levsky, SM Shenoy, RC Pezo, RH Singer - Science, 2002 - science.sciencemag.org
... Transcriptional regulation has been studied almost exclusively on **nucleic** acids extracted from cultured cells or ... 1B). The color combination, or spectral **barcode**, defined transcript identity (Fig. ... a direct comparison of data based on extracted RNA with **single-cell** expression levels ...
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Cited by 175 Related articles All 6 versions Cite Save

Immuno-PCR: high sensitivity detection of proteins by **nucleic** acid amplification
CM Niemeyer, M Adler, R Wacker - TRENDS in Biotechnology, 2005 - Elsevier
... In immuno-rolling circle amplification (RCA) technology, an **oligonucleotide** primer, covalently attached to the ... been intensively investigated for their material properties and applications in **nucleic** acid analysis ... from the gold nanoparticle, and the released DNA **barcode** is used to ...
Cited by 295 Related articles All 8 versions Cite Save

Molecular encoding of **nucleic** acid templates for PCR and other forms of sequence analysis
M McCloskey, C Laird, R Hansen, R Stoger... - US Patent App. 11/ ..., 2005 - Google Patents
... under suitable conditions to anneal the bar-coded **oligonucleotide** to the target **nucleic** acid molecule, wherein the bar-coded **oligonucleotide** comprises a first sequence complementary to the target **nucleic** acid molecule, a second sequence providing a random **barcode**, and a ...
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Nanoparticles and their biological and environmental applications
WT Liu - Journal of Bioscience and bioengineering, 2006 - Elsevier
... Usually, they are derivatized with different functional groups such as **nucleic** acid-targeted ... specific DNA duplex were released into solution and the amount of **oligonucleotide** released was ... can be further developed to achieve rapid bacterial detection at the **single-cell** level (35). ...
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Cancer nanotechnology: opportunities and challenges
M Ferrari - Nature reviews. Cancer, 2005 - search.proquest.com
... as a result of affinity binding and as a result of **nucleic**-acid hybridization ... precise map of the distribution of many molecular markers in a **single cell**, cell population or ... developed by Chad Mirkin and colleagues10.Dubbed bio-**barcode**, it involves **oligonucleotide**-modified gold ...
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Miniaturised **nucleic** acid analysis
PA Auroux, Y Koc, A Manz, PJR Day - Lab on a Chip, 2004 - pubs.rsc.org
... comparing poly(A) tails and random hexamers for **cDNA** synthesis were ... applied dielectrophoresis and electrical impedance spectroscopy for **single cell** manipulation within microfluidic ... DNAzyme, a catalytically active **oligonucleotide** capable of cleaving **nucleic** acid substrates ...
Cited by 251 Related articles All 3 versions Cite Save

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F Vigneault, AM Sismour, GM Church - Nature methods, 2008 - nature.com
... RNA or DNA-RNA chimera), and observed that the RNA and chimera adaptor successfully ligated to the 5' end of the synthetic miRNA whereas the DNA **oligonucleotide** did not ... **Nucleic** Acids Res. ... Systematic and **single cell** analysis of Xenopus Piwi-interacting RNAs and Xiwi ...
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The current status of **cDNA** cloning
M Harbers - Genomics, 2008 - Elsevier
... **cDNA** synthesis from whole RNA is primed by an **oligonucleotide** containing a T7 RNA ... procedure have been published to enable full-length **cDNA** cloning after **cDNA** tailing by ... An alternative approach to **single-cell cDNA** library preparation makes use of oligo(dT) primers linked ...
Cited by 37 Related articles All 7 versions Cite Save

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A Watson, A Mazumder, M Stewart... - Current opinion in ..., 1998 - Elsevier
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Mammalian RNAi: a practical guide
P Sandy, A Ventura, T Jacks - Biotechniques, 2005 - researchgate.net
... red label for control cells) and the products are hybridized to the **barcode oligonucleotide** array (4 ... The **cDNA** of a target gene flanked by recombination (att) sites is cloned downstream of ... cassettes can be directly injected into the perivi- telline space of **single-cell** mouse embryos ...
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From **single cell** gene-based diagnostics to diagnostic genomics: current applications and future perspectives
R Zhao - Clinical Laboratory Science, 2005 - search.proquest.com
... of genomic and proteomic approaches especially in pure cell types or even in the **single-cell** level, which ... **Nucleic** acid sequence based amplification (NASBA). ... reverse transcription from an RNA template by AMV reverse transcriptase and RNA amplification from **cDNA** by gene ...
Cited by 11 Related articles All 6 versions Cite Save

Methods, flow cells and systems for **single cell** analysis
R Joseph, JJ DiMeo, M Jarosz... - US Patent App. 12/ ..., 2009 - Google Patents
... Methods for preparing a **nucleic** acid sample from a **single cell** and capturing **nucleic** acids ... function as both surface capture and sequencing primers, especially when an **oligonucleotide** containing a ... Optionally, the sample **nucleic** acid fragments can be attached to a surface via ...
Cited by 15 Related articles All 4 versions Cite Save

Homogeneous methods for **nucleic** acid amplification and detection
RG Higuchi - US Patent 5,994,056, 1999 - Google Patents
... a sample is provided which contains, or is suspected of containing, a particular **oligonucleotide** sequence of ... For example, in a PCR method where the target **nucleic** acid is single-stranded DNA, such as mRNA, the target may be first reverse-transcribed into **cDNA**, prior to ...
Cited by 266 Related articles All 2 versions Cite Save

Multiplexed and microparticle-based analyses: Quantitative tools for the large-scale analysis of biological systems
JP Nolan, F Mandy - Cytometry Part A, 2006 - Wiley Online Library
... hybridization to any other **oligonucleotide** in the set (54, 55), Microspheres bearing such **oligonucleotide** tags offer ... The examples described earlier for immunoassays and **nucleic** acid analyses represent the best-developed uses of ... Quantitative **single cell** analysis and sorting. ...
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Microfluidic device for analyzing **nucleic** acids and/or proteins, methods of preparation and uses thereof

F Chatelain, FW Frueh - US Patent App. 10/139,100, 2002 - Google Patents

... a 3-ethoxy-aminopropyl-silanol group, from which will be extended the **oligonucleotide** chain terminating **...** [0093]. The present invention thus relates to a method for analyzing **nucleic** acids and **...** in very small amounts, such as when working with extracts obtained from a **single cell**. **...**

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Single-cell fluorescence imaging using metal plasmon-coupled probe 2: single-molecule counting on lifetime image

J Zhang, Y Fu, D Liang, K Nowaczyk, RY Zhao... - Nano ..., 2008 - ACS Publications

... Single-Cell Fluorescence Imaging Using Metal Plasmon-Coupled Probe 2: Single-Molecule Counting on Lifetime Image. **...**

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L1	14828	(barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 (distinct or different).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 08:57
L2	6654	(cdna or nucleic or dna or polynucleotide)same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 08:58
L3	0	2 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)same (different or distinct) same each.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 08:59
L4	1574	2 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)same (different or distinct).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:00
L5	52	4 and "single cell".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:00
L6	96	2 and "single cell".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:04
L7	13	6 and pool$3 near5 (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/13 09:05
S1	1445	illumina.as. or linnarsson.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 18:25
S2	6	S1 and "single cell" same (mrna or nucleic or dna or polynucleotide or	US-PGPUB; USPAT; USOCR;	OR	ON	2017/09/12 18:27

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		ribonucleic).clm.	FPRS; EPO; JPO; DERWENT; IBM_TDB			
S3	30651	"10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:39
S4	3	S3 and "single cell" same (mrna or nucleic or dna or polynucleotide or ribonucleic).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:40
S5	343	"single cell" same (mrna or nucleic or dna or polynucleotide or ribonucleic).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:41
S6	6	S5 and (mid or "multiplex identifier").clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:42
S7	4833	"single cell" near5 (mrna or nucleic or dna or polynucleotide or ribonucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:44
S8	219	S7 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 "single cell"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:46
S9	166	S8 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 (mrna or nucleic or dna or polynucleotide or ribonucleic)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:48
S10	118	S9 and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) near5 (distinct or different)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/12 21:48

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	4	("10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.) and ("single cell" or "one cell" or "individual cell") same (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 08:57
L2	89	("10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.) and (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:00
L3	4	2 and ("single cell" or "one cell" or "individual cell") same (nucleic or dna or polynucleotide or cdna).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:01
L4	1	2 and ("single cell" or "one cell" or "individual cell") same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:04
L5	1	("10X GENOMICS".as. or brenner.in. or mikawa.in. or osborne.in. or slatter.in. or "population genetics technologies".as.) and ("single cell" or "one cell" or "individual cell") same ("single cell" or "one cell" or "individual cell") same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:05
L6	176	("single cell" or "one cell" or "individual cell") same ("single cell" or "one cell" or "individual cell") same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:06
L7	36	6 and (nucleic or dna or cdna) same (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3) same (identif$6 or specific).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:09

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L8	671	"cell specific" near5 (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:20
L9	25	8 and (dna or nucleic or cdna or polynucleotide) near5 specific near5 (barcode or "bar code" or "multiplex identifier" or zipcode or tag or token or addressable or uniqu$3)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2017/09/14 09:22

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 09/20/2017
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

EXAMINER	
BHAT, NARAYAN KAMESHWAR	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
09/20/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

sfipdocketing@morganlewis.com
donald.mixon@morganlewis.com

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Office Action Summary	Application No. 14/792,094	Applicant(s) Brenner et al.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 8/30/2017 .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☑ Claim(s) 56-64 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☑ Claim(s) 56-64 is/are rejected.

8)☑ Claim(s) 63-64 is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 06 July 2015 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____ .

3) ☑ Interview Summary (PTO-413) Paper No(s)/Mail Date _____ .

4) ☐ Other: _____ .

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Continued Examination under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 8/30/2017 has been entered.

2. The present application is being examined under the pre-AIA first to invent provisions. The instant application is a continuation of 14/172,694 now U.S. Patent 9,102,980, which is a continuation of 14/021,790, now U.S. Patent 8,679,756, which is a continuation of 13/859,450 now U.S. Patent 8,563,274, which is a continuation of 13/622,872 now abandoned, which is a continuation of 13/387,343, now U.S. Patent 8,298,767, which is a national stage entry of PCT/IB2010/002243, filed on 08/13/2010, claiming priorities from provisional applications 61288792 filed on 12/21/2009, 61235595 filed on 08/20/2009.

It is noted that none of the provisional applications provide support for the limitation of "*second multiplex identifier sequences are all different from each other*". *Therefore the priority for the instant application is 08/13/2010*.

3. This office action is in response to the papers filed on 8/30/2017.

Track1 request

4. Track1 request filed by the applicant on December 22, 2016 has been granted by the office on January 10, 2017. Therefore instant application has been examined on

a priority basis. To maintain its status applicant is required to follow the requirements listed in the Track 1 grant document.

Interview summary

5. In view of instant application being part of family of patents, for compact prosecution, the examiner contacted the applicant's representative Ms. Talukder telephonically on 9/8/2017 to inform that further amendments are needed to overcome the potential rejections under 35 USC 112 first and second paragraphs and to fix the minor informalities.

The examiner suggested to further clarifications in claim 56 are needed to specify that the plurality of nucleic acid segments are made from each of the single isolated cell, wherein the plurality of nucleic acid segments from each single cell have cell specific multiplex identifier and different sizes of the nucleic acids from each cell have different multiplex identifier consistent with results schematically presented in figures 11 and 12 (top panel). The examiner also informed that claim 56 as recited is not clear how plurality of nucleic acid samples from plurality of single isolated cell being tagged with first and second multiplex identifiers capable identifying origin of nucleic acid sample each of the single isolated cell.

The amendments to include the step of isolating nucleic acids from each of the isolated single cell separately and appending nucleic acid sample with cell specific identifier were discussed. But no specific amendments to clarify how second MID (multiplex identifier) are different from each other were discussed in view of 35 USC 112 rejections are not being on the record. In view of time constraint to move the instant

application on an expedited basis on the part of the examiner and to get specific claim language approval from the applicant, the examiner informed that the office action will be issued to decide further course of action. No specific claim languages were agreed to during the interview.

Claim status

6. In the claim listing of 8/30/2017 claims 56-64 are pending in this application and are under prosecution. Claims 56-64 are new. Claims 1-55 are canceled. The claim amendments have been reviewed and entered.

Withdrawn Rejections and Response to the Remarks

6. The previous rejection of claims 40-55 under pre-AIA 35 USC 103(a) as being unpatentable over Gillevet in view of Wangh has been withdrawn in view of cancelation of said claims. The arguments regarding the teachings of Gillevet and Wangh have been fully considered (Remarks, pgs. 4 and 5). They are moot in view of withdrawn rejection necessitated by the cancelation of said claims.

7. All pending ODP rejections on the record have been withdrawn in view of cancellation of instant claims 11-44.

However, upon further consideration, new grounds of rejections are set forth in this office action as discussed below.

Claim Objections

8. Claims 63 and 64 are objected to because of the following informalities.

Claim 63 is objected over the recitation of "the segments of (a)" in line 1 and appears to be minor typographical error because step 'a' of claim 56 is drawn to nucleic acid samples not to segments.

Claim 64 is dependent from canceled claim 40 and appears to be a minor typographical error. For compact prosecution, claim 64 has been presumed to be dependent from claim 56. In response to this office action, applicant is suggested to identify the proper dependency of claim 64.

Appropriate corrections are required.

Claim Rejections - 35 USC § 112 first paragraph

Scope of Enablement Rejection

9. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

10. The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

11. Claims 56-64 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabled for nucleic acid sample

made from each of the single isolated cell, wherein the nucleic acid sample from each single isolated cell tagged with unique/distinct first multiplex identifier (first MID) sequence to track the origin of nucleic acid samples, wherein the different sized nucleic acid segments from plurality of nucleic acid samples from plurality of isolated single cells are tagged with different second MID sequences, to distinguish different sized nucleic acid segments (as shown in figures 11 and 13). However, the specification is not enabled for (a) providing a plurality of nucleic acid samples, each made from single isolated cells; (b) tagging a plurality of segments of the nucleic acid molecules in the samples of (a) with first and second multiplex identifier (MID) sequences, wherein the second MID sequences are all different from each other.

The instant specification discloses "In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more" (pg. 19, paragraph 2) and "In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined (pg. 19 paragraph 3) and "In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is

tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run" (pg. 20, paragraph 1, emphasis under lined by the examiner). In other words to enable the method of claim 56, reflex sequence method is essential for tagging different sized nucleic acid segments with different second MID sequences.

Therefore the instant specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, the invention requiring the steps of (a) providing a plurality of nucleic acid samples, each made from single isolated cells; (b) tagging a plurality of segments of the nucleic acid molecules in the samples of (a) with first and second multiplex identifier (MID) sequences, wherein the second MID sequences are all different from each other (recited in generality). Therefore instant specification is not enabled for carry out the method of claim 56 as discussed in detail below.

Per eMPEP 2164.01(a) there are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to:

(A) The breadth of the claims;

(B) The nature of the invention;

(C) The state of the prior art;

(D) The level of one of ordinary skill;

(E) The level of predictability in the art;

(F) The amount of direction provided by the inventor;

(G) The existence of working examples; and

(H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Breadth of Claims: In the instant case, claim 56 as recited encompass large number of single isolated cells, which will have very large number of nucleic acid samples and appending first MID and second MID sequences to large number of nucleic acid segments and sequencing appended segments which generate large number of sequences reads and not able to even analyze which first MID sequence of nucleic acid sample is from single isolated cell number 1 versus cell number "n" because of presence of large number of nucleic acid samples appended with first MID sequence. Also, instant claim 56 as recited encompasses a large genus of single isolated cells, plurality of nucleic acid samples and large number of nucleic acid segments tagged with first MID sequence and different second MID sequences.

Nature of the invention and state of the prior art: The instantly claimed invention is directed to analyze nucleic acids from single isolated cell and matching the nucleic acid sequences originating from each of the nucleic acid using reflex method to introduce a plurality of multiplex identifiers in plurality of nucleic acid segments.

Tagging nucleic acid samples with sample specific MID sequence or isolating single cells, tagging nucleic acid sample from each isolated single cell with specific first MID sequence to track the origin of nucleic acid sample from each of isolated single cell and pooling the nucleic acids and identifying the nucleic acid from each sample based on the sample or cell specific first MID sequence were known in the art at the time the claimed invention was made (Gillevet et al, arts of the record; Fig. 1 and Linnarsson, cited below, claims 1-5).

However, the step of tagging a plurality of segments of the nucleic acid molecules in the samples from plurality of single isolated cells with first and second multiplex identifier (MID) sequences, wherein: (i) the first MID sequences associated with a segment of a nucleic acid molecule of (a) uniquely identifies the single isolated cell from which the nucleic acid molecule is provided; and (ii) the second MID sequences are all different from each other was not established in the art prior to the claimed invention. Generation of large number of MID sequences were also known at the time of the claimed invention (454 multiplex identifier sequence, Roche bulletin, cited in the IDS of 8/30/17).

The level of one of ordinary skill: The level of skills required is very high as the claimed method requires isolating single cell, nucleic acids from the cell, tagging, and sequencing of the tagged nucleic acids and analysis of the sequence data to trace back the origin of nucleic acid from each cell.

The amount of direction provided by the inventor and the existence of working examples: The instant specification set forth steps to append tags to nucleic acid sample from each sample or a cell and isolating nucleic acids and appending the

nucleic acids with different multiplex identifier sequences using Reflex method (Fig. 1) and generating nucleic acid segments using primers/adaptors and specifically appending different multiplex identifiers to different sizes of fragments (Figs. 11 and 13 and their descriptions in the text).

The instant specification does not provide guidance regarding step 'b' of instant claim 56, especially tagging plurality of segments of the plurality of nucleic acid samples with first and different second MID sequences and identifying the origin of nucleic acid segments with specific isolated single cell.

The level of predictability in the art and the quantity of experimentation needed:

One having ordinary skill in the art would recognize that it is not possible to identify the source of nucleic acid from each sample when all of the plurality of the samples have the same first MID sequences. Therefore quantities of experimentations to be done are very large especially when the instant specification does not provide any guidance for addressing issues as discussed especially step 'b' of claim 56 in the previous sections.

Conclusion:

Courts have ruled that to be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation (In re Wright990 F.2d 1557, 1561; In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970)). It was also determined that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art. The amount of guidance needed to enable the invention is related to the amount of knowledge in the art as well as the

predictability in the art. Furthermore, the Court in Genentech Inc. v Novo Nordisk 42 USPQ2d 1001 held that it is the specification, not the knowledge of one skilled in the art that must supply the novel aspects of the invention in order to constitute adequate enablement.

Considering all the factors discussed above and lack of working example for the steps of claim 56 it is clear that the specification does not disclose a representative number of working examples. Thus, one skilled in the art would be lead to conclude that the applicant was not in possession of the claimed step 'b' encompassing large number of genus.

Therefore the claimed method is enabled for nucleic acid sample made from each of the single isolated cell, wherein the nucleic acid sample from each single isolated cell tagged with unique/distinct first multiplex identifier (first MID) sequence to track the origin of nucleic acid samples, wherein the different sized nucleic acid segments from plurality of nucleic acid samples from plurality of isolated single cells are tagged with different second MID sequences consistent with the reflex method of tagging MID sequences.

12. Claims 57-64 are rejected under pre-AIA 35 USC 112 first paragraph because they include all the limitations of claim 56.

For compact prosecution, applicant is suggested to identify support for the amendments in the instant specification.

Claim Rejections - 35 USC § 112-second paragraph

13. The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

14. The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

15. Claims 56-64 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

16. Claim 56 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being incomplete for omitting essential steps, such omission amounting to a gap between the steps (See MPEP § 2172.01). The omitted steps are: (i) the details of the steps of tagging first and second MID sequences to the plurality of segments of the nucleic acid molecules from the plurality of nucleic acid samples from plurality of single isolated cells, (ii) not specifying the step of tagging nucleic acid molecules from, for example, single isolated cell 1 with first MID sequences type 1 separately from the step of tagging nucleic acid molecules from single isolated cell 2 with first MID sequences type 2 and (iii) not specifying the step of how plurality of tagged segments of nucleic acid molecules with first MID sequences from plurality of nucleic acid samples uniquely identifies the nucleic acid molecules from single isolated cell because claim 56 does not specify if first MID sequences from isolated single cell 1 is different from first MID sequences from isolated single cell 2. Similarly the steps of tagging different second MID sequences to the type of nucleic acid molecules from plurality of nucleic acid samples are omitted.

If the intent is to identify the nucleic acid molecules from each of the single isolated cells with unique first MID sequence, it is suggested to include the additional steps, of tagging nucleic acid sample from each of single isolated cell separately with first MID sequence, wherein the first MID sequence is unique to each of the single isolated cell (i.e., first MID sequence from single isolated cell 1 is different from single isolated cell 2).

For claiming the step of second MID sequences are all different from each other, applicant is suggested to recite the steps associated with instant figures 11 and 13, wherein different sized nucleic acid segments from plurality of nucleic acid samples are tagged with different MID sequences, which is consistent with the novelty of the reflex method of the instant invention.

17. Claims 57-64 are also incomplete because they include all the limitations of claim 56, which is considered to be incomplete for the reasons discussed above.

18. Claims 58-62 are indefinite because it is not clear if "the MID sequences" include the first or the second or both MID sequences. Appropriate clarification is required.

Claim Rejections - 35 USC § 102

19. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

20. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

21. The changes made to 35 U.S.C. 102(e) by the American Inventors Protection Act of 1999 (AIPA) and the Intellectual Property and High Technology Technical Amendments Act of 2002 do not apply when the reference is a U.S. patent resulting directly or indirectly from an international application filed before November 29, 2000. Therefore, the prior art date of the reference is determined under 35 U.S.C. 102(e) prior to the amendment by the AIPA (**pre-AIPA** 35 U.S.C. 102(e)).

22. Claims 56-64 are rejected under pre-AIA 35 U.S.C. 102(e) as being anticipated by Linnarsson (WO 2010/117620 published Oct. 14, 2010, priority date Mar. 30, 2009, cited in the IDS of 8/30/17).

Regarding claims 56-58 and 60-61, Linnarsson teaches a method of preparing a nucleic acid libraries (i.e., cDNA libraries) from a plurality of single cells, the method comprising the steps of: releasing mRNA from each single cell to provide a plurality of individual mRNA samples, wherein the mRNA in each individual mRNA (i.e., nucleic acid) sample is from a single cell; synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell; pooling the tagged cDNA samples; and amplifying the pooled cDNA samples to generate a cDNA library comprising double-

stranded cDNA, wherein the method further comprises processing the cDNA library to obtain a library suitable for sequencing and sequencing the cDNA library to identify genes originated from the cell source (Figs. 1-4 and their legend, Linnarsson, claims 1-5 and 9-11, the instant steps of claims 56-58 and 60-62 taught by Linnarsson are underlined by the examiner).

The cell specific tag (e.g., see figure 3) in the nucleic acid sample from each of the single cell is the first Multiplex Identifier (first MID) sequences associated with a segment of a nucleic acid molecule of (a) uniquely identifies the single isolated cell from which the nucleic acid molecule is provided.

Linnarsson also teaches that the synthesis of the first strand of cDNA is directed by a cDNA synthesis primer (CDS) that includes an RNA complementary sequence (RCS) that is at least partially complementary to one or more mRNA in an individual mRNA sample (Linnarsson claim 3), wherein a template-switching oligonucleotide (TSO) is added to each individual mRNA sample, wherein said TSO comprises a portion which is at least partially complementary to a portion at the 3' end of the first strand of cDNA (Linnarsson claim 5), wherein the CDS and the TSO each include a different tag (Linnarsson claims 10 and 11). Different tags associated with cDNA segment of Linnarsson meets the limitation of second MID sequences are different from each other.

Regarding claim 59 requiring the MID sequences are appended with ligation, Linnarsson teaches ligating the adaptor to the nucleic acid segments that comprise MID sequences CDS and TSO (Fig. 8 and pg. 29, lines 3-11).

Regarding claim 62 requiring MID sequences are from 2 to 100 bases in length, Linnarsson teaches that the tag sequences (i.e., MID sequences) are from 4-20 nucleotides in length (Linnarsson claim 12), which is within the claimed range.

Regarding claims 63 and 64, Linnarsson teaches that the length is from 200 bp to 5 kb (Fig. 16), which is within the claimed range.

Double Patenting

23. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l) (1) - 706.02(l) (3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

24. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 9,410,149 in view of Linnarsson (WO 2010/117620 published Oct. 14, 2010, priority date Mar. 30, 2009, cited in the IDS of 8/30/17) for the following reasons.

Regarding instant claim 56, claims 1 and 10 of '149 patent disclose a method of analyzing genomic DNA, comprising: a) generating a first plurality of different segments of a first nucleic acid molecule of a contiguous region in the genomic DNA, wherein said contiguous region in the genomic DNA is represented by a plurality of nucleic acid molecules, and wherein each of said first plurality of different segments is appended to a first multiplex identifier (MID) sequence; b) generating a second plurality of different segments of a second nucleic acid molecule of said contiguous region, wherein each of said second plurality of different segments is appended to a second MID sequence and said first MID sequence and said second MID sequence are different; and c) sequencing the first and second plurality of different segments and the appended first and second MID sequences, respectively, to analyze the genomic DNA, wherein the genomic DNA is genomic DNA from a virus or a bacterium (the steps that are common between instant claim 56 and claims 1 and 10 of '149 patent are underlined by the examiner). One having ordinary skill in the art would recognize that genomic DNA from a bacterium meets the limitation of an isolated single cell.

Claims of '149 patent do not disclose step of providing a plurality of nucleic acid samples each made from single isolated cells and the identifier sequence that uniquely identifies the single isolated cell, which is taught by Linnarsson, who teaches a method of preparing a nucleic acid libraries (i.e., cDNA libraries) from a plurality of single cells,

the method comprising the steps of: releasing mRNA from each single cell to provide a plurality of individual mRNA samples, wherein the mRNA in each individual mRNA sample is from a single cell; synthesizing a first strand of cDNA from the mRNA in each individual mRNA sample and incorporating a tag into the cDNA to provide a plurality of tagged cDNA samples, wherein the cDNA in each tagged cDNA sample is complementary to mRNA from a single cell; pooling the tagged cDNA samples; and amplifying the pooled cDNA samples to generate a cDNA library comprising double-stranded cDNA, wherein the method further comprises processing the cDNA library to obtain a library suitable for sequencing and sequencing the cDNA library to identify genes originated from the cell source (Fig. 1 and its legend, Linnarsson, claims 1-5 and 9-11). Linnarsson also teaches pooling the samples simplifies handling of the samples from each single cell and reduces the time required to analyze gene expression in the single cells, which allows high throughput analysis of gene expression. Pooling the samples prior to amplification also provides the advantage that technical variation between samples is virtually eliminated (pg. 11, paragraph 1), thus providing motivation to include the step of including the tagging the nucleic acid sample for each of the isolated single cell and pooling the plurality of isolated single cell nucleic acids in the claims of '149 patent.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of including the tagging the nucleic acid sample for each of the isolated single cell and pooling the plurality of isolated single cell nucleic acids in the claims of '149 patent with a reasonable expectation of success with the expected benefit of allowing high throughput analysis of

gene expression and eliminating the technical variation between samples as taught by Linnarsson (pg. 11, paragraph 1). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including additional step of tagging nucleic acid sample with identifier sequence which is routinely practiced in the art as exemplified Linnarsson.

Regarding instant dependent claims 57-64, claims 1-13 of '149 patent in view of Linnarsson disclose the steps of said dependent claims and thus are not patentably distinct.

25. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 9,410,150 in view of Linnarsson (WO 2010/117620 as cited above) for the following reasons.

Regarding instant claim 56, claims 1, 10, 12, 13 and 19 of '150 patent disclose the steps of providing a first plurality of different segments of DNA from a first sample, wherein the sample is a single cell, wherein the first plurality of different segments of DNA are derived from a first nucleic acid molecule of a locus in genomic DNA and wherein each of said first plurality of different segments is appended to a first sequence tag; and b) a second plurality of different segments of DNA from the first sample, wherein the second plurality of different segments of DNA are derived from a second nucleic acid molecule of the locus and wherein each of said second plurality of different segments is appended to a second sequence tag and said first sequence tag and said second sequence tag are different, wherein the first plurality of different segments the second plurality of different segments further comprise a multiplex identifier (MID)

sequence that identifies the source of the segments of DNA wherein the MID sequence identifies an individual person, a tissue, a cell . Claim 10 of '150 patent discloses the step of wherein the first plurality of different segments and the second plurality of different segments comprise one or more of an amplification primer sequence and a sequencing primer sequence appended thereto (the steps that are common between instant claim 56 and claims 1, 12, 13 and 19 of '150 patent are underlined by the examiner). One having ordinary skill in the art would recognize that the sequencing of the nucleic acid segments using primer amounts to sequencing the tagged segments of to obtain the nucleotide sequences of the plurality of nucleic acids and the attached first and second MID sequences because the first and second sequence tag amount to two different second MID sequences.

Claims of '150 patent do not disclose step of providing a plurality of nucleic acid samples each made from single isolated cells and analyzing the nucleic acid samples, which is taught by Linnarsson, who teaches a method of preparing a nucleic acid libraries (i.e., cDNA libraries) from a plurality of single cells and sequencing the cDNA library to identify genes originated from the cell source (Fig. 1 and its legend, Linnarsson, claims 1-5 and 9-11). Linnarsson also teaches pooling the samples simplifies handling of the samples from each single cell and reduces the time required to analyze gene expression in the single cells, which allows high throughput analysis of gene expression. Pooling the samples prior to amplification also provides the advantage that technical variation between samples is virtually eliminated (pg. 11, paragraph 1), thus providing motivation to include the step of providing a plurality of nucleic acid

samples each made from single isolated cells and analyzing the nucleic acid samples in the claims of '150 patent.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the step of providing a plurality of nucleic acid samples each made from single isolated cells and analyzing the nucleic acid samples of Linnarsson in the claims of '150 patent with a reasonable expectation of success with the expected benefit of allowing high throughput analysis of gene expression and eliminating the technical variation between samples as taught by Linnarsson (pg. 11, paragraph 1). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including additional step of tagging nucleic acid sample with identifier sequence which is routinely practiced in the art as exemplified Linnarsson.

Regarding instant dependent claims 57-64 claims 1-19 of '150 patent in view of Linnarsson discloses the steps of said dependent claims and thus are not patentably distinct.

26. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12 of U.S. Patent No. 8,679,756 in view of Linnarsson (WO 2010/117620 as cited above) for the following reasons.

It is noted that the subject matter of claims 1-12 of '756 patent is similar to claims 1-13 of '149 patent. Therefore analysis is very similar. Thus it is maintained that steps of instant claims 56-64 are not significantly different from claims 1-12 of '756 patent in view of Linnarsson.

27. Claims 56-64 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11 of U.S. Patent No. 8,563,274 in view of Linnarsson (WO 2010/117620 as cited above) for the following reasons.

It is noted that the subject matter of claims 1-11 of '274 patent is similar to claims 1-13 of '149 patent. Therefore analysis is very similar. Thus it is maintained that steps of instant claims 56-64 are not significantly different from claims 1-11 of '274 patent in view of Linnarsson.

Conclusion

28. No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on MON-THURS 8.00 am -6.30pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on 5712720731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR.

Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

NARAYAN BHAT
Primary Examiner
Art Unit 1634

/NARAYAN BHAT/
Primary Examiner, Art Unit 1634

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/792,094	Brenner et al.
	Examiner	Art Unit
	NARAYAN BHAT	1634

CPC - Searched*		
Symbol	Date	Examiner
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Searched uisng key words in different CPC classes. See the search history in EAST.	1/6/16	Bhat N.

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

/NARAYAN BHAT/ Primary Examiner.Art Unit 1634	

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/792,094	Brenner et al.
	Examiner	Art Unit
	NARAYAN BHAT	1634

Search Notes		
Search Notes	**Date**	**Examiner**
Reviewed the copending applications for the inventors in PLAM and copending claims for potential ODP issues.	1/6/16	Bhat N.
Reviewed the prosecution histories of copending applications resulting in the issuance of patents.	1/6/16	Bhat N.
Reviewed the cited arts in the IDS of 10/9/15.	1/6/16	Bhat N.
searched for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.	1/6/16	Bhat N.
Updated the search for the topics in EAST. Search history is enclosed.	7/17/16	Bhat N.
C12Q 1/68, C12Q 1/6874, C07H 21/02 and C40B 30/04	1/6/16	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are enclosed.		
Updated the search for the topics in EAST. Search history is enclosed.	5/25/17	Bhat N.
Updated the search for the topics in EAST, Pubmed and Google scholar. Search histories are encloased.	09/13/2017	Bhat N.
Reviewed the arts filed in the IDS (8/30/17)	09/13/2017	Bhat N.

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**

/NARAYAN BHAT/ Primary Examiner.Art Unit 1634	

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Examiner-Initiated Interview Summary	Application No. 14/792,094	Applicant(s) Brenner et al.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA Status No

All participants (applicant, applicant's representative, PTO personnel):

(1) Examiner: Bhat. (3) _____.

(2) Representative: Ms. Talukder. (4) _____.

Date of Interview: 08 September 2017.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐ 101 ☐ 112 ☐ 102 ☐ 103 ☑ Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 56.

Identification of prior art discussed: None.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

See Continuation Sheet.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/NARAYAN BHAT/ Primary Examiner, Art Unit 1634	

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Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

In view of instant application being part of family of patents, for compact prosecution, the examiner contacted the applicant's representative Ms. Talukder telephonically on 9/8/2017 to inform that further amendments are needed to overcome the potential rejections under 35 USC 112 first and second paragraphs and to fix the minor informalities.

The examiner suggested to further clarifications in claim 56 are needed to specify that the plurality of nucleic acid segments are made from each of the single isolated cell, wherein the plurality of nucleic acid segments from each single cell have cell specific multiplex identifier and different sizes of the nucleic acids from each cell have different multiplex identifier consistent with results schematically presented in figures 11 and 12 (top panel). The examiner also informed that claim 56 as recited is not clear how plurality of nucleic acid samples from plurality of single isolated cell tagged with first and second multiplex identifiers capable identifying origin of nucleic acid sample from each of the single isolated cell.

The amendments to include the step of isolating nucleic acids from each of the isolated single cell separately and appending nucleic acid sample with cell specific identifier were discussed. But no specific amendments to clarify how second MID (multiplex identifier) are different from each other were discussed in view of 35 USC 112 rejections are not being on the record. In view of time constraint to move the instant application on an expedited basis on the part of the examiner and to get specific claim language approval from the applicant, the examiner informed that the office action will be issued to decide further course of action.

No specific claim languages were agreed to during the interview.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/792,094	07/06/2015	Sydney Brenner	104371-5016-US05	9697

43850 7590 05/24/2018

MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CALIFORNIA 94105
UNITED STATES OF AMERICA

EXAMINER	
BHAT, NARAYAN KAMESHWAR	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
05/24/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

donald.mixon@morganlewis.com
sfipdocketing@morganlewis.com

10x Genomics, Inc. Exhibit 2004
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Notice of Abandonment	Application No.	Applicant(s)
	14/792,094	Brenner et al.
	Examiner	Art Unit
	NARAYAN BHAT	1634

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☑ Applicant's failure to timely file a proper reply to the Office letter mailed on 20 September 2017.
   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
   (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 to the final rejection. (A proper reply under 37 CFR 1.113 to a final rejection consists only of:(1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) if this is utility or plant application, a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. Note that RCEs are not permitted in design applications.)
   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
   (d) ☑ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $ _____ is insufficient. A balance of $ _____ is due.
   The issue fee required by 37CFR 1.18 is $ _____ . The publication fee, if required by 37 CFR 1.18(d), is $ _____ .
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record or other party authorized under 37 CFR 1.33 (b). See 37 CFR 1.138(b).

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34) upon the filing of a continuing application.

6. ☐ The decision by the Board of Patent Appeals and Interference rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☑ The reason(s) below:

As discussed in the interview summary, applicant's representative Ms. Talukder did not respond to the message left on her voice mail on 4/18/18 by 4/21/18 and therefore the notice of abandonment has been issued.

	/NARAYAN K BHAT/ Primary Examiner, Art Unit 1634

Petitions to revive under 37 CFR 1.137, or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

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Examiner-Initiated Interview Summary	Application No. 14/792,094	Applicant(s) Brenner et al.	
	Examiner NARAYAN BHAT	Art Unit 1634	AIA Status No

All participants (applicant, applicant's representative, PTO personnel):

(1) Examiner: Bhat. (3) _____.

(2) Representative: Ms. Talukder. (4) _____.

Date of Interview: 18 April 2018.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐ 101 ☐ 112 ☐ 102 ☐ 103 ☑ Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 56.

Identification of prior art discussed: Arts of the record.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Foe compact prosecution the examiner contacted the applicant's representative, Ms. Talukder and a left a message on her voice mail on 4/18/18 to inquire if any response has been filed to the Non-final rejection mailed on 9/20/2017 and informed that if examiner did not get a reply by 4/21/18, the examiner will send a notice of abandonment. No response has been filed till 5/19/2018..

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

	/NARAYAN K BHAT/ Primary Examiner, Art Unit 1634

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PTO/SB/122 (04-15)
Approved for use through 01/31/2018. OMB 0651-0035
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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Application

Address to:
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Application Number	14/792,094
Filing Date	July 6, 2015
First Named Inventor	SYDNEY BRENNER
Art Unit	1634
Examiner Name	Narayan Kameshwar Bhat
Attorney Docket Number	104371-5016-US05

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I am the:

☐ Applicant/Inventor

☐ Assignee of record of the entire interest.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☒ Attorney or agent of record. Registration Number 61,368.

☐ Registered practitioner named in the application transmittal letter in an application without an executed oath or declaration. See 37 CFR 1.33(a)(1). Registration Number_____.

Signature	/Gargi Talukder/
Typed or Printed Name	Gargi Talukder
Date	August 10, 2018
Telephone	415.442.1000

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of _____forms are submitted.

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Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
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