FIG. 7

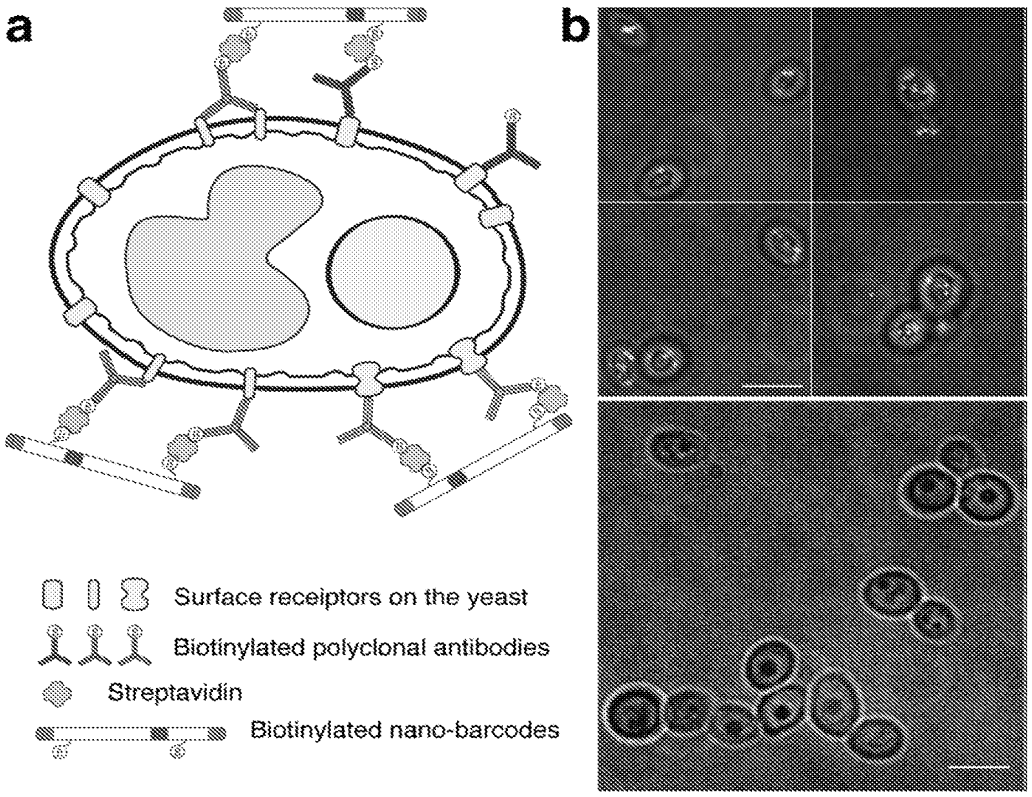
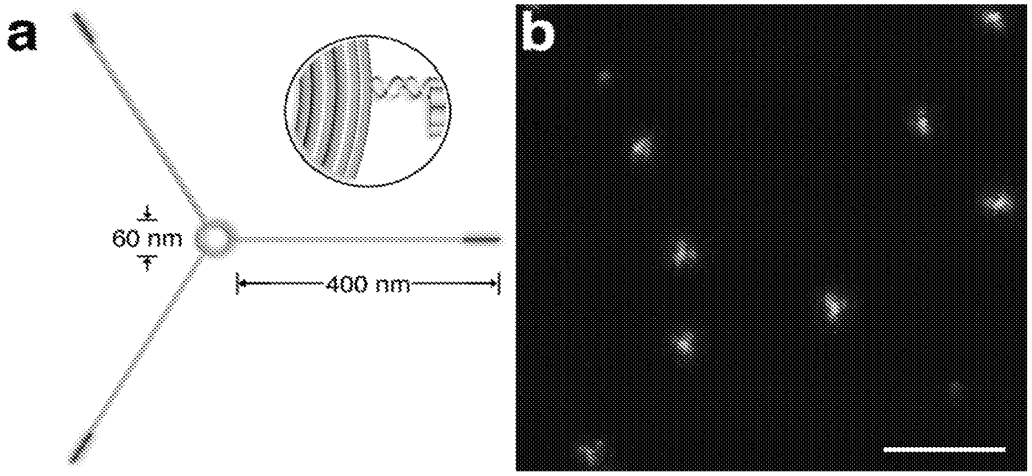

a
b
Surface receiptors on the yeast
Biotinylated polyclonal antibodies
Streptavidin
Biotinylated nano-barcodes

FIG. 8



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(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, QA, RO, RS, RU, RW, 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.

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[Continued on next page]

(54) **Title**: NUCLEIC ACID NANOSTRUCTURE BARCODE PROBES

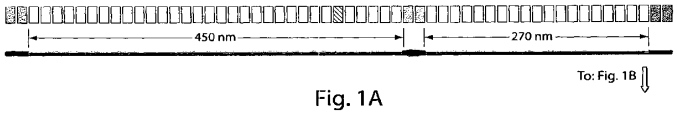


Fig. 1A

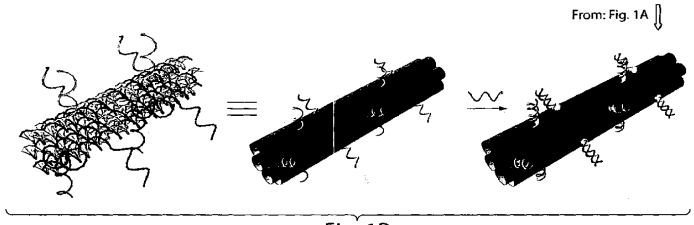


Fig. 1B

(57) **Abstract**: Provided herein are, inter alia, barcode probes comprised of transiently or stably fhiorescently labeled nucleic acid nanostructures that are fully addressable and able to be read using standard fluorescent microscope and methods of use thereof including methods of use as detectable labels for probes, e.g. in the multiplex detection of targets, based on the pattern and orientation of the labels.

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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C12Q B82Y

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/076128 A2 (NANOSTRING TECHNOLOGIES INC [US]; INST SYSTEMS BIOLOGY [US]; GEISS GAR) 5 July 2007 (2007-07-05) cited in the application page 3, lines 25-31; figure 1 page 20 - pages 26-27 figure 3 figures 6-7 page 30, lines 16-17 page 94, lines 8-14 page 49 - page 50 page 120, lines 18-20 page 52	1-14,16, 18-34
X	US 2007/048759 A1 (LUO DAN [US] ET AL) 1 March 2007 (2007-03-01) claims 1-2,6,11-14,18; example 1; tables 1,9	1,2,4-6, 8

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Knudsen, Henrik

2

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1634

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	STEINHAUER CHRISTIAN ET AL: "DNA origami as a nanoscopic ruler for super-resolution microscopy", ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, WILEY VCH VERLAG, WEINHEIM, vol. 48, no. 47, 1 January 2009 (2009-01-01), pages 8870-8873, XP002635459, ISSN: 1433-7851, DOI: 10.1002/ANIE.200903308 page 8871, right-hand column, last paragraph; figure 1b page 8872, right-hand column, lines 4-5	9-14,18, 21
X	OFER I. WILNER ET AL: "Covalently Linked DNA Nanotubes", NANO LETTERS, vol. 10, no. 4, 17 March 2010 (2010-03-17) , pages 1458-1465, XP55031369, ISSN: 1530-6984, DOI: 10.1021/nl100389v Scheme 1 figure 3B	9,14,15
X	RALF JUNGMANN ET AL: "Single-Molecule Kinetics and Super-Resolution Microscopy by Fluorescence Imaging of Transient Binding on DNA Origami", NANO LETTERS, vol. 10, no. 11, 19 October 2010 (2010-10-19), pages 4756-4761, XP55031396, ISSN: 1530-6984, DOI: 10.1021/nl103427w page 4758, right-hand column, last paragraph; figures 1c, 3	9,10, 13-18

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2

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1635

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHENXIANG LIN ET AL: "Self-Assembled Combinatorial Encoding Nanoarrays for Multiplexed Biosensing", NANO LETTERS, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 507-512, XP55031573, ISSN: 1530-6984, DOI: 10.1021/nl062998n figures 1b,2	1,2,4-6, 8
A	CHHABRA R ET AL: "DNA Self-assembly for Nanomedicine", ADVANCED DRUG DELIVERY REVIEWS, ELSEVIER BV, AMSTERDAM, NL, vol. 62, no. 6, 30 April 2010 (2010-04-30) , pages 617-625, XP027076629, ISSN: 0169-409X, DOI: 10.1016/J.ADDR.2010.03.005 [retrieved on 2010-03-15] abstract	1-34
A,P	QIAN MEI ET AL: "Stability of DNA Origami Nanoarrays in Cell Lysate", NANO LETTERS, vol. 11, no. 4, 13 April 2011 (2011-04-13) , pages 1477-1482, XP55031409, ISSN: 1530-6984, DOI: 10.1021/nl1040836 abstract	1-34
A,P	XIANGWEI ZHAO ET AL: "Advances of multiplex and high throughput biomolecular detection technologies based on encoding microparticles", SCIENCE CHINA CHEMISTRY, SP SCIENCE CHINA PRESS, HEIDELBERG, vol. 54, no. 8, 20 August 2011 (2011-08-20), pages 1185-1201, XP019942238, ISSN: 1869-1870, DOI: 10.1007/S11426-011-4343-6 page 1195 - page 1197	1-34
T	RALF JUNGMANN ET AL: "Nanoscale imaging in DNA nanotechnology", WILEY INTERDISCIPLINARY REVIEWS: NANOMEDICINE AND NANOBIOTECHNOLOGY, vol. 4, no. 1, 1 January 2012 (2012-01-01) , pages 66-81, XP55031394, ISSN: 1939-5116, DOI: 10.1002/wnan.173 the whole document	

2

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1636

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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(54) Title: DETECTION METHODS FOR TARGET DNA

(57) Abstract: The invention pertains to a safe, quick and reliable method of detecting the presence of a target DNA sequence in a sample. The invention also pertains to a system for detecting presence of a target DNA sequence in a biological sample. The system includes an ITC template that includes a probe binding sequence that binds to a corresponding ITC probe and a first and second flanking primer recognizing sequence that binds to a first and second primer, respectively. The system also includes an ITC probe that binds to the ITC template probe binding sequence. The ITC probe includes a first marker molecule. The system also includes a first primer that binds to the first flanking primer sequence and a second primer that binds to said second primer sequence. The system further includes a target DNA sequence probe, wherein the target DNA sequence probe comprises a second marker molecule.

DETECTION METHODS FOR TARGET DNA

BACKGROUND

[001] The availability of simplified and controlled tests for detecting exogenous DNA molecules is of great demand in infectious disease diagnosis and evaluating the response to treatment, evaluating biodistribution of vectors used in legitimate gene therapy clinical trials, and detection of illicit gene doping. Real-time PCR can detect specific DNA signals, even at a very low concentration with reliability and specificity. In addition, real-time PCR is amenable to automation and remote data collection for high throughput screening. Taqman real-time PCR, using specific sequence probes, provides an efficient method to detect exogenous DNA accurately and quantitatively. However, to determine with confidence if the PCR signal is not a false positive or a false negative requires multiple controls. In addition, for quantitative determination of copy number, external copy number standard curves are required and pose a risk of contaminating the laboratory.

[002] Several viral-based and non-viral based gene transfer systems are being developed and evaluated in human clinical trials. These systems have demonstrated the ability to safely and efficiently deliver therapeutic transgenes to a variety of tissues of animals and humans, and examples of therapeutic benefit in humans are increasing [1,2,3,4,5,6,7]. Recombinant adeno-associated viral (rAAV) vectors and naked plasmid are two such gene transfer systems capable of gene delivery to skeletal muscle of animals [8],[9,10,11,12] and humans [13,14,15,16]. Recently, as an alternative to direct injection, regional vascular infusion of vector to achieve skeletal muscle transduction has been reported for plasmid DNA [17,18] and for rAAV vectors [19,20].

[003] When developing legitimate gene transfer modalities for gene therapy and vaccination, the evaluation of the distribution of vector sequences

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1639

in pre-clinical animal studies and human clinical trials is required by regulatory agencies to determine the level of gene delivery to the target tissue and non-target tissues transduced collaterally, and to evaluate vector shedding into the environment. The type of vector, the delivery method, injection schedule, route of administration, and administrated dose are the main variables to impact biodistribution and shedding [20,21,22,23,24]. To date, traditional real-time PCR assays have been used to analyze rAAV vector distribution after administration in human trials [25] or animal models [9] [26] [27,28,29]. To control for the presence of different potential inhibitors for each tissue source (eg. matrix effects), a common practice is to analyze each sample in duplicate with one of the duplicates spiked with a known number of copies of a plasmid harboring the PCR target sequences.

[004] Sports organizations have increased their focus on developing efficient ways to curtail the illegitimate use of genes or genetic elements that have the capacity to enhance athletic performance, also referred to as gene doping [30]. These organizations recognize that gene doping has the potential to threaten the integrity of sport, undermine principles of fair play in sport, imposes potential harm to non-doping athletes, and involves major health risks to athletes [31].

SUMMARY

[005] One of the major challenges in standard real-time PCR analysis is how to eliminate the false negative signals which can be caused by inhibitors [33] or inefficient PCR conditions. Although multiplex Taqman PCR assays have been applied in an effort to address problems of reliability, such as by adding an extra primer-probe sets targeted to other endogenous DNA sequences (housekeeping genes), however, the sequence and secondary structure differences between primers and probe binding sites, and amplified

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1640

sequences contribute to different detection efficiencies. Competitive PCR methods can be used to quantify DNA copy number, however, the method is limited by the necessity of assembling multiple competitive reactions for a single determination and, most notably, the need for a post-PCR electrophore-sis-based detection and analysis step. In realizing these problems and difficulties of utilizing real-time PCR to detect exogenous DNA sequences, different Taqman PCR assays which use internal controls have been applied in an effort to address problems of reliability, such as by adding an extra primer-probe set targeted to other endogenous DNA sequences [34] or exogenous targets [34,35,36,37,38,39,40,41]. These previous approaches were primarily aimed at controlling sample adequacy, eliminating false negative results, performed in separate reactions, did not share the same primers, or preferentially amplified the target.

[006] In realizing these problems and difficulties of utilizing real-time PCR to detect exogenous DNA sequences, the inventors have developed a more efficient and simple detection system. According to one embodiment, the system includes the use of an internal threshold control (ITC) template and corresponding ITC probe. Sample DNA, a prescribed number of ITC template molecules, a single pair of primers, target probe, and ITC probe are added to one reaction. Marker signals, such as fluorescence emission signals are obtained simultaneously to determine the cycle thresholds (Ct) for amplification of the target and ITC sequences. Comparison of the Ct from the ITC and the Ct of the target is the parameter used to determine if a test sample is positive or negative for the presence of a homologous or nonhomologous exogenous DNA sequence.

[007] As will be discussed in more detail herein, such in reference to FIG. 3, for the ITC assays, if the Ct of the Target probe is less than or equal to the Ct of the ITC probe, then the sample is considered positive (meaning that

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1641

there is the same or more copies of the target sequence of interest relative to the ITC template). Additionally, if the Ct of the Target probe is greater than the Ct of the ITC probe, then the sample is considered negative. In a typical embodiment, the primers recognize both the target sequence and ITC template.

[008] The inventors have developed different ITC assay methodologies, which have validated the effectiveness of technology. For example, real-time PCR assays were developed to detect a performance enhancing transgene (erythropoietin, EPO) or vector backbone sequences in the presence of endogenous cellular sequences. EPO is a therapeutic gene that has been clinically evaluated and can potentially be illicitly used for gene doping. Two different ITC^{EPO} duplex assays target macaque and human EPO cDNA. These ITC^{EPO} duplex assays are performed in the presence of the cellular homologous genomic DNA locus, thus the ITC assay format is capable of distinguishing the cDNA carried in a gene transfer vector from the cellular host genomic DNA. By real-time PCR, the vector transgene is distinguishable from the genomic DNA sequence due to the absence of introns, and the vector backbone can be identified by heterologous gene expression control elements. In addition to developing the real-time PCR assays and ITC assay format, the steps involved in specimen collection, DNA extraction, DNA storage and DNA transport were validated. Bio-distribution of gene transfer vectors in legitimate gene therapy clinical trials is an important parameter to evaluate biosafety in humans. Thus, ITC assays might be applied to legitimate pre-clinical studies and clinical gene therapeutic trials to determine the presence or absence of gene transfer vector sequences in different tissues.

[009] Embodiments of the technology are also capable of being used for detection of human infectious agents such as viruses or bacteria that have

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1642

sequences that are nonhomologous to human genomic DNA. Those skilled in the art will appreciate in view of the teachings herein that certain embodiments of the invention can be used to determine the presence of a target sequence of interest in a sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[010] Figure 1. Internal Threshold Control (ITC) assay format. Panel (A) ITC^{EPO} duplex assay format. The EPO probe specifically detects the EPO cDNA harbored in rAAV vectors. The EPO ITC probe recognizes only the synthetic ITC^{EPO} template. The EPO primers recognize the EPO cDNA, the ITC^{EPO} template, and the genomic EPO locus. **Panel (B)** ITC^{CMV} duplex assay format. The CMV probe specifically detects the CMV immediate early promoter region in the pSSV9-MD2-cmEPO plasmid. The CMV ITC probe recognizes only the synthetic ITC^{CMV} template. The CMV primers recognize the CMV promoter and the ITC^{CMV} template.

[011] Figure 2. ITC duplex assay equivalence testing. All three ITC duplex assays were tested in the presence of same amount of target and ITC template and 500ng naïve gDNA. Five copies of pSSV9-MD2-cmEPO plasmid and ITC^{cmEPO} template were amplified using the ITC^{cmEPO} duplex assay. 10 copies of pShuttle-CAG-hEPO-pA plasmid and ITC^{hEPO} template were amplified using the ITC^{hEPO} duplex assay. 10 copies of pSSV9-MD2-cmEPO plasmid and ITC^{CMV} template were amplified using the ITC^{CMV} duplex assay. Each reaction was repeated 15 times. Similarities in mean Ct values were analyzed by SAS9.2.

[012] Figure 3. ITC duplex assay competition testing. The copy number of the ITC template was held at 5 copies (cmEPO ITC) or 10 copies (hEPO or CMV ITC) in each reaction, while the target template was titrated from 5 or 10 copies to 100 copies. Each reaction was repeated 5 times in the

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1643

presence of 500ng naïve gDNA to obtain the mean Ct value. **Panel (A)** ITC^{cmEPO} duplex assay, **Panel (B)** ITC^{hEPO} duplex assay and **Panel (C)** ITC^{CMV} duplex assay.

[013] Figure 4. ITC duplex assay interference testing. Target plasmid (◆) and ITC template (○) were amplified in one reaction in the presence of 500ng naïve gDNA to test the influence on the Ct between the two DNA targets. The total copy number of the plasmid and ITC template was maintained at 100. The target plasmid copy number is 10, 25, 50, 75, 90 and the ITC template copy number is 90, 75, 50, 25, 10 from left to right. Each reaction was repeated 5 times to obtain the mean. **Panel (A)** ITC^{cmEPO} duplex assay **Panel (B)** ITC^{hEPO} duplex assay and **Panel (C)** ITC^{CMV} duplex assay.

[014] Figure 5. Alignment between the macaque and human Epo genes. The location of the macaque assay (across the exon 2-3 boundary) is similar to the homologous location of the human assay (across the exon 3-4 boundary).

DETAILED DESCRIPTION

[015] The inventors have developed a system that enables the straightforward, accurate and efficient detection of exogenous sequences in a biological sample. Embodiments of the invention include the detection of genes that can be used in gene doping and may also be implemented to detect and monitor the efficacy, safety and spread of vectors and other constructs utilized for legitimate gene therapy. In certain specific embodiments, the invention pertains to methods, genetic constructs, and systems that are engineered to identify transfected or transduced cDNA sequences that pertain and can be homologous to endogenous genomic DNA. In alternative embodiments, such as, but not limited to, those related to monitoring the use of viral vectors for gene transfer or detection of infectious

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1644

agents, the invention pertains to detection of any foreign DNA material including, but not limited to, transfected, transduced or infected exogenous DNA material.

[016] In one embodiment, the invention pertains to a method of detecting exogenous DNA material in a subject. The term "exogenous DNA" means DNA that would not otherwise be in a subject but for the intentional transfection or transduction of exogenous DNA into the subject or infection. The method includes obtaining a specimen and preparing a DNA-containing sample from said subject and conducting real-time PCR on the DNA sample in the presence of a prescribed amount of an ITC template, primers for a target DNA sequence; a target probe that binds to the target DNA sequence and an ITC probe. Examples of a sample include, but are not limited to, blood, urine, mucous, hair, semen, or tissue sample. The method involves then determining whether the target DNA sequence amount is equal to or greater than the ITC template amount. In the case of determining Ct, the lower the Ct the higher the amount of copies are present in the sample.

[017] In a specific embodiment, the target DNA sequence pertains to an exon-exon junction of a cDNA that is homologous to an endogenous gene. In a more specific embodiment, the endogenous gene is erythropoetin.

[018] In a typical embodiment, the ITC template and the target DNA sequence comprise primer binding sequences that are homologous. In the context of the primer binding sequences, the term "homologous" means that sequences are sufficiently the same so as to each be recognized by the same primers.

[019] The inventors have determined that the sensitivity of their method is exceptional and can reliably detect the presence of approximately 5 copies of the target or ITC template in the presence of 500ng of genomic DNA (equivalent to 75,000 diploid genomes). This means that the ITC assay

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1645

format can determine whether there are approximately 5 or more copies of illicit genes in a sample from a subject. In a more specific embodiment, approximately in the context of copy number means 1-3 copies more or less. Thus, approximately 5 or more copies may be 2 or more, 3 or more, 4 or more, 6 or more 7 or more copies, etc. This level of sensitivity dramatically increases the ability of determining whether someone has undergone prohibited gene doping, or the presence of vector sequences in legitimate gene transfer, or presence of infectious agents.

[020] In most cases, the method embodiments are utilized to detect foreign DNA in a human, but can be used for non-human animals as well. In other words, this can be used to monitor gene doping in race horses or other race animals, and the presence of vector sequences in tissues analyzed in pre-clinical studies used to support human clinical trials It also could be used by the USDA to monitor whether meat or plant based materials have been genetically modified.

[021] In a more specific embodiment, the ITC probe includes a fluorophore that generates a first color signal and the target probe comprises a fluorophore that generates a second color signal. Detection can be conducted by determining whether the Ct of the target is less than or equal to the Ct of the ITC template (positive), or greater than the Ct of the ITC (negative).

[022] In yet another embodiment, the invention pertains to a system for detecting foreign DNA material in a subject. The system includes a real-time PCR instrument that includes a receptacle for holding a PCR reaction containing the DNA sample; and a known amount of copies of an ITC template, primers for a target DNA sequence; target probe specific to said target DNA sequence and an ITC probe disposed in said receptacle.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1646

[023] Another embodiment of the invention pertains to an ITC template. The template comprises a probe binding sequence that binds to a corresponding ITC probe and flanking sequences that hybridize to the primers. The ITC template's flanking primer recognizing sequences are homologous to primer sites of a separate target DNA sequence, however, the target DNA sequence lacks the ITC probe binding sequence. This prevents cross-interaction between the ITC probe and the target DNA sequence.

[024] *General Features of one ITC embodiment*

a. Primers are the same for ITC template and target sequence
b. Primers are separated by a similar distance in ITC template and target sequence
c. The ITC probe and Target Probe have similar Tm's.
d. Two different probes (dyes) are detected
e. Readout is simple

[025] A further embodiment of the invention pertains to a system for detecting presence of a target DNA sequence in a biological sample. The system includes an ITC template that includes a probe binding sequence that binds to a corresponding ITC probe and a first and second flanking primer recognizing sequence that bind to a first and second primer, respectively. The system also includes an ITC probe that binds to the ITC template probe binding sequence. The ITC probe includes a first marker molecule. The system also includes a first primer that binds to the first flanking primer sequence and a second primer that binds to said second primer sequence. The system further includes a target DNA sequence probe, wherein the target DNA sequence probe comprises a second marker molecule.

[026] *General features/advantages of a method embodiment*:

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1647

--Single tube: DNA sample + master mix (including primers, ITC probe, Target probe, and ITC template)

--Specific and sensitive, all samples INTERNALLY controlled

--Fast: 2 to 3 hours (including set up and analysis)

--No standard curve titration tubes (NO EXTERNAL STDS) needed

--High throughput: many samples analyzed at once

--Automated: data captured by PCR machine

--Can Transfer results to centralized database(s). Database(s) can be accessible by multiple remote users.

--No cumbersome manipulations

(such as 2 PCR reactions for nested PCR or Gel electrophoresis analysis)

--Reduced risk of contamination (false positives)

--No positive control plasmid needed

--uracil-N-glycosylase (UNG) can be used in specific embodiments which acts to prevent the reamplification of carryover PCR products in subsequent analyses

[027] Examples

[028] Detection of EPO

[029] A. Materials and Methods

[030] ***Recombinant human and macaque EPO plasmid***

[031] The pShuttle-CAG-hEPO-pA plasmid contains the human erythropoietin (hEPO) cDNA under the control of the CAG promoter and the SV40 polyA (pA) sequence. The pSSV9-MD2-cmEPO rAAV vector plasmid harbors the macaque Epo (cmEPO) transgene under the control of the CMV

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1648

promoter and SV40 pA. The integrity of the plasmids was verified by complete sequencing.

[032] ***rAAV Vector Production***

[033] The rAAV1 and rAAV8: rAAV-MD2-cmEPO vectors were made by transient transfection of 293 cells and purified by cesium chloride density gradients followed by extensive dialysis against phosphate-buffered saline. Appropriate quality control was performed to evaluate viral vector purity, vector genome titer, and infectious titer.

[034] ***rAAV vector administration to macaque skeletal muscle***

[035] Experiments were conducted on captive-bred cynomolgus macaques purchased from BioPrim (Baziège, France). Animals were prescreened for the presence of anti-AAV1 or 8 antibodies, SV40, and other pathogens. For direct rAAV IM injections, the total dose was split over one or two pre-tattooed injection sites along the Tibialis Anterior muscle in a maximal volume of 600µl. Mac 3 was injected with 2.5E10 vg/kg rAAV1-MD2-cmEPO vector. Mac 4 was injected with 2.5E11 vg/kg rAAV1-MD2-cmEPO vector. Mac 5 was injected with 5E9 vg/kg rAAV8-MD2-cmEPO vector. Mac 6 was injected with 2.5E10 vg/kg rAAV8-MD2-cmEPO vector. All injections and blood samples were collected under ketamine-induced anesthesia (10mg/kg).

[036] ***DNA extraction from macaque and human white blood cells***

[037] Human (naïve) and macaque (naïve and rAAV transduced) whole blood was collected and DNA extracted from the WBC pellet using the Gentra Puregene kit (cat # 158467) from Qiagen. Concentration and purity of the gDNA was determined using a nano-spectrophotometer from Implen. Integrity of the DNA was verified by migration of 3µg of total DNA on a 0.8% agarose gel, followed by Ethidium Bromide staining, and for macaque DNA by real-time PCR of the endogenous macaque ε-globin gene.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1649

[038] ***ITC assay Development***

[039] ***Primer-Probe design***

[040] Primers and probes were designed using ABI Primer Express 3.0 based on the cytomegalovirus (CMV) immediate early promoter in the pSSV9-MD2-cmEPO plasmid, the human EPO gene (NC_000007.13), or macaque EPO gene (NC_007860.1). Because of the constraints of the EPO Exon-Exon junction sequences and the required Tm, screening of multiple primer-probes combinations was required. Primer and probe sequences were screened *in silico* against the human genome and the macaque genome (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). Primers, probes and ITC templates were quantified using a nano- spectrophotometer from Implen.

[041] **a. ITC^{cmEPO} duplex assay**

[042] The cmEPO primer-probe assay targets the cmEPO Exon2-3 junction, harbored in pSSV9-MD2-cmEPO plasmid. The ITC^{cmEPO} duplex assay was designed with forward primer 5'AATGAGAATATCACCGTCCCAGAC3', reverse primer 5'AGCTTCTGAGAGCAGGGCC3', cmEPO probe 6FAM-AAGAGGATGGAGG TCGG-MGBNFQ and ITC probe 6VIC-CGGCCATTTTCCA-MGBNFQ. The ITC probe targets ITC^{cmEPO} template of two complimentary synthetic single strand DNAs that were annealed. The sequence of the forward ITC template sequence is 5'GAATGAGAATATCACCGTCCCAGACACCAAAGTTAACTT CTATGCCTGGAAGACGGCCATTTTCCAAGCAGGCTGTAGAAGTCTGGCA GGGCCTGGCCCTGCTCTCAGAAGCTGACGT3' and the sequence of the complementary sequence is 5'CAGCTTCTGAGAGCAGGGCCAGGCCCTGCCAGACTTCTACA

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1650

GCCTGCTTGGAAAATGGCCGTCTTCCAGGCATAGAAGTTAACTTTGGTG TCTGGGACGGTGATATTCTCATTCTGCA.3'.

[043] b. ITC^{hEPO} duplex assay

[044] The hEPO primer-probe assay targets the hEPO Exon3-4 junction, harbored in pShuttle-CAG-hEPO-pA plasmid. The ITC^{hEPO} duplex assay was designed with forward primer 5'TGAATGAGAATATCACTGTCCCAGAC3', reverse primer 5'CTTCCGACAGCAGGGCC3', hEPO probe 6FAM-AAGAGGATG GAGGTCGG-MGBNFQ and ITC probe VIC-CGGCC ATTTTCCA-MGBNFQ. The ITC probe targets ITC^{hEPO} template of two complementary synthetic single strand DNAs that were annealed. The sequence of the forward ITC template is 5'GTGAATGAGAATATCACTGTCCCAGACACCAAAGTTAACTTC TATGCCTGGAAGACGGCCATTTTCCAAGCAGGCTGTAGAAGTCTGGCAG GGCCTGGCCCTGCTGTC GGAAGGACGT3' and the sequence of the complementary ITC sequence is 5'CCTTCCGACAGCAGGGCCAGGCCCTGCCAGACTTCTACAGC CTGCTTGGAAAATGGCCGTCTTCCAGGCATAGAAGTTAACTTTGGTGTCT GGGACAGTGATATTCTC ATTCACTGCA3'.

[045] c. ITC^{CMV} duplex assay

[046] The CMV primer-probe assay targets the CMV immediate early promoter junction, harbored in pSSV9-MD2-cmEPO plasmid. The ITC^{CMV} duplex assay was designed with forward primer 5'AATGGGCGGTAGGCGTGTA3', reverse primer 5'CGATCTGACGGTTCACTAAACG3', CMV probe 6FAM-TGGGAGGT CTATATAAGC–MGBNFQ and ITC probe VIC-CG GCCATTTTCCA-MGBNFQ. The CMV ITC probe targets ITC^{CMV} template of two complementary synthesized single strand DNAs that were annealed. The

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1651

sequence of the forward ITC template is 5'CCGATCTGACGGTTCACTAAACGAGCTCTTGGAAAATGGCCGCCGTAC ACGCCTACCGCCCATTCTGCA3' and the sequence of the complementary sequence is 5'GAATGGGCGGTAGGCGTGTACGGCGGCCATTTTCCAAGAGCTCGTTTA G TGAA CCGTCAGATCGGACGT3'.

[047] ***Traditional real-time PCR program***

[048] Taqman Real-time PCR conditions were optimized with primers and their corresponding fluorescent probes. The concentrations of 250nM probe and 900nM primers were found to be optimal. 500ng of each DNA sample was amplified in a final volume of 25µL containing 1x TaqMan® Universal PCR Master Mix (Applied Biosystems cat# 4304437). Amplification was performed using an ABI StepOnePlus PCR machine with an initial incubation at 50°C for 2min, a denaturation at 95°C for 10min, then 40 cycles of denaturation at 94°C for 15s and an annealing/extension step at 60°C for 1min. During thermal cycling, emission from each sample was recorded and ABI StepOne software v2.0 processed the raw fluorescence data to produce threshold cycle (Ct) values for each sample.

[049] ***ITC duplex assay real-time PCR program***

[050] 500ng of macaque gDNA sample (for ITC^{cmEPO} duplex assay) or human gDNA (for ITC^{hEPO} and ITC^{CMV} duplex assays) was amplified in a final volume of 30µL containing 1x TaqMan® Universal PCR Master Mix (Applied Biosystems cat# 4304437) or 1x TaqMan® Fast Virus 1-Step Master Mix with reverse transcriptase (Applied Biosystems cat# 4444432). 5 copies or 10 copies of the corresponding ITC template were added in each reaction system. DMSO (Sigma D2650) was added in the final master mix to increase the assay sensitivity. Amplification was performed using an ABI StepOnePlus PCR machine with an initial incubation at 50°C for 2min, a denaturation at

SUBSTITUTE SHEET (RULE 26)

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1652

95℃ for 10min, then 40 cycles of denaturation at 94℃ for 15s and an annealing/extension step at 60℃ for 1min. During thermal cycling, 6FAM and VIC fluorescence emissions were recorded and ABI StepOne software v2.0 processed the raw 6FAM fluorescence data to produce threshold cycle (Ct) values for testing samples and VIC fluorescence data for ITC templates.

[051] ***Statistical Analyses***

[052] **a. SAS Analyses**

SAS 9.2 software was utilized to analyze data from ITC assays. SAS T-Test procedure was applied to compare the Ct values from each ITC assays. SAS GLM procedure was used to perform One-Way ANOVA and Two-Way ANOVA analyses.

b. Equivalence Testing

Independent sample equivalence testing was performed [42]. The null hypothesis (H_0) is $Ct_{Target} > Ct_{ITC}$ and $Ct_{Target} < Ct_{ITC}$; The alternative hypothesis (H_A) is $Ct_{Target} = Ct_{ITC}$. The tolerance limit (Δ) was set to 0.5 Ct to evaluate the parity of the Ct's from the two different real-time PCR reactions in the duplex assay. The confidence level (α) was set to 0.10. The critical t-value is $t_{\alpha, 2*n-2}$ which is obtained from the Student's t-test distribution table. The observed t-values are calculated:

$$t_{ci} = \frac{d - (-\Delta)}{S_d}$$

$$t_{cs} = \frac{\Delta - d}{S_d}$$

d is the difference in the mean Ct between the target sequence and corresponding ITC template. S_d is the pooled standard deviation of the two independent samples:

$$s_d = \sqrt{\frac{(n-1)s_1^2 + (n-1)s_2^2}{(2n-2)}} * \sqrt{\frac{2n}{(n*n)}}$$

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1653

n: Number of replicates

S_1: Standard deviation of Ct_{target}.

S_2: Standard deviation of Ct_{ITC}.

The rejection rule is: if t_{ci} and $t_{cs} > t_{\alpha, 2*n-2}$, then reject H_0.

[053] B. Results

[054] A novel real-time PCR-based approach for detecting exogenous DNA sequences has been developed. The system includes the use of an internal threshold control (ITC) template and corresponding ITC probe. The implementation of the ITC involves maintaining the melting temperature (Tm) of the ITC probe similar to the Tm of the probe used for the target, having the distance between the primer hybridization sites on the ITC template similar to the distance in the target, and labeling the ITC probe with a fluorescent dye different than the target probe. In this duplex assay format, the target and ITC template are co-amplified by the same primers, but are detected by two different probes each with a different fluorescent dye. Sample DNA, a prescribed number of ITC template molecules set near the limit of sensitivity, target primers, target probe and ITC probe are amplified in one reaction. Fluorescence emission signals are obtained by the real-time PCR machine simultaneously to determine the cycle thresholds (Ct) for amplification of the target and ITC sequences. Comparison of Ct from the ITC and Ct of the target is the parameter used to determine if test samples are positive or negative for the targeted DNA sequence. For the ITC duplex assays, if the Ct of the target probe is less than or equal to the Ct of the ITC probe, then the sample is

SUBSTITUTE SHEET (RULE 26)

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1654

considered a true positive (meaning that there is the same or more copies of the gene of interest relative to the ITC template). Additionally, if the Ct of the Target probe is greater than the Ct of the ITC probe, then the sample is considered negative.

[055] **Figure 1** represents the features and rationale of applying the ITC to detect exogenous sequences that are either homologous or non-homologous with genomic DNA. Two different ITC^{EPO} duplex assays were developed that target macaque or human EPO cDNA. These ITC^{EPO} duplex assays are performed in the presence of the endogenous homologous genomic locus, thus the ITC assay needs to distinguish the cDNA carried in a gene transfer vector from the cellular host genomic DNA. For this, the EPO primer-probe assays were designed to target an EPO Exon-Exon junction (Exon 2-3 junction for cmEPO and Exon 3-4 junction for hEPO). The synthetic ITC^{EPO} template includes a different probe binding site and maintains the flanking EPO sequences including the EPO primer binding sites. As a consequence, the EPO primers recognize the EPO cDNA in the viral vector, along with the ITC^{EPO} template, and macaque or human gDNA. However, the EPO probe (6FAM dye) specifically detects the EPO cDNA, and the EPO ITC probe (VIC dye) specifically detects the EPO ITC template and neither can detect genomic DNA (**Figure 1A**).

[056] To demonstrate the applicability of the ITC duplex assay approach to address infectious agent detection, we also developed an ITC duplex assay in which the cytomegalovirus (CMV) promoter, used in the pSSV9-MD2-cmEPO plasmid, was targeted. The CMV promoter PCR target developed here is homologous to the promoter found in most CMV strains, including: the Towne strain (Genbank AY315197) that is the basis of the National Institute of Standards and Technology (NIST) Reference plasmid, the Merlin strain (Genbank AY446894) used in the World Helath Organization

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1655

(WHO) reference standard (National Institute for Biological Standards and Control (NIBSC) product # 09/162), the JP strain (Genbank GQ221975) from a clinical specimen, and cell culture strains HAN38 (Genbank GQ396662) and VR1814 (Genbank GU179289). Since the heterologous CMV sequences are targeted, there is no competition with the host genomic DNA (**Figure 1B**).

[057] Traditional real-time PCR assays to detect the hEPO cDNA and CMV promoter were first developed using an approach similar to our previously published assay for cmEPO cDNA [32]. Design of the corresponding synthetic ITC template for each of these targets requires that (1) the distance between the primers is similar to the distance between the same primer sites on the target; (2) the target probe and ITC probe have similar Tm; (3) the PCR products have similar Tm; (4) there is no homology between the target probe and ITC template nor between ITC probe and target sequences. For each assay the primers and probes were verified *in silico* to ensure that there was no cross hybridization between primers, primers and probes, primers/probe sets and EPO cDNA or genomic DNA or ITC template. The PCR products of each individual PCR assay were analyzed by agarose gel electrophoresis and demonstrated a single band (data not shown). The performance of each individual assay was evaluated.

[058] Performance and detection limits of individual assays

[059] Traditional real-time PCR was performed to evaluate the lower limit of quantitation and linearity of the six individual assays. A titration of the pSSV9-MD2-cmEpo plasmid in the presence of naïve macaque genomic DNA illustrates that the cmEPO assay is capable of detecting 5 copies. Likewise, a titration of the ITC^{cmEPO} template illustrates that the ITC^{cmEPO} assay is capable of detecting 5 copies in the presence of naïve macaque genomic DNA. The sensitivities of the individual hEPO, ITC^{hEPO}, CMV, ITC^{CMV} assays in the presence of naïve human genomic DNA is 10 copies. The linearity of all the

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1656

six assays is above 0.98 over an 8 log dynamic range from 10 to 1E9 copies (**Table 1**). The reduction in efficiencies seen in the presence of gDNA most likely reflects competition for primers. To evaluate the accuracy of detecting the sequences at the lower limit of quantitation, 15 replicates were performed for cmEPO and ITC^{cmEPO} assays at 5 copies and at 10 copies for the other four assays and the means and standard errors of copy number and Ct were calculated (**Table 2**).

[060] Specificity of the individual assays

[061] The specificity of the individual assays was tested (**Table 3**). A lack of signal at 40 cycles was defined as "negative" and a Ct signal before 40 cycles of amplification was considered "positive". 500ng of naïve macaque or human gDNA were used as samples. **Table 3** demonstrates that no false positive signals were detected in 20 replicates of each of the six individual assays caused by either non-targeted exogenous or endogenous DNA sequences, or laboratory contamination. To confirm that we are detecting the vector genome harboring the EPO cDNA and not residual endogenous mRNA, we obtained the copy number signal with our standard master mix and this signal was not influenced by RNase. Furthermore, when a master mix that utilizes a reverse transcriptase step was used, endogenous mRNA was detected and this RT-dependent signal was reduced with an RNase A pre-treatment (data not shown).

[062] Equivalence testing of ITC duplex assays

[063] Each of the three individual target assays was paired with the corresponding ITC assay to create the three ITC duplex assays. The ITC duplex assay format includes the requirement that same copy number of target sequences and corresponding ITC templates give similar Ct's. In cases where the P-value shows a statistically significant difference, such as when the Ct of the target is significantly higher or lower than the Ct of the ITC, then

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1657

the Student's t-test can be used. However, when the Ct of the target is equal to the Ct of the ITC, the equivalency needs to be confirmed statistically to be able to designate the sample as a true positive. Equivalence testing of our three ITC duplex assays was performed. Each assay was used to detect target DNA sequences near their limit of sensitivity in the presence of an equal amount of ITC template and 500ng of naïve gDNA (**Figure 2**). Fifteen replicates were performed and the Ct's were recorded and analyzed. First, large P-values (cmEPO P=0.5816, hEPO P=0.6783, CMV P= 0.4819) were obtained by the Student's t-test suggesting that no significant differences exist between each target Ct and the corresponding ITC Ct from all three duplex assays. To determine if the Ct values were statistically equivalent, equivalence testing was conducted (see Materials and Methods), where the tolerance limit was set as 0.5 Ct (equal to 1.5% of the max Ct of 37), the confidence level (α) was set to 0.10, and the critical t-value is $t_{0.10, 28}$ =1.31. The calculated t_{ci}, and t_{cs} of the three assays are 2.8 and 1.7 for cmEPO, 2.7 and 2.1 for hEPO, and 1.6 and 3.0 for CMV. All of these t-values are greater than the critical t-value of 1.31, demonstrating that the Ct from a target DNA and the Ct from an equal amount of corresponding ITC template in each duplex assay are statistically equivalent.

[064] Assay Precision

[065] As shown in **Table 2**, the reproducibility of the assays was evaluated. Coefficients of variation (CV) from all six assays are within 3.2%. Furthermore, to determine the precision of the ITC^{hEPO} duplex assay, an additional experiment was conducted where eight replicates of the ITC^{hEPO} duplex assay were performed on three consecutive days using hEPO plasmid DNA in the presence of naïve human gDNA. The intra-assay CV is 1.3% and the inter-assay CV is 2.8%. These CVs of less than 3% demonstrate that the ITC^{hEPO} assay is highly reproducible. Moreover, one-way ANOVA was

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1658

performed to determine the relationship between testing days and Ct from the individual hEPO and ITC^{hEPO} PCR reactions, and shows that each Ct does not vary significantly over the testing days (data not shown).

[066] Evaluation of assay interference

[067] The target probe and its corresponding ITC probe are designed to detect two unique DNA sequences in one reaction. However, the two amplification systems share the same pair of primers, thus, possible competition between the target PCR reaction and the ITC PCR reaction was analyzed for each of the three ITC duplex assays. Experiments were performed where the copy number of the ITC template was held at 5 copies (cmEPO ITC) or 10 copies (hEPO or CMV ITC) in each reaction, while the target template was titrated from 5 or 10 copies to 100 copies, which is at the upper range of rAAV copies seen in 500ng of macaque WBC gDNA at late timepoints following intramuscular injection [32]. As shown in **Figure 3**, the observed Ct from the ITC probe is stable while the Ct from the corresponding target probe increases according to the decrease in target copy number in the presence of 500ng naïve gDNA. All three assays show the same pattern, which demonstrates that the target template does not interfere with the ITC detection in this copy number range. In the cases where the target copy number is over 100 copies, then an unequivocal signal will be detected by the target probe, even if an ITC signal is not detected, and will warrant further analysis of the sample.

[068] In addition, testing was performed to evaluate the dynamic range of all the three ITC duplex assays (**Figure 4**). The total number of the two templates was maintained at 100 copies. For each duplex assay, a plasmid harboring the target was titrated reciprocally with the ITC template in the presence of 500ng naïve gDNA. The Ct of both probes illustrates that the Ct values for each template changed only with the template amount, without

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1659

interference from the other template. As a result, if no signal is obtained from the ITC template it is most likely due to an inhibitor present in the sample, and the test will be invalid and require further analysis.

[069] Transduced Macaque blood sample testing

[070] The performance of the ITC assay format was evaluated on the WBC DNA samples taken from macaques transduced *in vivo* with rAAV vectors and previously analyzed by traditional real-time PCR, where the actual copy number was determined at each timepoint [32]. The ITC^{cmEPO} duplex assay was conducted on both rAAV1 and rAAV8 *in vivo* samples. For the ITC testing, a 500ng DNA sample and 5 copies of ITC^{cmEPO} template were amplified simultaneously in the presence of the cmEPO primers, the cmEPO probe, and the cmEPO ITC probe, and both fluorescence signals were recorded to obtain the Ct's. Each sample was tested repeatedly 5 times in order to acquire the mean and standard error for statistical analysis. Comparing the mean of cmEPO Ct and ITC^{cmEpo} Ct, the tested samples are defined to be positive (cmEPO Ct is less than or equal to the ITC^{cmEpo} Ct) or negative (cmEPO Ct is greater than ITC^{cmEpo} Ct with P-Value < 0.05). As shown in **Table 4**, the ITC^{cmEPO} duplex assay results from both rAAV1 and rAAV8 injected animals are consistent with the previous absolute copy number data. Samples having more than two copies per 500ng DNA are positive in the ITC duplex assay, meanwhile, the pre-injected samples test negative. The Student's t-test was applied and P-values of <0.001 were obtained when the absolute value of the difference between cmEPO cDNA and ITC^{cmEPO} template copy numbers is larger than five, demonstrating that this Ct difference is statistically significant. In addition, there is little to no competition of the target with the ITC, since the Ct's of the ITC for both animals at all vector copy numbers is ~37 (CV=1.5%). Furthermore, the

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1660

samples do not appear to contain inhibitors since the Ct=37 is similar to the Ct seen in TE (in the absence of gDNA, **Table 2**).

[071] Testing of human WBC gDNA spiked with plasmid DNA

[072] The plasmid pShuttle-CAG-hEPO-pA harboring the hEPO cDNA was spiked into 500ng naïve human gDNA at the same copy numbers detected in the rAAV1 injected macaque shown in **Table 4**, and ten copies of ITC^{hEPO} template was added to each reaction. **Table 5** shows that spiking with 188, 13 and 8 copies of pShuttle-CAG-hEPO-pA were designated as positive, meanwhile 0 and 2 copies were designated negative when compared to the ITC^{hEPO} Ct.

[073] The ITC duplex assay format is also capable of being used to detect human infectious agents such as viruses or bacteria that have sequences that are non-homologous to human genomic DNA. Cytomegalovirus causes many human infections [43], and the viral load in blood is very important for clinicians to evaluate patients' prognosis. As a proof of concept, The CMV immediate early promoter in plasmid pSSV9-MD2-cmEPO was used as a PCR target for two reasons. The first is to compare the sensitivity of the EPO intron-spanning PCR to a target that has no competition with human genomic DNA, and the second is to demonstrate the applicability to using the ITC approach for infectious disease diagnosis and treatment monitoring. As can be seen in **Tables 1 and 2**, the CMV and hEPO assays had similar 10 copy sensitivities.

[074] For the ITC^{hEPO} and ITC^{CMV} duplex assays, we determined the difference in the Ct's between the target sequence and corresponding ITC template at the 95% confidence interval. For this purpose, the ITC templates were held constant at 10 copies and a titration of 5, 10 and 20 copies of target plasmid were added to evaluate the ability to determine positive (10 and 20 copies) from negative (5 copies) samples. Four different naïve human gDNA

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1661

samples spiked with 5, 10 and 20 copies of hEPO or CMV target plasmid were amplified in the presence of 10 copies of their corresponding ITC template. The Ct's were analyzed by Student's t-test and One-Way ANOVA. **Table 6** shows that, with all four human gDNA sources, the Ct from 5 copies of hEPO or CMV target sequence is significantly larger than 10 copies of corresponding ITC template, the Ct from 20 copies is significantly smaller than 10 copies of ITC template, and no statistical difference (evaluated by equivalence testing – data not shown) was seen when the target and ITC copy numbers were equal at 10 copies each. Two-Way ANOVA analysis was performed and showed that the copy number detected was independent of the different gDNA samples (data not shown).

[075] The human Epo PCR assay was designed in a homologous region as the macaque Epo PCR assay (Figure 5). The human Epo ITC assay format was also tested by titrating the two DNA molecules (plasmid target and ITC^{hEPO} template, Figure 4B). The Ct of both amplifications illustrated that Ct values changed only with the target template amount but was not affected by the other template, demonstrating that no obvious interference exists. As a result, there is a high confidence that an absence of ITC signal would indicate the presence of an inhibitor in the sample.

[076] Discussion

[077] Reported herein is the development of a real-time PCR assay format for detecting homologous and non-homologous exogenous DNA. These tests are useful for infectious disease diagnosis, gene therapy clinical trial safety, and gene doping surveillance where the control of false negative and false positive results, and the assurance of true positive results is required. These procedures facilitate the procurement, preparation, and testing of samples to detect exogenous DNA sequences in a user-friendly

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1662

format. Furthermore, the ITC assay format described here is ideal for clinical testing labs since 1) it is a "single tube" assay [sample DNA + master mix (2 primers, 2 probes and ITC template)], 2) it is specific and sensitive with samples internally controlled, 3) it is fast: 2 to 3 hours (including set up and analysis), 4) it requires no standard curve titration tubes (no external standards), 5) it is high throughput, capable of analyzing many samples at once, 6) it is automated: data captured by PCR machine and results can be transferred to centralized database(s), 7) no additional manipulations are needed for analysis such as gel electrophoresis, and 8) it reduces risk of laboratory contamination (a source of false positives) since no positive control plasmid is needed and uracil-N-glycosylase (UNG) prevents the re-amplification of carryover PCR products in subsequent analyses.

[078] When designing and validating an ITC duplex assay, in addition to considering GC content, primer and probe Tm, and amplicon length, additionally, lack of cross complementary of the primers and two probes, the targets or amplicons, or any combination of these is needed [44]. Moreover, since both assays are amplified in a single tube, amplification competes for the same dNTPs and polymerase. One more challenge in the detection of cDNAs in the presence of gDNA is that the assay must detect the exon-exon junction of the cDNA, which greatly restricts the choice of primers and probes.

[079] The individual assays described here have a sensitivity of 10 copies or better in the presence of 1.5E5 cellular genome copies and maintain linearity over 8 logs. The two probes in each duplex assay give similar Ct values when detecting the same amount of their respective sequences, and this was supported statistically using equivalence testing. Interference and competition testing has shown that target probe, ITC probe, target template and ITC template will not inhibit each other in the range of 10-100 copies.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1663

Furthermore, similar amplification of the PCR targets was achieved in individual PCR reactions compared with the duplex PCR reactions.

[080] The maintenance of rAAV sequences in WBC of nonhuman primates [32] and humans [25] provides an easily accessible target for the surveillance of gene doping. Our previous experiments in the nonhuman primate were designed to test the feasibility of detecting vector sequences in blood long-term following IM injection and to gain insight into testing humans. We have designed the ITC duplex PCR format described in this paper with the expectation that athlete samples will be sent to testing laboratories for preparation and PCR analysis, and then the test results will then be sent to central databases for analysis and trending. The methods developed to detect EPO also provide the basis to detect other prohibited gene doping targets.

[081] Previous studies have introduced internal controls using cellular housekeeping genes to control for possible PCR inhibitory factors, but they were not used to set a copy number threshold of the exogenous sequence target [45] [46]. Moreover, competitive PCR methods are used to quantify DNA copy number, however, this approach is limited by the necessity of assembling multiple competitive reactions for a single determination and, most notably, the need for a post-PCR detection and analysis step [47]. On the other hand, each ITC duplex assay detects two different DNA sequences in one reaction by real-time PCR, with the Ct from the ITC probe being a threshold to evaluate if the samples are positive or negative. The ITC^{cmEPO} duplex assay testing on blood samples from macaques transduced *in vivo* with rAAV vectors shows consistent results with our previous quantitative data [32]. Furthermore, to simulate the testing of human samples, the ITC templates were held constant and the corresponding hEPO or CMV target plasmid was titrated in the presence of naïve human genomic DNA to evaluate the ability to determine positive from negative samples.

SUBSTITUTE SHEET (RULE 26)

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1664

[082] ITC assays can be used to detect heterologous sequences (infectious agents) or exogenously added homologous cDNA sequences (gene transfer vectors). Thus, ITC assays can be applied to pre-clinical animal biodustribution studies and legitimate human gene therapy clinical trials to determine the presence or absence of gene transfer vector sequences in different tissues, where the ITC can control for different types of inhibitors potentially present in different tissue samples. Likewise, molecular tests for infectious disease diagnosis, prognosis, and evaluation of response to therapy could benefit from an ITC assay approach. The ITC assay format is also applicable to gene doping surveillance testing as a means to deter the illegitimate use of gene transfer vectors for athletic performance. Compared to traditional real-time PCR, the ITC assay format has advantages for detecting exogenous DNA.

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1666

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1667

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IPR2024-01177
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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1669

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1670

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[0131] It should be borne in mind that all patents, patent applications, patent publications, technical publications, scientific publications, and other references referenced herein are hereby incorporated by reference in this application in order to more fully describe the state of the art to which the present invention pertains.

[0132] It is important to an understanding of the present invention to note that all technical and scientific terms used herein, unless defined herein, are intended to have the same meaning as commonly understood by one of ordinary skill in the art. The techniques employed herein are also those that are known to one of ordinary skill in the art, unless stated otherwise. For purposes of more clearly facilitating an understanding the invention as disclosed and claimed herein, the following definitions are provided.

[0133] While a number of embodiments of the present invention have been shown and described herein in the present context, such embodiments are provided by way of example only, and not of limitation. Numerous variations, changes and substitutions will occur to those of skilled in the art without materially departing from the invention herein. For example, the present invention need not be limited to best mode disclosed herein, since

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1671

other applications can equally benefit from the teachings of the present invention. Also, in the claims, means-plus-function and step-plus-function clauses are intended to cover the structures and acts, respectively, described herein as performing the recited function and not only structural equivalents or act equivalents, but also equivalent structures or equivalent acts, respectively. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the following claims, in accordance with relevant law as to their interpretation.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1672

Table 1: Individual assay linearity and efficiency

Assay	Linearity (R^2) In the absence of 500ng gDNA	Linearity (R^2) In the presence of 500ng gDNA*	Efficiency (%) In the absence of 500ng gDNA	Efficiency (%) In the presence of 500ng gDNA
cmEPO	0.992	0.989	95	82
ITC^{cmEPO}	0.989	0.998	91	86
hEPO	0.999	0.998	92	88
ITC^{hEPO}	0.996	0.993	89	86
CMV	0.996	1.000	99	97
ITC^{CMV}	0.997	0.989	98	94

*macaque gDNA with cmEPO and ITC^{cmEPO} assays, and human gDNA with hEPO, ITC^{hEPO}, CMV and ITC^{CMV} assays.

Table 2: Individual assay sensitivity

Assay	Positive Control	Mean* Copy Number (s.e.)	Mean* Ct (s.e.)	CV (%) of Ct
cmEPO	5 copies pDNA with 500ng gDNA (macaque)	6.7 (2.5)	37.24 (0.22)	2.2
	5 copies pDNA with TE	5.9 (3.4)	37.47 (0.28)	2.9
ITC^{cmEPO}	5 copies ITC template with 500ng gDNA (macaque)	6.5 (2.7)	37.22 (0.24)	1.5
	5 copies ITC template with TE	5.5 (3.8)	37.14 (0.31)	3.2
hEPO	10 copies pDNA with 500ng gDNA (human)	9.6 (3.3)	36.74 (0.14)	1.5
	10 copies pDNA with TE	10.8 (2.8)	36.45 (0.10)	1.1
ITC^{hEPO}	10 copies ITC template with 500ng gDNA (human)	11.8 (3.9)	36.55 (0.14)	1.6
	10 copies ITC template with TE	11.1 (2.4)	36.73 (0.10)	0.9
CMV	10 copies pDNA with 500ng gDNA (human)	11.9 (5.3)	36.29 (0.18)	2.0
	10 copies pDNA with TE	11.1 (4.1)	36.35 (0.14)	1.5
ITC^{CMV}	10 copies ITC template with 500ng gDNA (human)	10.7 (4.3)	36.64 (0.16)	1.7
	10 copies ITC template with TE	11.8 (4.0)	36.55 (0.13)	1.4

*From fifteen replicates of plasmid (pDNA) or ITC template. Standard error (in parenthesis).

Table 3: Individual assay specificity

Assay	Negative Control	False positive	False Positive Rate (%)
cmEPO	500ng gDNA (macaque)	0/20	0
ITC^{cmEPO}	500ng gDNA (macaque)	0/20	0
hEPO	500ng gDNA (human)	0/20	0
ITC^{hEPO}	500ng gDNA (human)	0/20	0
CMV	500ng gDNA (human)	0/20	0
ITC^{CMV}	500ng gDNA (human)	0/20	0

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1673

Table 4: ITC^{cmEPO} duplex assay testing of WBC samples from NHP transduced IM *in vivo*

rAAV1

Time Points	Mean* cmEPO Ct (s.e.)	Mean ITC^{cmEPO} Ct** (s.e.)	Positive/Negative	Actual Copy Number***
Pre-injection.	40.00$^{\#}$ (0)	37.00 (0.29)	Negative (P-value <0.0001)	0
3 Days p.i.	32.81 (0.10)	37.21 (0.15)	Positive (P-value <0.0001)	188
7 Days p.i.	35.92 (0.27)	37.48 (0.29)	Positive (P-value =0.0019)	13
14 Days p.i.	36.94 (0.51)	37.46 (0.40)	Positive (P-value =0.4350)	8
10 Weeks p.i.	38.49 (0.49)	37.67 (0.13)	Positive (P-value =0.1066)	2
16 Weeks p.i.	38.32 (0.72)	37.18 (0.73)	Positive (P-value = 0.2913)	3
23 Weeks p.i.	39.91 (0.09)	37.18 (0.23)	Negative (P-value <0.0010)	0

rAAV 8

Time Points	Mean* cmEPO Ct (s.e.)	Mean ITC^{cmEPO} Ct** (s.e.)	Positive/Negative	Actual Copy Number***
Pre-injection.	40.00$^{\#}$ (0)	37.43 (0.24)	Negative (P-value <0.0001)	0
3 Days p.i.	35.70 (0.49)	36.67 (0.12)	Positive (P-value <0.0875)	10
7 Days p.i.	36.68 (0.35)	36.99 (0.44)	Positive (P-value = 0.5923)	7
14 Days p.i.	37.44 (0.43)	37.43 (0.14)	Positive (P-value = 0.9795)	8
10 Weeks p.i.	40.00 (0)	36.82 (0.33)	Negative (P-value< 0.0001)	0
16 Weeks p.i.	38.02 (0.68)	36.83 (0.68)	Positive (P-value = 0.2528)	2
23 Weeks p.i.	38.17 (0.54)	37.30 (0.19)	Positive (P-value = 0.1432)	3

*From five replicates of each 500ng *in vivo* sample. s.e. = standard error (in parenthesis).
**From an input of five copies of ITC^{cmEPO} template.
***The actual copy number is from our previous study [32].
#The number of cycles of the real-time PCR program was set to 40.

Table 5: ITC^{hEPO} duplex assay testing

Copy Number of Spiked Plasmid	Mean* hEPO Ct (s.e.)	Mean ITC^{hEPO} Ct** (s.e.)	Positive/Negative
0	40.00$^{\#}$ (0)	36.93 (0.42)	Negative (P-value <0.0001)
188	32.60 (0.44)	36.48 (0.33)	Positive (P-value <0.0001)
13	36.63 (0.28)	37.12 (0.27)	Positive (P-value =0.2426)
8	36.88 (0.28)	36.84 (0.28)	Positive (P-value =0.9172)
2	39.80 (0.20)	37.32 (0.37)	Negative (P-value <0.0001)

*From five replicates of pShuttle-CAG-hEPO-pA plasmid DNA spiked into 500ng naïve human gDNA. Standard error (in parenthesis).
**From an input of 10 copies of ITC^{hEPO} template.
#The number of cycles of the real-time PCR program was set to 40.

Table 6: Plasmid spiking of different naïve human gDNA samples

Human gDNA	5:10*		P-Value	10:10**		P-Value	20:10***		P-Value
	Mean§ hEPO Ct	Mean ITC^{hEPO} Ct		Mean§ hEPO Ct	Mean ITC^{hEPO} Ct		Mean§ hEPO Ct	Mean ITC^{hEPO} Ct	
1	37.36 (0.84)	36.25 (0.53)	0.0368	36.80 (0.41)	36.70 (0.83)	0.8853	35.36 (0.46)	36.95 (0.50)	0.0482
2	38.03 (0.40)	36.25 (0.24)	0.0052	36.88 (0.32)	36.99 (0.58)	0.8663	35.17 (0.39)	36.41 (0.14)	0.0168
3	37.45 (0.85)	36.39 (0.38)	0.0253	36.94 (0.44)	37.14 (0.83)	0.7840	35.18 (0.44)	37.46 (0.59)	0.0143
4	37.62 (0.33)	36.24 (0.10)	0.0040	36.91 (0.35)	37.00 (0.79)	0.8924	35.34(0.45)	36.97 (0.50)	0.0425

Human gDNA	5:10*		P-Value	10:10**		P-Value	20:10***		P-Value
	Mean† CMV Ct	Mean ITC^{CMV} Ct		Mean† CMV Ct	Mean ITC^{CMV} Ct		Mean† CMV Ct	Mean ITC^{CMV} Ct	
1	38.88 (0.35)	36.27 (0.08)	<0.0001	36.77 (0.08)	36.62 (0.12)	0.3259	36.21 (0.09)	36.65 (0.11)	0.0172
2	38.37 (0.32)	36.70 (0.12)	0.0011	36.56 (0.14)	36.97 (0.15)	0.3333	36.11 (0.11)	36.69 (0.08)	0.0025
3	38.55 (0.42)	36.82 (0.13)	0.0045	36.78 (0.07)	36.74 (0.13)	0.7807	36.23 (0.14)	36.91 (0.13)	0.0081
4	38.83 (0.32)	36.73 (0.18)	0.0005	36.67 (0.12)	36.67 (0.08)	0.9355	36.06 (0.23)	36.71 (0.04)	0.0183

§From five replicates of pShuttle-CAG-hEPO-pA plasmid DNA spiked into 500ng naïve human gDNA. Standard error (in parenthesis).
†From five replicates of pSSV9-MD2-cmEPO plasmid DNA spiked into 500ng naïve human gDNA. Standard error (in parenthesis).
*5 copies of plasmid and 10 copies of ITC template.
**10 copies of plasmid and 10 copies of ITC template
***20 copies of plasmid and 10 copies of ITC template
#The number of cycles of the real-time PCR program was set to 40.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1674

CLAIMS

What is claimed is:

1. A method of detecting exogenous DNA material in a subject, said method comprising:

obtaining a DNA containing sample from said subject;

conducting real-time PCR on said sample in the presence of a prescribed amount of an ITC template, primers for a target DNA sequence; a target probe that binds to said target DNA sequence and an ITC probe; and

determining whether said target DNA sequence amount is equal to or greater than said ITC template amount.

2. The method of claim 1, wherein said target DNA sequence is an exon-exon junction of DNA homologous to an endogenous gene.

3. The method of claim 2, wherein said endogenous gene is erythropoietin (EPO), darbepoetin (dEPO), hypoxia-inducible factor (HIF) stabilizers, Chorionic Gonadotrophin (CG), Luteinizing Hormone (LH); Insulins; Corticotrophins; Growth Hormone (GH), Insulin-like Growth Factor-1 (IGF-1), insulin-like growth factor-2 (IGF2), Fibroblast Growth Factors (FGFs), Hepatocyte Growth Factor (HGF), Mechano Growth Factors (MGFs), myogenin, peroxisome proliferator-activated receptor delta (PPARd), calcineurin-A-alpha, chorionic somatomammo-tropin hormone 1 (CSHI), chorionic somato- mammo-tropin hormone 1/2 (CSH1/CSH2), chorionic somatomammo-tropin hormone 2 (CSH2), chorionic somatomammo-tropin hormone-like 1 (CSHLI), myostatin inhibitor. Platelet-Derived Growth Factor (PDGF), Vascular-Endothelial Growth Factor (VEGF) as well as any other growth factor affecting muscle, tendon or ligament protein

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1675

synthesis/degradation, vascularisation, energy utilization, regenerative capacity or fibre type switching; and other substances with similar biological effect(s).

4. The method of claim 1, wherein said ITC template and said target DNA sequence comprise primer binding sequences recognized by said primers.

5. The method of claim 1, wherein the distance between primer binding sequences for the ITC template is the same for the target DNA sequence +/- 50bp.

6. The method of claim 1, wherein said prescribed amount is 3-5 or more copies of said ITC template.

7. The method of claim 6, wherein said prescribed amount is 3-100 copies.

8. The method of claim 6, wherein said prescribed amount is 3-10 copies.

9. The method of claim 1, wherein said subject is a human or non-human mammal.

10. The method of claim 1, wherein said subject is an organism .

11. The method of claim 1, wherein said target DNA sequence is homologous or nonhomologous to the host genome.

12. The method of claim 1, wherein said ITC probe comprises a first marker molecule.

13. The method of claim 12, wherein said first marker molecule is a fluorophore of a first color.

14. The method of claim 1, wherein said target probe comprises a second marker molecule.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1676

15. The method of claim 14, wherein said second marker molecule is a fluorophore of a second color.

16. The method of claim 1, wherein said ITC probe comprises a fluorophore that generates a first color signal and said target probe comprises a fluorophore that generates a second color signal.

17. A system for detecting foreign DNA material in a subject, said system comprising:

a real-time PCR instrument comprising a receptacle for holding a DNA containing sample; and

3 or more copies of an ITC template, primers for a target DNA sequence; target probe specific to said target DNA sequence and an ITC probe disposed in said receptacle.

18. An ITC template comprising a probe binding sequence that binds to a corresponding ITC probe and flanking primer recognizing sequences that binds to primers, wherein said primers also hybridize to primer sites of a separate target DNA sequence, said target DNA sequence lacking the ITC probe binding sequence.

19. The ITC template of claim 18, comprising a first and second primer sequence that flanks said probe binding sequence.

20. A system for detecting presence of a target DNA sequence in a biological sample, said system comprising:

an ITC template comprising a probe binding sequence that binds to a corresponding ITC probe and a first and second flanking primer recognizing sequence that binds to a first and second primer, respectively;

an ITC probe that binds to said probe binding sequence in the ITC template, said ITC probe comprising a first marker molecule;

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1677

a first primer that binds to said first flanking primer sequence in the ITC template and binds to sequences in the target DNA;

a second primer that binds to said second primer sequence in the ITC template and binds to sequences in the target DNA; and

a target DNA sequence probe, said target DNA sequence probe comprising a second marker molecule.

21. The system of claim 20, wherein said first marker molecule is a fluorophore of a first color and said second marker molecule is a fluorophore of a second color.

22. The system of claim 20, wherein said first and second marker molecules are different.

23. A kit comprising an ITC template according to claim 18 and an ITC probe.

24. The method according to claim 4, wherein said primers comprise a primer pair a first PCR primer can hybridize at stringent conditions to a first exon on the first strand of the target DNA sequence, and the second PCR primer can hybridize at stringent conditions to a second exon on the strand of the target DNA complementary to said first strand, wherein said second exon is located adjacent to said first exon.

25. The method of claim 2, wherein the target probe hybridizes with a first segment to a first exon and simultaneously with a second segment to a second exon of said target DNA sequence (intron-spanning probe).

26. A method according to claim 25, wherein intron- spanning PCR probe is designed hybridizes to such regions of said first and said second exons on said target DNA, which are conserved among splice variants of such genes from which the coding sequence of the target DNA derives.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1678

27. The method of claim 1, wherein determining comprises (i) determining a Ct of said target probe to obtain a first Ct and (ii) determining a Ct of said ITC probe to obtain a second Ct, wherein when first Ct is equal or lower than said second Ct the sample contains said target DNA sequence.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1679

Figure 1A

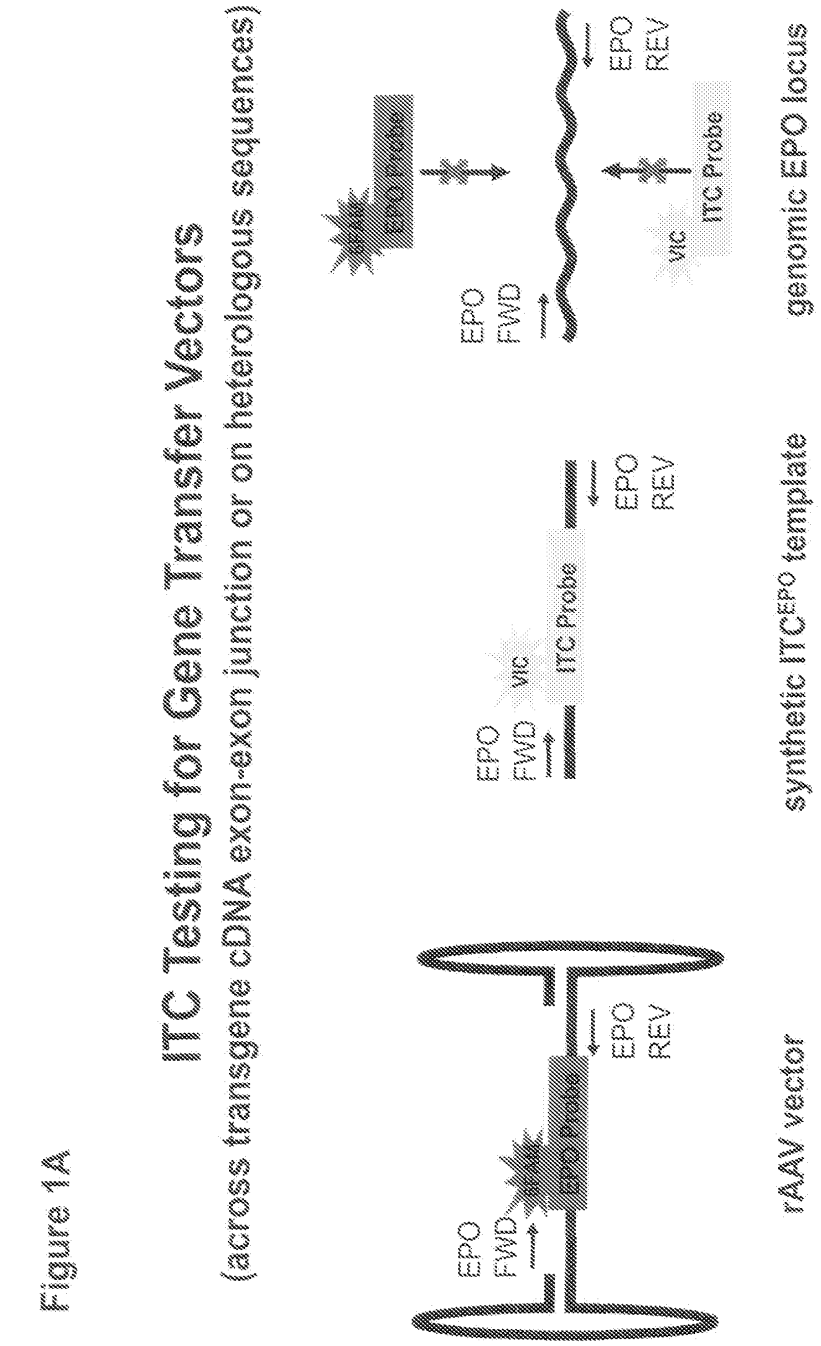
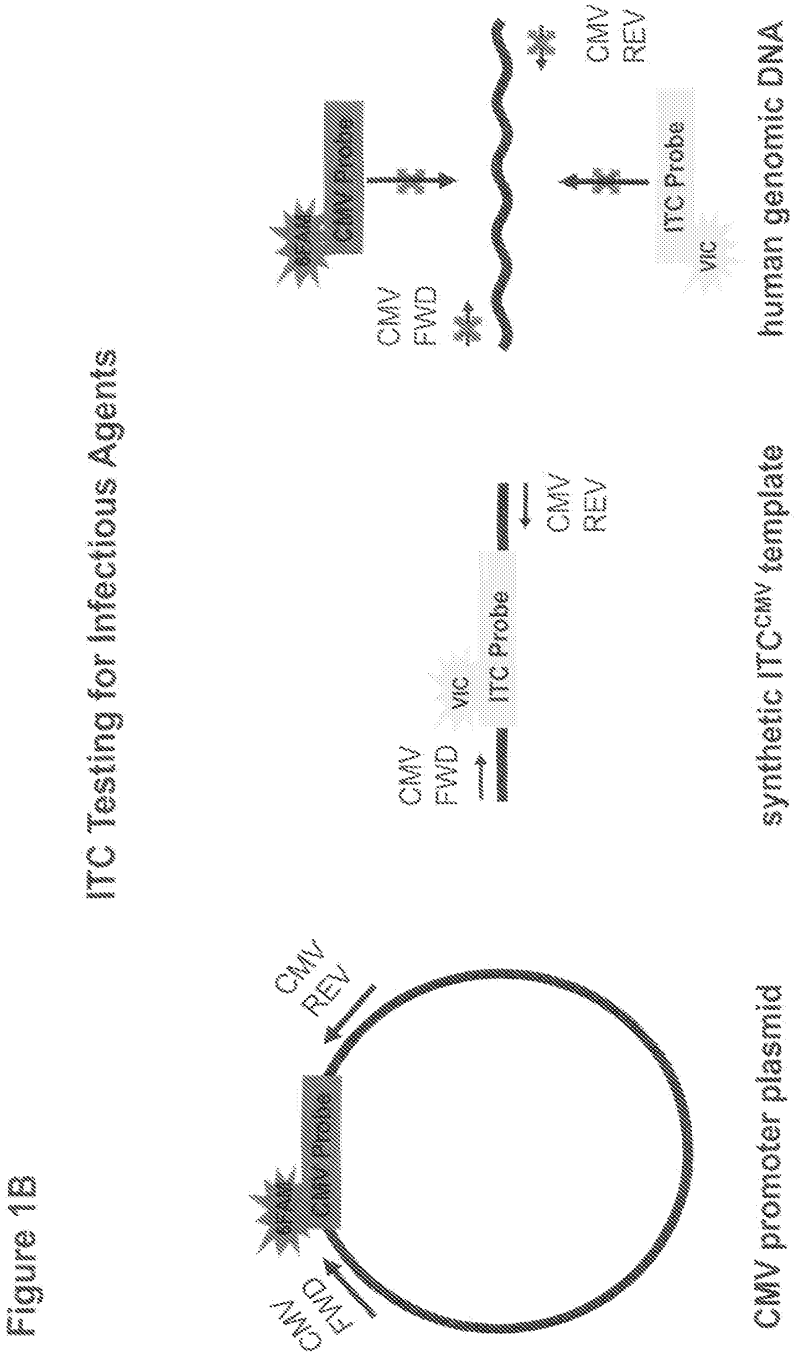

ITC Testing for Gene Transfer Vectors
(across transgene cDNA exon-exon junction or on heterologous sequences)
EPO FWD
ITC Probe
EPO REV
rAAV vector
EPO FWD
vic
ITC Probe
EPO REV
synthetic ITC^EPO template
EPO FWD
ITC Probe
EPO REV
vic
genomic EPO locus

Figure 1B


ITC Testing for Infectious Agents
CMV REV
CMV FWD
CMV promoter plasmid
(Infectious agent example)
CMV FWD
VIC
ITC Probe
CMV REV
synthetic ITC^CMV template
CMV Probe
CMV FWD
CMV REV
ITC Probe
VIC
human genomic DNA

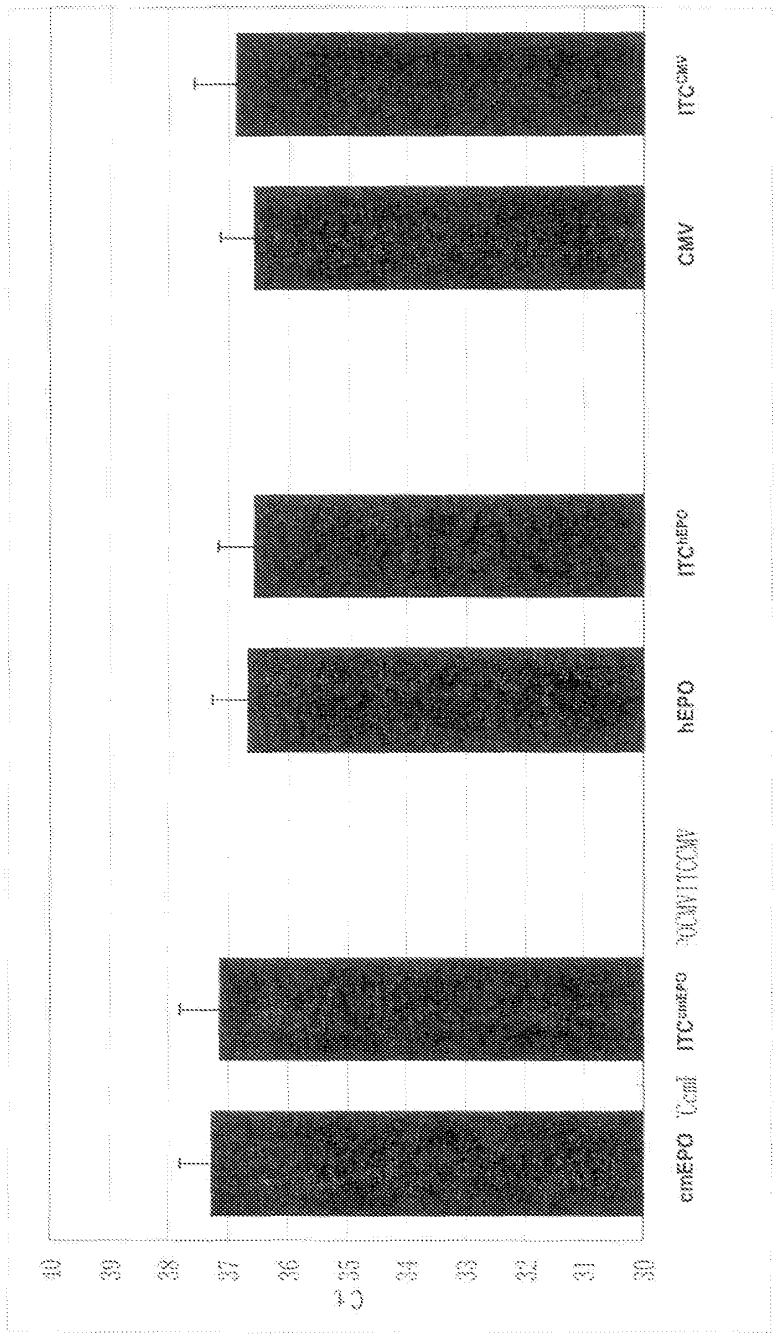

pmEPO (Cmt)
ITCmEPO
ITCmEPO
hEPO
ITCmEPO
CMV
ITCmEPO
Ct
40
39
38
37
36
35
34
33
32
31
30

Figure 2

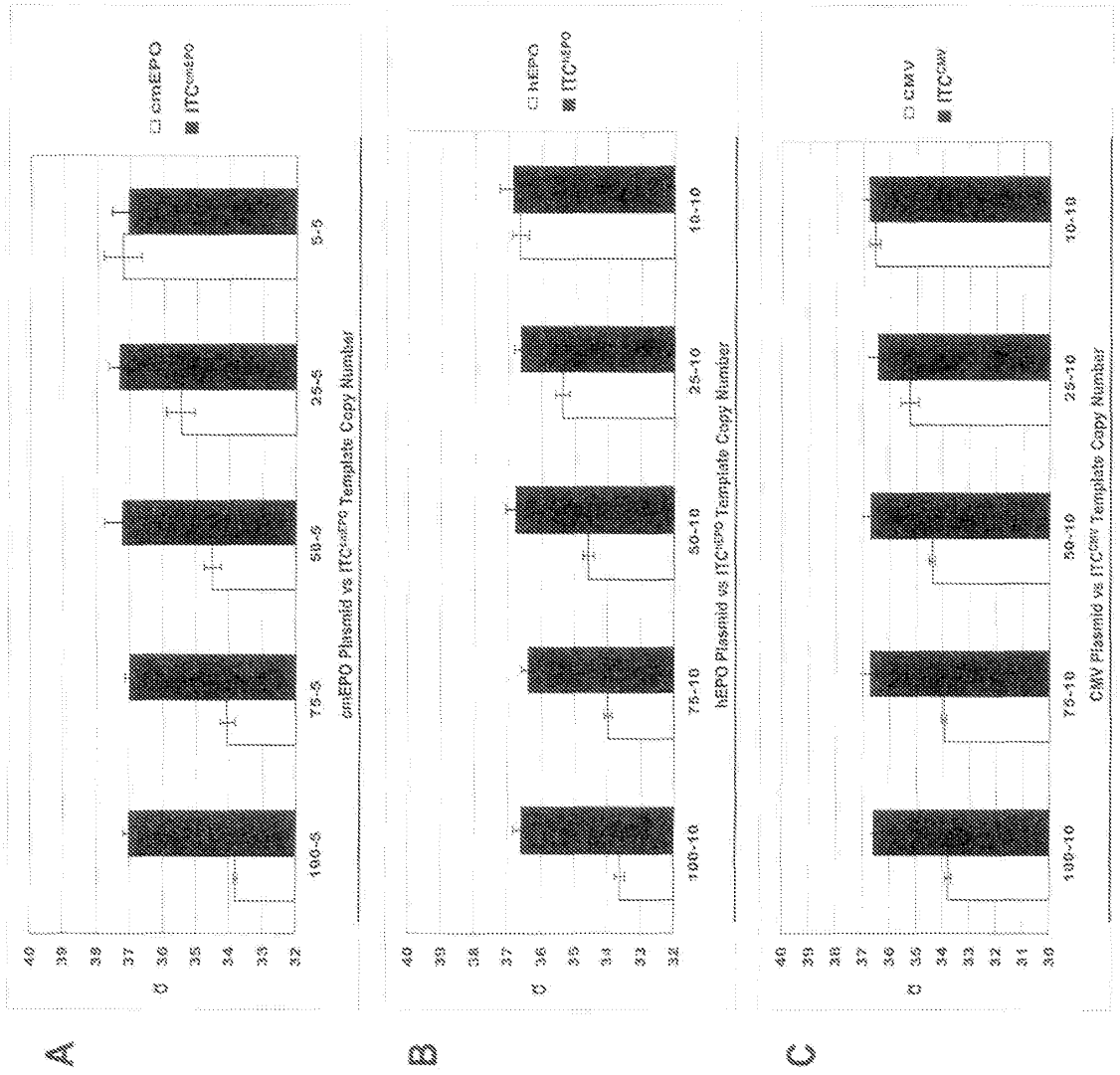
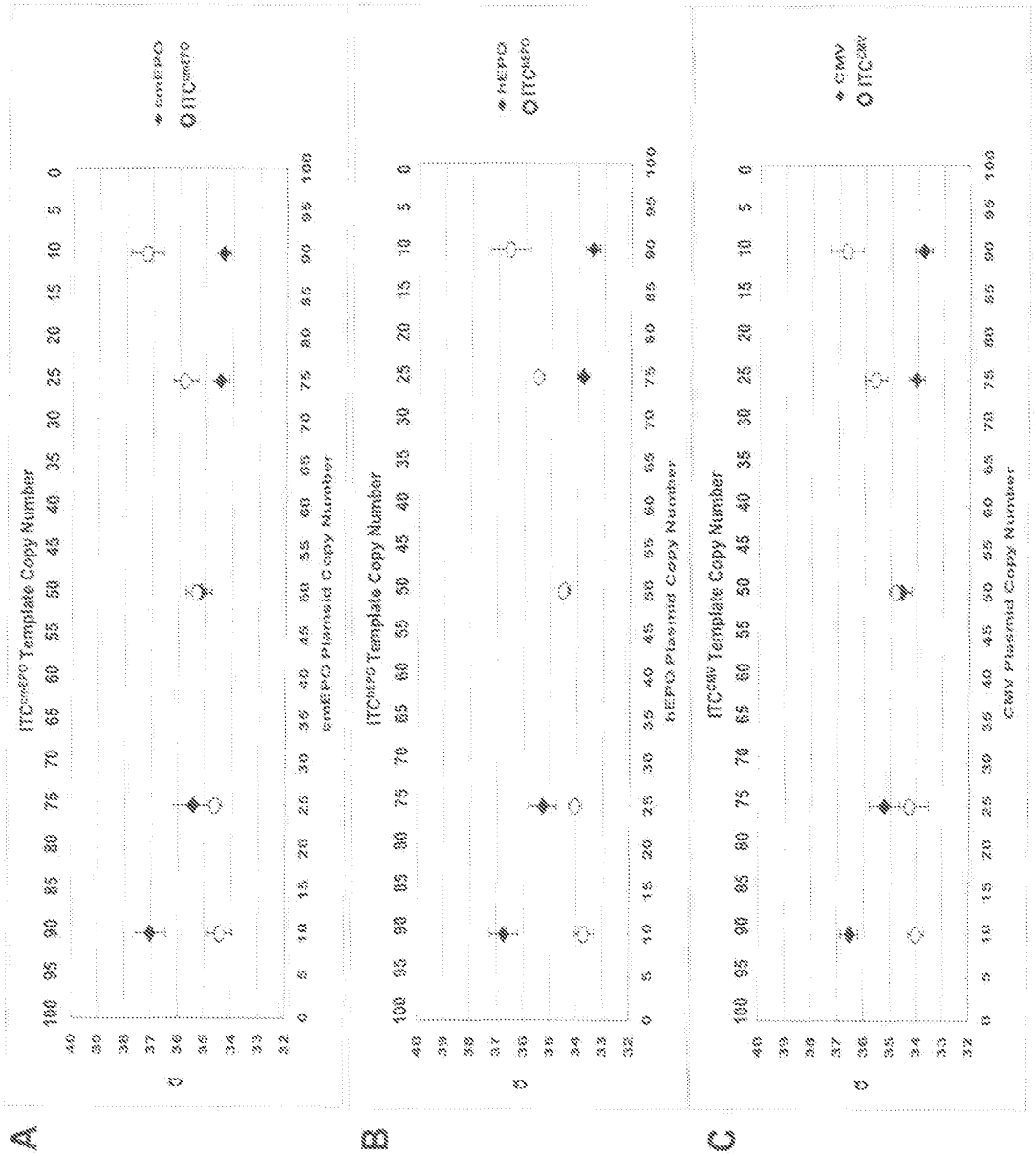
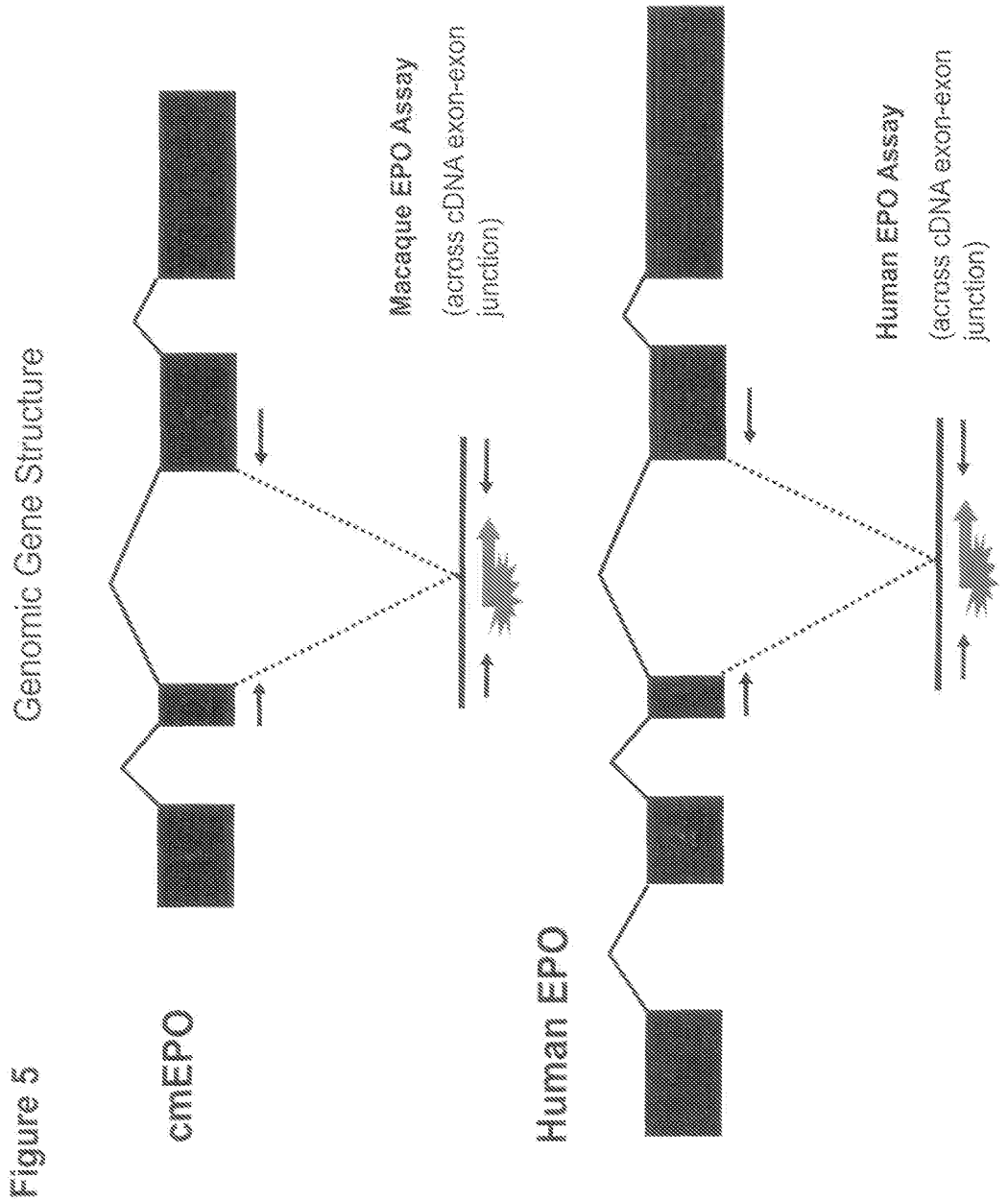

A
B
C
Ct
emEPO
ITCemEPO
hEPO
ITChEPO
CMV
ITCCMV
100-10
75-10
50-10
25-10
10-10
100-5
75-5
50-5
25-5
5-5
emEPO Plasmid vs ITCemEPO Template Copy Number
hEPO Plasmid vs ITChEPO Template Copy Number
CMV Plasmid vs ITCCMV Template Copy Number

Figure 3

Figure 4


A
B
C
ITC^cmEPO Template Copy Number
cmEPO Plasmid Copy Number
cmEPO
ITC^cmEPO
ITC^hEPO Template Copy Number
hEPO Plasmid Copy Number
hEPO
ITC^hEPO
ITC^CMV Template Copy Number
CMV Plasmid Copy Number
CMV
ITC^CMV


Figure 5
Genomic Gene Structure
cmEPO
Macaque EPO Assay
(across cDNA exon-exon junction)
Human EPO
Human EPO Assay
(across cDNA exon-exon junction)

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(54) Title: COMPOSITIONS AND METHODS FOR DIAGNOSING CANCER

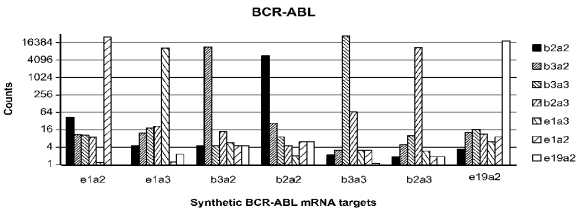


FIG. 1A

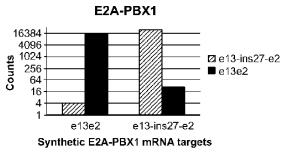


FIG. 1B

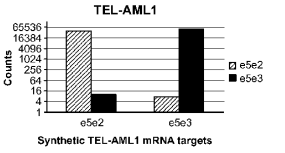


FIG. 1C

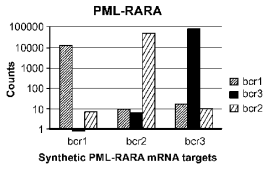


FIG. 1D

(57) Abstract: The application describes methods for diagnosing subjects with leukemia by detecting fusion genes associated with the onset of leukemia.

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COMPOSITIONS AND METHODS FOR DIAGNOSING CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application claims the benefit of U.S. Provisional Application No. 61/468,361, filed March 28, 2011, the contents of which are incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[02] This disclosure relates generally to the field of cancer biology, and specifically, to the fields of detection and identification of specific cancer cell phenotypes and correlation with relevant patient prognosis.

BACKGROUND OF THE INVENTION

[03] Recurrent chromosomal translocations characterize a substantial proportion of leukemias, resulting in the formation of unique fusion genes. The identification of particular fusion gene abnormalities is of considerable diagnostic and prognostic importance. There is a need in the art for reliable diagnostic compositions for the detection and diagnosis of leukemias and sub-types of leukemias based on gene fusion.

SUMMARY OF THE INVENTION

[04] Leukemia is often accompanied by the formation of fusion genes. These chromosomal abnormalities are caused by the reciprocal translocation of portions of genes at different chromosomal locations forming a new gene characterized by the fusion of a nucleic acid sequence from a first and a second gene. For example, the *BCR-ABL* fusion gene is caused by a reciprocal translocation of parts of chromosome 9 and 22. Specifically, the translocated regions are 9p34 and 22q11. This joins a portion of the *ABL1* gene on chromosome 9 to a part of the *BCR* ("breakpoint cluster region") of chromosome 22. The presence of this and other fusion genes is correlated with the onset and progression of various types of leukemia. This disclosure presents compositions and methods for the diagnosis and prognosis of leukemia based on the detection of fusion genes.

[05] The disclosure provides a composition for the diagnosis of leukemia. In one embodiment, this composition includes one or more target-specific nucleic acid probes. Each of

the one or more target-specific probes includes a target-specific region that specifically binds to one target nucleic acid analyte; and a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe. The label has a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid. One or more targets of the one or more target-specific probes include a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.

[06] The fusion genes can be caused by the fusion of two or more genes at the onset of leukemia. The fusion gene can be *BCR-ABL, AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*. The fusions can be between varying exons of the upstream and downstream genes. The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia.

[07] In certain embodiments, at least one of the one or more target-specific probes has a target that is the sequence of the junction where the two genes are fused. These target-specific probes can also include a target-specific region with one or more mismatches to the target of the target-specific region.

[08] In other specific embodiments, the label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[09] The disclosure also provides a composition for the diagnosis of leukemia and different sub-types of leukemia. In one embodiment, the composition comprises a probe pair including a first probe and a second probe. The first probe is made up, at least in part, of a complex including a first molecule. The first molecule includes a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal. The first molecule also includes a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal. The first molecule also includes a first target-specific sequence attached to the first molecule. The second probe is made up, at least in part, of a second molecule including a second target-specific sequence and an affinity tag. The first target-specific sequence and the second target-specific sequence bind to different regions of the same target

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1689

molecule. The target molecule is a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia. Also, when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule.

[10] In certain embodiments, the fusion gene results from the fusion of at least a portion of a first gene and at least a portion of a second gene. In this situation, the first target-specific sequence can hybridize to a portion of the first gene and the second target-specific sequence can hybridize to a portion of the second gene. The first target-specific sequence can hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

[11] In other embodiments, the first target-specific sequence can also hybridize to a portion of the first gene and a portion of the second gene. In this situation, the first target-specific sequence can hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[12] The second target-specific sequence can also hybridize to a portion of the first gene and a portion of the second gene. In this situation, the first target-specific sequence can hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[13] The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia. The fusion gene can be *BCR-ABL, AML-ETO, CBFB-MYH11, E2A-PBX1, MLL-AF4, PML-RARA*, or *TEL-AML1*.

[14] In certain embodiments, each of said label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[15] The disclosure also provides a method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject. In one embodiment, the method includes providing a sample from the subject and contacting the sample with one or more target-specific probes. Each of the one or more target-specific probes includes

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1690

a target-specific region that binds to one target nucleic acid analyte; and a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe. The label has a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid. One or more targets of the one or more target-specific probes include a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia. When the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia in a subject is determined.

[16] The fusion gene can be *BCR-ABL, AML-ETO, CBFB-MYH11, E2A-PBX1, MLL-AF4, PML-RARA*, or *TEL-AML1*. The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia.

[17] In certain embodiments, at least one of the one or more target-specific probes can have a target that is the sequence of the junction of the two genes that are fused. The one or more target-specific probes can include a target-specific region with one or more mismatches to the target of the target-specific region.

[18] In other embodiments, each of the label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[19] The disclosure also provides a method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject. In one embodiment, the method includes providing a sample from the subject and contacting the sample with a probe pair. The probe pair includes a first probe and a second probe. The first probe is made up, at least in part, of a complex including a first molecule with a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal. The first probe also includes a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal. The first probe also includes a first target-specific sequence attached to the first molecule. The second probe is made up, at least in part, of a second molecule, comprising a second target-specific sequence and an affinity tag. The first target-specific sequence and the second target-specific sequence bind to different regions of the

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1691

same target molecule. The target molecule is a fusion gene or mRNA transcribed from said fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia. When said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule. Also, when the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia is determined.

[20] In certain embodiments, the fusion gene can include the fusion of at least a portion of a first gene and at least a portion of a second gene. The first target-specific sequence can hybridize to a portion of the first gene and the second target-specific sequence hybridizes to a portion of the second gene. The first target-specific sequence can also hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

[21] In other embodiments, the first target-specific sequence can hybridize to a portion of the first gene and a portion of the second gene. The first target-specific sequence can also hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[22] In other embodiments, the second target-specific sequence can hybridize to a portion of the first gene and a portion of the second gene. The first target-specific sequence can also hybridize to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene. At least one of the target-specific sequences can include a region with one or more mismatches to the target of the target-specific sequence.

[23] The leukemia can be chronic myelogenous leukemia, acute myeloid leukemia or acute lymphoblastic leukemia. The fusion gene can be *BCR-ABL, AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.

[24] Each of said label monomers can be a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety. The unique label for each target-specific probe can be composed of 4 or 5 label monomers.

[25] The disclosure also provides a kit. In one embodiment, the kit includes one or more reporter probes and one or more capture probes. The reporter probe and the capture probe each

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1692

bind specifically to a fusion gene. The fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.

[26] The fusion gene can be *BCR-ABL, AML-ETO, CBFB-MYH11, E2A-PBX1, MLL-AF4, PML-RARA*, or *TEL-AML1*. The kit can include one or more reporter probes and one or more capture probes from Table 2.

Brief Description of the Drawings

[27] Figures 1A is a bar graph showing counts for probes for the *BCR-ABL* gene fusion when exposed to gene fragments of the *BCR-ABL* gene fusion and non-specific targets.

[28] Figure 1B is a bar graph showing counts for probes for the *E2A-PBX1* gene fusion when exposed to gene fragments of the *E2A-PBX1* gene fusion and non-specific targets.

[29] Figure 1C is a bar graph showing counts for probes for the *TEL-AML1* gene fusion when exposed to gene fragments of the *TEL-AML1* gene fusion and non-specific targets.

[30] Figure 1D is a bar graph showing counts for probes for the *PML-RARA* gene fusion when exposed to gene fragments of the *PML-RARA* gene fusion and non-specific targets.

[31] Figure 2A is a bar graph showing the relative expression of various gene fusions in the SUP B13 cell line.

[32] Figure 2B is a bar graph showing the relative expression of various gene fusions in the K562 cell line.

[33] Figure 2C is a bar graph showing the relative expression of various gene fusions in the NB4 cell line.

[34] Figure 2D is a bar graph showing the relative expression of various gene fusions in the Kasumi cell line.

[35] Figure 2E is a bar graph showing the relative expression of various gene fusions in the KCL-22 cell line.

[36] Figure 2F is a bar graph showing the relative expression of various gene fusions in the MV4-11 cell line.

Detailed Description of the Invention

[37] This disclosure describes compositions and methods for the diagnosis of leukemia, diagnosis of the subtype of leukemia present in a subject and determination of the genetic

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1693

aberration underlying leukemia in a subject. The onset and progression of leukemia is associated with the formation of several fusion genes. These fusion genes include *BCR-ABL, AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*. The compositions disclosed herein are nucleic acid probes used to detect the presence of these fusion genes.

[38] The detection of these fusion genes can be used to detect the presence of leukemia and also what type of leukemia is present. For example, a *BCR-ABL* fusion gene is associated with chronic myelogenous leukemia. *AML-ETO*, *CBFB-MYH11*, *MLL-AF4*, and *PML-RARA* fusion genes are associated with acute myeloid leukemia. *E2A-PBX1* and *TEL-AML1* fusion genes are associated with acute lymphoblastic leukemia. In addition, specific isoforms of a given fusion may indicate a specific sub-type of disease.

[39] The presence of certain gene fusions in certain types of leukemia is associated with differing prognoses in the outcome of the disease for a subject. Thus, the detection of various gene fusions can be used to provide a prognosis for a subject with leukemia.

[40] Preferably, the nucleic acid probes used according to the methods of the disclosure are nanoreporters. A fully assembled and labeled nanoreporter comprises two main portions, a target-specific sequence that is capable of binding to a target molecule, and a labeled region which emits a "code" of signals (the "nanoreporter code") associated with the target-specific sequence.

[41] Upon binding of the nanoreporter to the target molecule, the nanoreporter code identifies the target molecule to which the nanoreporter is bound.

Nanoreporters

[42] Many nanoreporters, referred to as singular nanoreporters, are composed of one molecular entity. However, to increase the specificity of a nanoreporter and/or to improve the kinetics of its binding to a target molecule, a preferred nanoreporter is a dual nanoreporter composed of two molecular entities, each containing a different target-specific sequence that binds to a different region of the same target molecule. In a dual nanoreporter, at least one of the two nanoreporter probes is labeled. This labeled nanoreporter probe is referred to herein as a "reporter probe". The other nanoreporter probe is not necessarily labeled. Such unlabeled components of dual nanoreporters are referred to herein as "capture probes" and often have affinity tags attached, such as biotin, which are useful to immobilize and/or stretch the complex containing the dual nanoreporter and the target molecule to allow visualization and/or imaging of

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1694

the complex. When both probes are labeled or both have affinity tags, the probe with more label monomer attachment regions is referred to as the reporter probe and the other probe in the pair is referred to as a capture probe.

[43] For both single and dual nanoreporters, a fully assembled and labeled nanoreporter probe comprises two main portions, a target-specific sequence that is capable of binding to a target molecule, and a labeled portion which provides a "code" of signals associated with the target-specific sequence. Upon binding of the nanoreporter probe to the target molecule, the code identifies the target molecule to which the nanoreporter is bound.

[44] Nanoreporters are modular structures. In some embodiments, the nanoreporter comprises a plurality of different detectable molecules. In some embodiments, a labeled nanoreporter, is a molecular entity containing certain basic elements: (i) a plurality of unique label attachment regions attached in a particular, unique linear combination, and (ii) complementary polynucleotide sequences attached to the label attachment regions of the backbone. In some embodiments, the labeled nanoreporter comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or more unique label attachment regions attached in a particular, unique linear combination, and complementary polynucleotide sequences attached to the label attachment regions of the backbone. In some embodiments, the labeled nanoreporter comprises 6 or more unique label attachment regions attached in a particular, unique linear combination, and complementary polynucleotide sequences attached to the label attachment regions of the backbone. A nanoreporter probe further comprises a target-specific sequence, also attached to the backbone.

[45] The term label attachment region includes a region of defined polynucleotide sequence within a given backbone that may serve as an individual attachment point for a detectable molecule. In some embodiments, the label attachment regions comprise designed sequences.

[46] In some embodiments, the label nanoreporter also comprises a backbone containing a constant region. The term constant region includes tandemly-repeated sequences of about 10 to about 25 nucleotides that are covalently attached to a nanoreporter. The constant region can be attached at either the 5' region or the 3' region of a nanoreporter, and may be utilized for capture and immobilization of a nanoreporter for imaging or detection, such as by attaching to a solid substrate a sequence that is complementary to the constant region. In certain aspects, the constant region contains 2, 3, 4, 5, 6, 7, 8, 9, 10, or more tandemly-repeated sequences, wherein the repeat sequences each comprise about 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25,

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1695

26, 27, 28, 29, 30, or more nucleotides, including about 12-18, 13-17, or about 14-16 nucleotides.

[47] The nanoreporters described herein can comprise synthetic, designed sequences. In some embodiments, the sequences contain a fairly regularly-spaced pattern of a nucleotide (e.g. adenine) residue in the backbone. In some embodiments, a nucleotide is spaced at least an average of 8, 9, 10, 12, 15, 16, 20, 30, or 50 bases apart. In some embodiments, a nucleotide is spaced at least an average of 8 to 16 bases apart. In some embodiments, a nucleotide is spaced at least an average of 8 bases apart. This allows for a regularly spaced complementary nucleotide in the complementary polynucleotide sequence having attached thereto a detectable molecule. For example, in some embodiments, when the nanoreporter sequences contain a fairly regularly-spaced pattern of adenine residues in the backbone, whose complement is a regularly-spaced pattern of uridine (U) residues in complementary RNA segments, the in vitro transcription of the segments can be done using an aminoallyl-modified uridine base, which allows the covalent amine coupling of dye molecules at regular intervals along the segment. In some embodiments, the sequences contain about the same number or percentage of a nucleotide (e.g. adenine) that is spaced at least an average of 8, 9, 10, 12, 15, 16, 20, 30, or 50 bases apart in the sequences. This allows for similar number or percentages in the complementary polynucleotide sequence having attached thereto a detectable molecule. Thus, in some embodiments, the sequences contain a nucleotide that is not regularly-spaced but that is spaced at least an average of 8, 9, 10, 12, 15, 16, 20, 30, or 50 bases apart. In some embodiments, 20%, 30%, 50%, 60%, 70%, 80%, 90% or 100% of the complementary nucleotide is coupled to a detectable molecule. For instance, in some embodiments, when the nanoreporter sequences contain a similar percentage of adenine residues in the backbone and the in vitro transcription of the complementary segments is done using an aminoallyl-modified uridine base, 20%, 30%, 50%, 60%, 70%, 80%, 90% or 100% of the aminoallyl-modified uridine base can be coupled to a detectable molecule. Alternatively, the ratio of aminoallyl-modified uridine bases and uridine bases can be changed during the in vitro transcription process to achieve the desired number of sites which can be attached to a detectable molecule. For example, in vitro transcription process can take place in the presence of a mixture with a ratio of 1/1 of uridine to aminoallyl-modified uridine bases, when some or all the aminoallyl-modified uridine bases can be coupled to a detectable molecule.

[48] In some embodiments, the nanoreporters described herein have a fairly consistent melting

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1696

temperature (Tm). Without intending to be limited to any theory, the Tm of the nanoreporters described herein provides for strong bonds between the nanoreporter backbone and the complementary polynucleotide sequence having attached thereto a detectable molecule, therefore, preventing dissociation during synthesis and hybridization procedures. In addition, the consistent Tm among a population of nanoreporters allows for the synthesis and hybridization procedures to be tightly optimized, as the optimal conditions are the same for all spots and positions. In some embodiments, the sequences of the nanoreporters have a 50% guanine/cytosine (G/C), with no more than three G's in a row. Thus, in some embodiments, the disclosure provides a population of nanoreporters in which the Tm among the nanoreporters in the population is fairly consistent. In some embodiments, the disclosure provides a population of nanoreporters in which the Tm of the complementary polynucleotide sequences when hybridized to its label attachment regions is about 80° C, 85° C, 90° C, 100° C or higher. In some embodiments, the disclosure provides a population of nanoreporters in which the Tm of the complementary polynucleotide sequences when hybridized to its label attachment regions is about 80° C or higher.

[49] In some embodiments, the nanoreporters described herein have minimal or no secondary structures, such as any stable intra-molecular base-paring interaction (e.g. hairpins). Without intending to be limited to any theory, the minimal secondary structure in the nanoreporters provides for better hybridization between the nanoreporter backbone and the polynucleotide sequence having attached thereto a detectable molecule. In addition, the minimal secondary structure in the nanoreporters provides for better detection of the detectable molecules in the nanoreporters. In some embodiments, the nanoreporters described herein have no significant intra-molecular pairing under annealing conditions of 75° C, 1 X SSPE. Secondary structures can be predicted by programs known in the art such as MFOLD. In some embodiments, the nanoreporters described herein contain less than 1% of inverted repeats in each strand, wherein the inverted repeats are 9 bases or greater. In some embodiments, the nanoreporters described herein contain no inverted repeats in each strand. In some embodiments, the nanoreporters do not contain any inverted repeat of 9 nucleotides or greater across a sequence that is 1100 base pairs in length. In some embodiments, the nanoreporters do not contain any inverted repeat of 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain less than 1% of inverted repeats in each strand, wherein the inverted

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1697

repeats are 9 nucleotides or greater across a sequence that 1100 base pairs in length. In some embodiments, the nanoreporters described herein contain less than 1% of inverted repeats in each strand, wherein the inverted repeats are 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain a skewed strand specific content such that one strand is CT-rich and the other is GA-rich.

[50] The disclosure also provides unique nanoreporters. In some embodiments, the nanoreporters described herein contain less that 1% of direct repeats. In some embodiments, the nanoreporters described herein contain no direct repeats. In some embodiments, the nanoreporters do not contain any direct repeat of 9 nucleotides or greater across a sequence that 1100 base pairs in length. In some embodiments, the labeled nanoreporters do not contain any direct repeat of 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain less than 1% of direct repeats in each strand, wherein the direct repeats are 9 nucleotides or greater across a sequence that 1100 base pairs in length. In some embodiments, the nanoreporters described herein contain less than 1% of direct repeats in each strand, wherein the direct repeats are 7 nucleotides or greater across any 100 base pair region. In some embodiments, the nanoreporters described herein contain less than 85, 80, 70, 60, 50, 40, 30, 20, 10, or 5% homology to any other sequence used in the backbones or to any sequence described in the REFSEQ public database. In some embodiments, the nanoreporters described herein contain less than 85% homology to any other sequence used in the backbones or to any sequence described in the REFSEQ public database. In some embodiments, the nanoreporters described herein contain less than 20, 16, 15, 10, 9, 7, 5, 3, 2 contiguous bases of homology to any other sequence used in the backbones or to any sequence described in the REFSEQ public database. In some embodiments, the nanoreporters described herein have no more than 15 contiguous bases of homology and no more than 85% identity across the entire length of the nanoreporter to any other sequence used in the backbones or to any sequence described in the REFSEQ public database.

[51] In some embodiments, the sequence characteristics of the nanoreporter probes described herein provide sensitive detection of a target molecule. For instance, the binding of the nanoreporter probes to target molecules which results in the identification of the target molecules can be performed by individually detecting the presence of the nanoreporter. This can be performed by individually counting the presence of one or more of the nanoreporter molecules in

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1698

a sample.

[52] The complementary polynucleotide sequences attached to a nanoreporter backbone serve to attach detectable molecules, or label monomers, to the nanoreporter backbone. The complementary polynucleotide sequences may be directly labeled, for example, by covalent incorporation of one or more detectable molecules into the complementary polynucleotide sequence. Alternatively, the complementary polynucleotide sequences may be indirectly labeled, such as by incorporation of biotin or other molecule capable of a specific ligand interaction into the complementary polynucleotide sequence. In such instances, the ligand (e.g., streptavidin in the case of biotin incorporation into the complementary polynucleotide sequence) may be covalently attached to the detectable molecule. Where the detectable molecules attached to a label attachment region are not directly incorporated into the complementary polynucleotide sequence, this sequence serves as a bridge between the detectable molecule and the label attachment region, and may be referred to as a bridging molecule, e.g., a bridging nucleic acid.

[53] The nucleic-acid based nanoreporter and nanoreporter-target complexes described herein comprise nucleic acids, which may be affinity-purified or immobilized using a nucleic acid, such as an oligonucleotide, that is complementary to the constant region or the nanoreporter or target nucleic acid. As noted above, in some embodiments the nanoreporters comprise at least one constant region, which may serve as an affinity tag for purification and/or for immobilization (for example to a solid surface). The constant region typically comprises two or more tandemly-repeated regions of repeat nucleotides, such as a series of 15-base repeats. In such exemplary embodiments, the nanoreporter, whether complexed to a target molecule or otherwise, can be purified or immobilized by an affinity reagent coated with a 15-base oligonucleotide which is the reverse complement of the repeat unit.

[54] Nanoreporters, or nanoreporter-target molecule complexes, can be purified in two or more affinity selection steps. For example, in a dual nanoreporter, one probe can comprise a first affinity tag and the other probe can comprise a second (different) affinity tag. The probes are mixed with target molecules, and complexes comprising the two probes of the dual nanoreporter are separated from unbound materials (e.g., the target or the individual probes of the nanoreporter) by affinity purification against one or both individual affinity tags. In the first step, the mixture can be bound to an affinity reagent for the first affinity tag, so that only probes comprising the first affinity tag and the desired complexes are purified. The bound materials are

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1699

released from the first affinity reagent and optionally bound to an affinity reagent for the second affinity tag, allowing the separation of complexes from probes comprising the first affinity tag. At this point only full complexes would be bound. The complexes are finally released from the affinity reagent for the second affinity tag and then preferably stretched and imaged. The affinity reagent can be any solid surface coated with a binding partner for the affinity tag, such as a column, bead (e.g., latex or magnetic bead) or slide coated with the binding partner. Immobilizing and stretching nanoreporters using affinity reagents is fully described in U.S. Publication No. 2010/0161026, which is incorporated by reference herein in its entirety.

[55] The sequence of signals provided by the label monomers associated with the various label attachment regions of the backbone of a given nanoreporter allows for the unique identification of the nanoreporter. For example, when using fluorescent labels, a nanoreporter having a unique identity or unique spectral signature is associated with a target-specific sequence that recognizes a specific target molecule or a portion thereof. When a nanoreporter is exposed to a mixture containing the target molecule under conditions that permit binding of the target-specific sequence(s) of the nanoreporter to the target molecule, the target-specific sequence(s) preferentially bind(s) to the target molecule. Detection of the nanoreporter signal, such as the spectral code of a fluorescently labeled nanoreporter, associated with the nanoreporter allows detection of the presence of the target molecule in the mixture (qualitative analysis). Counting all the label monomers associated with a given spectral code or signature allows the counting of all the molecules in the mixture associated with the target-specific sequence coupled to the nanoreporter (quantitative analysis). Nanoreporters are thus useful for the diagnosis or prognosis of different biological states (e.g., disease vs. healthy) by quantitative analysis of known biological markers. Moreover, the exquisite sensitivity of single molecule detection and quantification provided by the nanoreporters described herein allows for the identification of new diagnostic and prognostic markers, including those whose fluctuations among the different biological states is too slight detect a correlation with a particular biological state using traditional molecular methods. The sensitivity of nanoreporter-based molecular detection permits detailed pharmacokinetic analysis of therapeutic and diagnostic agents in small biological samples.

[56] Many nanoreporters, referred to as singular nanoreporters, are composed of one molecular entity. However, to increase the specificity of a nanoreporter and/or to improve the

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1700

kinetics of its binding to a target molecule, a nanoreporter can be a dual nanoreporter composed of two molecular entities, each containing a different target-specific sequence that binds to a different region of the same target molecule. In a dual nanoreporter, at least one of the two molecular entities is labeled. The other molecular entity need not necessarily be labeled. Such unlabeled components of dual nanoreporters may be used as capture probes and optionally have affinity tags attached, such as biotin, which are useful to immobilize and/or stretch the complex containing the dual nanoreporter and the target molecule to allow visualization and/or imaging of the complex. For instance, in some embodiments, a dual nanoreporter with a 6-position nanoreporter code uses one 6-position coded nanoreporter (also referred to herein as a reporter probe) and a capture probe. In some embodiments, a dual nanoreporter with a 6-position nanoreporter code can be used, using one capture probe with an affinity tag and one 6-position nanoreporter component. In some embodiments an affinity tag is optionally included and can be used to purify the nanoreporter or to immobilize the nanoreporter (or nanoreporter-target molecule complex) for the purpose of imaging.

[57] In some embodiments, the nucleotide sequences of the individual label attachment regions within each nanoreporter are different from the nucleotide sequences of the other label attachment regions within that nanoreporter, preventing rearrangements, such recombination, sharing or swapping of the label polynucleotide sequences. The number of label attachment regions to be formed on a backbone is based on the length and nature of the backbone, the means of labeling the nanoreporter, as well as the type of label monomers providing a signal to be attached to the label attachment regions of the backbone. In some embodiments, the complementary nucleotide sequence of each label attachment region is assigned a specific detectable molecule.

[58] The disclosure also provides labeled nanoreporters wherein one or more label attachment regions are attached to a corresponding detectable molecule, each detectable molecule providing a signal. For example, in some embodiments, a labeled nanoreporter according to the disclosure is obtained when at least three detectable molecules are attached to three corresponding label attachment regions of the backbone such that these labeled label attachment regions, or spots, are distinguishable based on their unique linear arrangement. A "spot," in the context of nanoreporter detection, is the aggregate signal detected from the label monomers attached to a single label attachment site on a nanoreporter, and which, depending on the size of the label attachment

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1701

region and the nature (e.g., primary emission wavelength) of the label monomer, may appear as a single point source of light when visualized under a microscope. Spots from a nanoreporter may be overlapping or non-overlapping. The nanoreporter code that identifies that target molecule can comprise any permutation of the length of a spot, its position relative to other spots, and/or the nature (e.g., primary emission wavelength(s)) of its signal. Generally, for each probe or probe pair described herein, adjacent label attachment regions are non-overlapping, and/or the spots from adjacent label attachment regions are spatially and/or spectrally distinguishable, at least under the detection conditions (e.g., when the nanoreporter is immobilized, stretched and observed under a microscope, as described in U.S. Publication No. 2010/0112710, incorporated herein by reference).

[59] Occasionally, reference is made to a spot size as a certain number of bases or nucleotides. As would be readily understood by one of skill in the art, this refers to the number of bases or nucleotides in the corresponding label attachment region.

[60] The order and nature (e.g., primary emission wavelength(s), optionally also length) of spots from a nanoreporter serve as a nanoreporter code that identifies the target molecule capable of being bound by the nanoreporter through the nanoreporter's target specific sequence(s). When the nanoreporter is bound to a target molecule, the nanoreporter code also identifies the target molecule. Optionally, the length of a spot can be a component of the nanoreporter code.

[61] Detectable molecules providing a signal associated with different label attachment regions of the backbone can provide signals that are indistinguishable under the detections conditions ("like" signals), or can provide signals that are distinguishable, at least under the detection conditions (e.g., when the nanoreporter is immobilized, stretched and observed under a microscope).

[62] The disclosure also provides a nanoreporter wherein two or more detectable molecules are attached to a label attachment region. The signal provided by the detectable molecules associated with said label attachment region produces an aggregate signal that is detected. The aggregate signal produced may be made up of like signals or made up of at least two distinguishable signals (e.g., spectrally distinguishable signals).

[63] In one embodiment, a nanoreporter includes at least three detectable molecules providing like signals attached to three corresponding label attachment regions of the backbone and said three detectable molecules are spatially distinguishable. In another embodiment, a nanoreporter

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1702

includes at least three detectable molecules providing three distinguishable signals attached to three neighboring label attachment regions, for example three adjacent label attachment regions, whereby said at least three label monomers are spectrally distinguishable.

[64] In other embodiments, a nanoreporter includes spots providing like or unlike signals separated by a spacer region, whereby interposing the spacer region allows the generation of dark spots, which expand the possible combination of uniquely detectable signals. The term "dark spot" refers to a lack of signal from a label attachment site on a nanoreporter. Dark spots can be incorporated into the nanoreporter code to add more coding permutations and generate greater nanoreporter diversity in a nanoreporter population. In one embodiment, the spacer regions have a length determined by the resolution of an instrument employed in detecting the nanoreporter.

[65] In other embodiments, a nanoreporter includes one or more "double spots." Each double spot contains two or more (e.g., three, four or five) adjacent spots that provide like signals without being separated by a spacer region. Double spots can be identified by their sizes.

[66] A detectable molecule providing a signal described herein may be attached covalently or non-covalently (e.g., via hybridization) to a complementary polynucleotide sequence that is attached to the label attachment region. The label monomers may also be attached indirectly to the complementary polynucleotide sequence, such as by being covalently attached to a ligand molecule (e.g., streptavidin) that is attached through its interaction with a molecule incorporated into the complementary polynucleotide sequence (e.g., biotin incorporated into the complementary polynucleotide sequence), which is in turn attached via hybridization to the backbone.

[67] A nanoreporter can also be associated with a uniquely detectable signal, such as a spectral code, determined by the sequence of signals provided by the label monomers attached (e.g., indirectly) to label attachment regions on the backbone of the nanoreporter, whereby detection of the signal allows identification of the nanoreporter.

[68] In other embodiments, a nanoreporter also includes an affinity tag attached to the reporter probe backbone, such that attachment of the affinity tag to a support allows backbone stretching and resolution of signals provided by label monomers corresponding to different label attachment regions on the backbone. Nanoreporter stretching may involve any stretching means known in the art including but not limited to, means involving physical, hydrodynamic or

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1703

electrical means. The affinity tag may comprise a constant region.

[69] In other embodiments, a nanoreporter also includes a target-specific sequence coupled to the backbone. The target-specific sequence is selected to allow the nanoreporter to recognize, bind or attach to a target molecule. The nanoreporters described herein are suitable for identification of target molecules of all types. For example, appropriate target-specific sequences can be coupled to the backbone of the nanoreporter to allow detection of a target molecule. Preferably the target molecule is DNA (including cDNA), RNA (including mRNA and cRNA), a peptide, a polypeptide, or a protein.

[70] One embodiment of the disclosure provides increased flexibility in target molecule detection with label monomers described herein. In this embodiment, a dual nanoreporter comprising two different molecular entities, each with a separate target-specific region, at least one of which is labeled, bind to the same target molecule. Thus, the target-specific sequences of the two components of the dual nanoreporter bind to different portions of a selected target molecule, whereby detection of the spectral code associated with the dual nanoreporter provides detection of the selected target molecule in a biomolecular sample contacted with said dual nanoreporter.

[71] The disclosure also provides a method of detecting the presence of a specific target molecule in a biomolecular sample comprising: (i) contacting said sample with a nanoreporter as described herein (e.g., a singular or dual nanoreporter) under conditions that allow binding of the target-specific sequences in the dual nanoreporter to the target molecule and (ii) detecting the spectral code associated with the dual nanoreporter. Depending on the nanoreporter architecture, the dual nanoreporter may be labeled before or after binding to the target molecule.

[72] The uniqueness of each nanoreporter probe in a population of probe allows for the multiplexed analysis of a plurality of target molecules. For example, in some embodiments, each nanoreporter probe contains six label attachment regions, where each label attachment region of each backbone is different from the other label attachment regions in that same backbone. If the label attachment regions are going to be labeled with one of four colors and there are 24 possible unique sequences for the label attachment regions and each label attachment region is assigned a specific color, each label attachment region in each backbone will consist of one of four sequences. There will be 4096 possible nanoreporters in this example. The number of possible nanoreporters can be increased, for example, by increasing the number of colors, increasing the

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1704

number of unique sequences for the label attachment regions and/or increasing the number of label attachment regions per backbone. Likewise the number of possible nanoreporters can be decreased by decreasing the number of colors, decreasing the number of unique sequences for the label attachment regions and/or decreasing the number of label attachment regions per backbone.

[73] In certain embodiments, the methods of detection are performed in multiplex assays, whereby a plurality of target molecules are detected in the same assay (a single reaction mixture). In a preferred embodiment, the assay is a hybridization assay in which the plurality of target molecules are detected simultaneously. In certain embodiments, the plurality of target molecules detected in the same assay is, at least 2, at least 5 different target molecules, at least 10 different target molecules, at least 20 different target molecules, at least 50 different target molecules, at least 75 different target molecules, at least 100 different target molecules, at least 200 different target molecules, at least 500 different target molecules, or at least 750 different target molecules, or at least 1000 different target molecules. In other embodiments, the plurality of target molecules detected in the same assay is up to 50 different target molecules, up to 100 different target molecules, up to 150 different target molecules, up to 200 different target molecules, up to 300 different target molecules, up to 500 different target molecules, up to 750 different target molecules, up to 1000 different target molecules, up to 2000 different target molecules, or up to 5000 different target molecules. In yet other embodiments, the plurality of target molecules detected is any range in between the foregoing numbers of different target molecules, such as, but not limited to, from 20 to 50 different target molecules, from 50 to 200 different target molecules, from 100 to 1000 different target molecules, from 500 to 5000 different target molecules, and so on and so forth.

[74] In certain embodiments, nanoreporters for detecting the presence of fusion genes contain both a reporter probe and a capture probe that hybridize to the fusion gene at different positions. In one embodiment, the target-specific regions of the capture and reporter probe hybridize to sequences on either side of the border of the fusion between the two fused genes. Optionally, the target-specific regions hybridize to regions adjacent to each other on either side of the border of fusion. The sequences to which the target-specific regions of the reporter and capture probes hybridize can also be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs apart.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1705

[75] In another embodiment, the reporter probe's target-specific region hybridizes to a sequence that spans the fusion junction while the target-specific region of the capture probe hybridizes to a sequence upstream or downstream of the reporter probe's target. In another embodiment, the capture probe's target-specific region hybridizes to a sequence that spans the fusion junction while the target-specific region of the reporter probe hybridizes to a sequence upstream or downstream of the reporter probe's target. Optionally, the target sequences of the reporter and capture probes are adjacent. The sequences to which the target-specific regions of the reporter and capture probes hybridize can also be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs apart. In certain embodiments, the target-specific region of a probe has 1, 2, 3, 4, 5 or more mismatches introduced into the target-specific sequence. The sequences which come together to form the junction of a fusion gene can be GC rich, resulting in a high melting temperature for the probe on both sides of the junction. Thus, in certain situations, the probe has an increased propensity to cross-hybridize with isoforms containing the same sequence as only one side of the junction, rather than requiring the presence of both sequences for successful hybridization. Introduction of certain mismatches into the probe produces a decreased melting temperature and higher specificity. In preferred embodiments, probes that hybridize to a sequence that encompasses the junction of the fusion genes have 1, 2, 3, 4, 5, or more mismatches. Additional disclosure regarding nanoreporters can be found in International Publication Nos. WO 07/076129 and WO 07/076132, the contents of which are incorporated herein in their entireties. Further, the term nucleic acid probes and nanoreporters can include the rationally designed (*e.g.* synthetic sequences) described in International Publication No. WO 2010/019826, incorporated herein by reference in its entirety.

[76] Examples of various probes and their targets are provided below in Table 1.

Table 1

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1706

Gene Fusion	Isoform	Reporter Probe	SEQ ID NO:
BCR-ABL	b2a2	TGGCCGCTGAAGGGCTT CTTCCTTATTGATGGTCA	1
BCR-ABL	b3a2	TGGCCGCTGAAGGGCTT TTGAACTCTGCTTAAATC	2
BCR-ABL	e1a2	TGGCCGCTGAAGGGCTT CTGCATCTCCATGGAAGG (*)	3
BCR-ABL	e19a2	TGGCCGCTGAAGGGCTT TGACGTCGAAAGCTGCCT (*)	4
BCR-ABL	b3a3	GACCCGGAGCTTTTCAC TTGAACTCTGCTTAAATC	5
BCR-ABL	b2a3	GACCCGGAGCTTTTCAC CTTCCTTATTGATGGTCA	6
BCR-ABL	e1a3	GACCCGGAGCTTTTCAC CTGCATCTCCATGGAAGG (*)	7
AML-ETO		CTTCACATCCACAGGTGAGTCTGGCATTGTGGAGTGCTTCTCAGTACGAT (+)	8
CBFb-MYH11	e5e12	TCCAGGGCCCGCTTGGACTTCTCCAGCTCATGGAC (+)	9
CBFb-MYH11	e5e8	CGGCCTCGTTAAGCATCCCTGTGACGCTCTCAACTTCATT (+)	10
CBFb-MYH11	e5e7	AGCGTCTGCTTATTCTTGTCTAGGTTCGCCTTGGC (+)	11
E2A-PBX1	e13-insertion-n27-e2	AAAAGGTTCCAGGTGACCGAACACTTTCAT CACTG	12
E2A-PBX1	e13e2	TCCTCGGATACTCAAAA CACTGTAGGAGTCGGGAG	13
MLL-AF4	e8e7	AGATGTATCATATTGTT CTGATTCTGGTGGTGGAG	14
MLL-AF4	e9e5	CATGAATGGGTCATTTC CTTTTCTTTTGGTTTTTG	15
MLL-AF4	e9e4	TCATTGGAGTAGGTCTG CTTTTCTTTTGGTTTTTG	16
MLL-AF4	e10e5	CATGAATGGGTCATTTC CTTAAAGTCCACTCTGAT	17
MLL-AF4	e10e4	TCATTGGAGTAGGTCTG CTTAAAGTCCACTCTGAT	18
MLL-AF4	e11e5	CATGAATGGGTCATTTC CTATACATGCCCACTACT (*)	19
MLL-AF4	e11e4	TCATTGGAGTAGGTCTG CTATACATGCCCACTACT (*)	20
PML-RARA	bcr1 (e6e3)	TCTGGGTCTCAATGG CTGACTCCCAAGCGCCACTG (*)	21
PML-RARA	bcr3 (e3e3)	TCTGGGTCTCAATGG CTTTCCACTGGGTGATGCAA (*)	22
PML-RARA	bcr2 (e6partial_e3)	GCTCTGGGTCTCAATGG TTCCTATGACGGGACTCC (*)	23
TEL-AML1	e5e2	TCCCCTAGGGCCACCAC CTGCTATTCTCACAATGG (*)	24
TEL-AML1	e5e3	CAGAGTGAAGCTTTTCC CTGCTATTCTACCAATGG (*)	25
Gene Fusion	**Isoform**	**Capture Probe**	**SEQ ID NO:**
BCR-ABL	b2a2	GCGGAATGCTGTGGACAGTCTGGAGTTTCACACACGAGTT	26
BCR-ABL	b3a2	CAGTGGCTGAGTGGACGATGACATTCAGAAACCCATAGAGCCCCGGAGAC	27
BCR-ABL	e1a2	CGCCCTCGCCATCGTTGGGCCAGATCTGCCCGGTCTTGCG	28
BCR-ABL	e19a2	TCAGTGCCTGGATGTCCGTGGCCACACCGGACACG	29
BCR-ABL	b3a3	CAGTGGCTGAGTGGACGATGACATTCAGAAACCCA	30
BCR-ABL	b2a3	GCGGAATGCTGTGGACAGTCTGGAGTTTCACACAC	31
BCR-ABL	e1a3	CGCCCTCGCCATCGTTGGGCCAGATCTGCCCGGTCTTGCG	32
AML-ETO		TTCGAGGTTCTCGGGGCCCATCCACTGTGATTTTGATGGCTCTGTGGTAG	33
CBFb-MYH11	e5e12	CAGTTACTGCCAGCAGCTGTGAAACTCTCACCTCCATTTC	34
CBFb-MYH11	e5e8	CAGTTACTGCCAGCAGCTGTGAAACTCTCACCTCCATTTC	35

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1707

CBFb-MYH11	e5e7	CAGTTACTGCCAGCAGCTGTGAAACTCTCACCTCCATTTC	36
E2A-PBX1	e13-insertion-n27-e2	TAGGAGTCGGGAGGCCGAGACAGGTCAGGGAGGGT	37
E2A-PBX1	e13e2	GCCGAGACAGGTCAGGGAGGGTGCCTGGCTGGCTG	38
MLL-AF4	e8e7	GCTGCTTTTTCTTGGGCTCACTAGGAGTGGTTTTG	39
MLL-AF4	e9e5	TTTTACAGGGATACTTGGGCGGGGAGCCACTTTTT	40
MLL-AF4	e9e4	TTTTACAGGGATACTTGGGCGGGGAGCCACTTTTT	41
MLL-AF4	e10e5	CCTGTGGACTCCATCTGCTGGAATTTTTTGCTTAG	42
MLL-AF4	e10e4	CCTGTGGACTCCATCTGCTGGAATTTTTTGCTTAG	43
MLL-AF4	e11e5	GGCACAGAGAAAGCAAACCACCCTGGGTGTTATAG	44
MLL-AF4	e11e4	GGCACAGAGAAAGCAAACCACCCTGGGTGTTATAG	45
PML-RARA	bcr1 (e6e3)	GCCACGTGGTTGCTGTTGGGCAGGAAGACCTCACTTCCTAT	46
PML-RARA	bcr3 (e3e3)	GAGCTGAGGTCCTGCAGGCGCACCTTGAACTCGTCGAAGC	47
PML-RARA	bcr2 (e6partial_e3)	TGGGGCTAGGCGGTCCATCCAGGTGGGGTGGTGAG	48
TEL-AML1	e5e2	GCATGGCGTGCTCTTCAGGCGGGGAGACAGAGACC	49
TEL-AML1	e5e3	GCATGGCGTGCTCTTCAGGCGGGGAGACAGAGACC	50

[77] Probes in Table 1 marked with (*) are mismatch junction probes that hybridize to the sequence across the gene fusion junction. Each has one, two or three mismatches shown underlined. These mismatches stop the probe from inappropriately hybridizing to a transcript which has only half of the relevant target sequence (for example, the wild-type transcript of one of the fusion genes), by lowering the amount of partial sequence complementarity so that stable hybridization can only occur with the true target. Such mismatches are only necessary in cases where the Tm of the sequence of one exon at the junction is high enough to be stable under standard hybridization conditions, and the sequence cannot simply be shortened because the shorter probe will not retain enough sequence specificity.

[78] Probes in Table 1 marked with (+) are border probes. These probes do not hybridize to a sequence that crosses the gene fusion junction. The target sequence of these reporter probes is adjacent to the target sequence of the capture probes. Each probe only hybridizes to a sequence on one side of the gene fusion junction. The probe pair (capture and reporter) meets at the border of the fusion, with the capture probe sitting in the upstream exon and the reporter probe sitting fully in the downstream exon.

[79] The unmarked sequences in Table 1 are probes with target sequences that cross the gene fusion junction, but do not have mismatches. The sequences of this class of probes are the exact reverse complement of the target sequence. Gaps in the sequence represent the fusion border;

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1708

the probe sequence is actually contiguous across this gap. In these probe pairs, the reporter probe spans the fusion junction, and the capture probe sits adjacent in the upstream exon.

[80] The disclosure also describes a panel of probes. These probes include probes for the detection of fusion genes from several gene families from various leukemia subtypes. The panel can contain probes for detection of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more fusion genes. The genes can be from 1, 2, 3, 4, 5, 6, 7 or more gene families. The fusion genes can be present in any leukemia subtype including CML, AML or ALL.

[81] The fusion events, or translocations, usually involve only one of the pair of each chromosome, leaving one intact copy, or "wild-type" copy, of each gene also present in the cell. The panel of probes can also include probes specific for mRNAs produced by the wild-type copies of the relevant genes, allowing the level of expression of the intact version of each gene involved in the translocation to be determined. The panel can include probes specific for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more wild-type mRNAs.

[82] The panel of probes can also include probes specific for mRNAs that are clinically relevant for the detection and diagnosis of leukemia. These markers for leukemia can include WT1, FLT3, BAALC, PRAME, MLLT11, ERG, MN1, RHAMM, HOX11L2, EVI1, N-RAS, RB1 and TP53.

[83] The panel of probes can also include probes specific for housekeeper mRNAs. The results produced from these probes can be used as an internal control to estimate the amount of sample present in the assay. Probes for 1, 2, 3, 4 or more housekeeper mRNAs can be included in the panel.

[84] An example of a panel of probes for the detection and/or diagnosis of leukemia or specific subtypes of leukemia is shown below in Table 2.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1709

Table 2.

Gene Type/Fusion Gene Family	Fusion Gene Isoform	Target Sequence (5'-3')	SEQ ID NO:	Reporter Probe (5'-3')	SEQ ID NO:	Capture Probe (5'-3')	SEQ ID NO:
Housekeeper Genes	GUSB	UCUGUGGCCAACGAGCCUGCGUCCCACCUAGAAU CUGCUGGCUACUACUUGAAGAUGGUGAUCGCUCA CACCAAAUCCUUGGA (SEQ ID NO:)	51	TCCAAGGATTTGGT GTGAGCGATCACCA TCTTCAAGTAGTAG CCAGC	52	AGATTCTAGGTGGG ACGCAGGCTCGTTG GCCACAGA	53
	TBP	CGCCGGCUGUUUAACUUGCGCUUCCGCUGGCCCAU AGUGAUCUUUGCAGUGACCCAGCAGCAUCACUGU UUCUUGGCGUGUGAAGAUAACCCAAGGAAUUG	54	CAATTCCTTGGGTT ATCTTCACACGCCA AGAAACAGTGATGC TGCTGGGT	55	CACTGCAAAGATCA CTATGGGCCAGCGG AAGCGAAGTTAAA CAGCCGGCG	56
	ABL1	CUGUGAUUGACUUCAAUUGCUGACUUGUGGAGAU GCAGCGAAUGUGAAAUCCCACGUAUAUGCCAUUU CCCUCUACGCUCGCUGACCGUUCUGGAAGAUC	57	GATCTTCCAGAACG GTCAGCGAGCGTAG AGGGAAATGGCATA TACGTGGG	58	ATTTCACATTCGCT GCATCTCCACAAGT CAGCAATTGAAGTC AATCACAG	59
	BCR	CUGGGAGACAGGGUGAAGGGAGUGGUUUUUAUGA ACUUAACUUAGAGUCUAAAAGAUUUCUACUGGAU CACUUGUCAAGAUGCGCCCUCUCUGGGGAGAA	60	TTCTCCCCAGAGAG GGCGCATCTTGACA AGTGATCCAGTAGA AATCTTTT	61	AGACTCTAAGTTAA GTTCATAAAAACCA CTCCCTTCACCCTG TCTCCCAG	62
	SOCS2	GGAACGGCACUGUUCACCUUUAUCUGACCAAACC GCUCUACACGUCAGCACCAUCUCUGCAGCAUCUC UGUAGGCUCACCAUUAACAAAUGUACCGGUGC	63	GCACCGGTACATTT GTTAATGGTGAGCC TACAGAGATGCTGC AGAGATGG	64	TGCTGACGTGTAGA GCGGTTTGGTCAGA TAAAGGTGAACAG TGCCGTTCC	65
Leukemia Biomarkers	WT1	UCAGAGAGCAAGGCAUCGGGGGUGAAUCUUGUCU AACAUUCCCGAGGUCAGCCAGGCUGCUAACCUGG AAAGCAGGAUGUAGUUCUGCCAGGCAACUUUU	66	AAAAGTTGCCTGGC AGAACTACATCCTG CTTTCCAGGTTAGC AGCCTGGC	67	TGACCTCGGGAATG TTAGACAAGATTCA CCCCCGATGCCTTG CTCTCTGA	68
	FLT3	AAAUGGAAAACCAGGACGCCCUGGUCUGCAUAUC UGAGAGCGUUCCAGAGCCGAUCGUGGAAUGGGUG CUUUGCGAUUCACAGGGGGAAAGCUGUAAAGA	69	TCTTTACAGCTTTCC CCCTGTGAATCGCA AAGCACCCATTCCA CGATCGG	70	CTCTGGAACGCTCT CAGATATGCAGACC AGGGCGTCCTGGTT TTCCATTT	71
	BAALC	AAGAAUCACAAAGAACUGUGUCAACUAGCAGAGA GUCCAAGCAGAAGGGCAGAUGGACUUCUUCAGUG UCCUUCACGGCACUGGAUCCCAUCAAAGAACC	72	GGTTCTTTGATGGG ATCCAGTGCCGTGA AGGACACTGAAGAA GTCCATCT	73	GCCCTTCTGCTTGG ACTCTCTGCTAGTT GACACAGTTCTTTG TGATTCTT	74
	PRAME	GGUCUUUGAUGAGUGUGGGAUCACGGAUGAUCAG CUCCUUGCCCUCCUGCCUUCCCUGAGCCACUGCUC	75	TTAAGGTTGTAAGC TGGGAGCAGTGGCT	76	GGCAAGGAGCTGA TCATCCGTGATCCC	77

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1710

	CCAGCUUACAACCUUAA		CAGGGAAGGCAGG AG		ACACTCATCAAAGA CC	
MLLT11	GGAAUAGGACUAGGUUUAUUUACCCAUUGUGAGG GUAGAGAGGCGAGUCUGGAGGAGCAGGGAUUGGG AGAAGGGGGUGGAAAAAUACUCUGAUUCUUAAA	78	TTTAAGAATCAGAG TATTTTTCCACCCCT TCTCCCAATCCCCTG CTCCTCC	79	AGACTCGCCTCTCT ACCCTCACAATGGG TAAATAAACCTAGT CCTATTCC	80
ERG	GACCUCAUCAUUAUGUGGGGGCUUUGUUCUCCAC AGGGUCAGGUAAGAGAUGGCCUUCUUGGCUGCCA CAAUCAGAAAUCACGCAGGCAUUUUGGGUAGG	81	CCTACCCAAAATGC CTGCGTGATTTCTG ATTGTGGCAGCCAA GAAGGCCA	82	TCTCTTACCTGACC CTGTGGAGAACAA AGCCCCCACATAAT GATGAGGTC	83
MN1	AGGGUGACGAACCAAGGAGCCGUCGACUCGCUGG AAUACAAUUACCCGGGCGAGGCGCCCUCGGGACA UUUUGACAUGUUUUCGCCCU	84	AGGGCGAAAACATG TCAAAATGTCCCGA GGGCGCCTCGCCCG G	85	GTAATTGTATTCCA GCGAGTCGACGGCT CCTTGGTTCGTCAC CCT	86
RHAMM	UUGAAACCGGUAGGGAGUGAUAAUCCGCAUUCAG UUGUCGAGGAGUGCCAGUCACCUUCAGUUUCUGG AGCUGGCCGUCAACAUGUCCUUUCCUAAGGCG	87	CGCCTTAGGAAAGG ACATGTTGACGGCC AGCTCCAGAAACTG AAGGTGAC	88	TGGCACTCCTCGAC AACTGAATGCGGAT TATCACTCCCTACC GGTTTCAA	89
HOX11	ACCACACAUCCCCAGCCCAAUCCAGGUACGCACAGA CAGGUUUUCACAUAAAUGCAGCCCAUUUCUCCAG AACCCAUUUGAGGGGUGGGGGGGUGUUAAUU	90	AATTAACACCCCCC CACCCCTCAAATGG GTTCTGGAGAAATG GGCTGCAT	91	TTATGTGAAAACCT GTCTGTGCGTACCT GGATTGGGCTGGG ATGTGTGGT	92
HOX11L2	GCGGCGCUCGCCAAGUCCCUCAAAAUGACGGACG CGCAGGUCAAGACCUGGUUCCAAAACCGGAGGAC CAAGUGGCGG	93	CCGCCACTTGGTCC TCCGGTTTTGGAAC CAGGTCTTGACC	94	TGCGCGTCCGTCAT TTTGAGGGACTTGG CGAGCGCCGC	95
EVI1	GGGGCAGGACUAGGAAUAUGGACCAAAAGGAAGA UCGAAGUAGGUGAAAAGUUUGGGCCUUAUGUGGG AGAGCAGAGGUCAAACCUGAAAGACCCCAGUU	96	AACTGGGGTCTTTC AGGTTTGACCTCTG CTCTCCCACATAAG GCCCAAAC	97	TTTTCACCTACTTC GATCTTCCTCTTTGG TCCATATTCCTAGT CCTGCCCC	98
N-RAS	CCCUGGUCCUGACUUCCCCUGGAGGAGAAGUAUUC CUGUUGCUGUCUUCAGUCUCACAGAGAAGCUCCU GCUACUUCCCCAGCUCUCAGUAGUUUAGUAC	99	GTACTAAACTACTG AGAGCTGGGGAAGT AGCAGGAGCTTCTC TGTGAGAC	100	TGAAGACAGCAAC AGGAATACTTCTCC TCCAGGGAAGTCA GGACCAGGG	101
RB1	CCUAUCUCCGGCUAAAUACACUUUGUGAACGCCU UCUGUCUGAGCACCCAGAAUUAGAACAUAUCAUC UGGACCCUUUUCCAGCACACCCUGCAGAAUGA	102	TCATTCTGCAGGGT GTGCTGGAAAAGGG TCCAGATGATATGT TCTAATTC	103	TGGGTGCTCAGACA GAAGGCGTTCACA AAGTGTATTTAGCC GGAGATAGG	104
TP53	CAGGGCUCACUCCAGCCACCUGAAGUCCAAAAAG GGUCAGUCUACCUCCCGCCAUAAAAAACUCAUGU UCAAGACAGAAGGGCCUGACUCAGAC	105	GTCTGAGTCAGGCC CTTCTGTCTTGAAC ATGAGTTTTTTATG	106	TAGACTGACCCTTT TTGGACTTCAGGTG GCTGGAGTGAGCCC	107

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1711

				GCGGGAGG		TG	
Wild Type Transcripts	PML	GAGGUGGGGCUGCAGCACUUCCUCAGCUUUCUGA GCUCCAUGCGCCGCCCUAUCUUGGCCUGCUACAA GCUGUG	108	CACAGCTTGTAGCA GGCCAAGATAGGGC GGCGCATGGAG	109	CTCAGAAAGCTGA GGAAGTGCTGCAG CCCCACCTC	110
	RARA	CUGGGGCCCAUCUAGGAGUGGCAUCUUUUUUGGU GCCCUGAAGGCCAGCUCUGGACCUUCCCAGGAAA AGUGCCAG	111	CTGGCACTTTTCCTG GGAAGGTCCAGAGC TGGCCTT	112	CAGGGCACCAAAA AAGATGCCACTCCT AGATGGGCCCCAG	113
	ETO (RUNX 1)	CAGCCAUGAAGAACCAGGUUGCAAGAUUUAAUGA CCUCAGGUUUGUCGGUCGAAGUGGAAGAGGGAAA AGCUUCACUCUGACCAUCACUGUCUUCA	114	TGAAGACAGTGATG GTCAGAGTGAAGCT TTTCCCTCTTCCACT TCG	115	ACCGACAAACCTG AGGTCATTAAATCT TGCAACCTGGTTCT TCATGGCTG	116
	ETV6 (TEL)	CGCCACUACUACAAACUAAACAUUAUCAGGAAGG AGCCAGGACAAAGGCUUUUGUUCAGGUUUAUGAA AACCCCAGAUGAAAUCAUGAGUG	117	CACTCATGATTTCA TCTGGGGTTTTCAT AAACCTGAACAAAA GCCTTTGT	118	CCTGGCTCCTTCCT GATAATGTTTTAGTT TGTAGTAGTGGCG	119
	TCF3 (E2A)	CUUGGAGCAGCAAGUGCGAGAGCGGAACCUGAAU CCCAAAGCAGCCUGUUUGAAACGGCGAGAAGAGG AAAAG	120	CTTTTCCTCTTCTCG CCGTTTCAAACAGG CTGCTTTGG	121	GATTCAGGTTTCCGC TCTCGCACTTGCTG CTCCAAG	122
	PBX1	CGGGAGGAAGCAGGACAUUGGAGACAUUUUACAG CAAAUUAUGACCAUCACAGACCAGAGUUUGGAUG AGGCGCAGGCCAGAAAACAUGCUUUAAACUGC	123	GCAGTTTAAAGCAT GTTTTCTGGCCTGC GCCTCATCCAAACT CTGGTCTG	124	TGATGGTCATAAATT TGCTGTAAAATGTC TCCAATGTCCTGCT TCCTCCCG	125
	CBFB	CAGUGUUGCCCAGGCUGGUCUCGAACUCCUGGCA UCAAGCGAUCCUCCUGCCUUAGCCUCCCAGAGUA CUG	126	CAGTACTCTGGGAG GCTAAGGCAGGAGG ATCGCTTG	127	ATGCCAGGAGTTCG AGACCAGCCTGGG CAACACTG	128
	MYH11	CUGCUAGAAAAAUCACGGCAAUUCGCCAAGCCA GAGACGAGAGGACAUUCCACAUCUUUUACUACAU GAUUGCUGGAGCCAAGAGAAGAUGAGAAGUG	129	CACTTCTCATCTTCT CCTTGGCTCCAGCA ATCATGTAGTAAAA GATGTGG	130	AATGTCCTCTCGTC TCTGGCTTGGCGAA TTGCCCGTGATTTT TCTAGCAG	131
	MLL	CAAAAUGAGUGGACACAUGUAAAUUGUGCUUUGU GGUCAGCGGAAGUGUUUGAAGAUGAUGACGGAUC ACUAAAGAAUGUGCAUAUGGCUGUGAUCAG	132	CTGATCACAGCCAT ATGCACATTCTTTA GTGATCCGTCATCA TCTTCA	133	AACACTTCCGCTGA CCACAAAGCACAA TTTACATGTGTCCA CTCATTTTG	134
	AFF1 (AF4)	UGAACUGAAACCACUGCCGGAGGACUAUCGACAG CAGACCUUUGAAAAAACAGACUUGAAAGUGCCUG CCAAAGCCAAGCUCACCAAACUGAAGAUGCCU	135	AGGCATCTTCAGTT TGGTGAGCTTGGCT TTGGCAGGCACTTT CAAGTCTG	136	TTTTTTCAAAGGTC TGCTGTCGATAGTC CTCCGGCAGTGGTT TCAGTTCA	137
	BCR-ABL	AACUCGUGUGUGAAACUCCAGACUGUCCACAGCA UUCCGCUGACCAUCAAUAAGGAAGAAGCCCUUCA	138	TGGCCGCTGAAGGG CTTCTTCCTTATTGA	139	GCGGAATGCTGTGG ACAGTCTGGAGTTT	140

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1712

		GCGGCCA		TGGTCA		CACACACGAGTT	
		GUCUCCGGGGCUCUAUGGGUUUCUGAAUGUCAUC GUCCACUCAGCCACUGGAUUUAAGCAGAGUUCAA AAGCCCUUCAGCGGCCA	141	TGGCCGCTGAAGGG CTTTTGAACTCTGCT TAAATC	142	CAGTGGCTGAGTGG ACGATGACATTCAG AAACCCATAGAGC CCCGGAGAC	143
	b3a2	CGCAAGACCGGGCAGAUCUGGCCCAACGAUGGCG AGGGCGCCUUCCAUGGAGACGCAGAAGCCCUUCA GCGGCCA	144	TGGCCGCTGAAGGG CTTCTGCΔTCTCCAT GGAAGG	145	CGCCCTCGCCATCG TTGGGCCAGATCTG CCCGGTCTTGCG	146
	e1a2	CGUGUCCGGUGUGGCCACGGACAUCCAGGCACUG AAGGCAGCCUUCGACGUCAAAGCCCUUCAGCGGC CA	147	TGGCCGCTGAAGGG CTTTGACGTCGAAΔ GCTGCCT	148	TCAGTGCCTGGATG TCCGTGGCCACACC GGACACG	149
	e19a2	UGGGUUUCUGAAUGUCAUCGUCCACUCAGCCACU GGAUUUAAGCAGAGUUCAAGUGAAAAGCUCCGGG UC	150	GACCCGGAGCTTTT CACTTGAACTCTGC TTAAATC	151	CAGTGGCTGAGTGG ACGATGACATTCAG AAACCCA	152
	b3a3	GUGUGUGAAACUCCAGACUGUCCACAGCAUUCCG CUGACCAUCAAUAAGGAAGGUGAAAAGCUCCGGG UC	153	GACCCGGAGCTTTT CACCTTCCTTATTGA TGGTCA	154	GCGGAATGCTGTGG ACAGTCTGGAGTTT CACACAC	155
	b2a3	CGCAAGACCGGGCAGAUCUGGCCCAACGAUGGCG AGGGCGCCUUCCAUGGAGACGCAGGUGAAAAGCU CCGGGUC	156	GACCCGGAGCTTTT CACCTGCΔTCTCCA TGGAAGG	157	CGCCCTCGCCATCG TTGGGCCAGATCTG CCCGGTCTTGCG	158
	e1a3		159	CTTCACATCCACAG GTGAGTCTGGCATT GTGGAGTGCTTCTC AGTACGAT	160	TTCGAGGTTCTCGG GGCCCATCCACTGT GATTTTGATGGCTC TGTGGTAG	161
AML-ETO		CUACCACAGAGCCAUCAAAAUCACAGUGGAUGGG CCCCGAGAACCUCGAAAUCGUACUGAGAAGCACU CCACAAUGCCAGACUCACCUGUGGAUGUGAAG					
	e5e12	GAAAUGGAGGUGAGAGUUUCACAGCUGCUGGCAG UAACUGGUCCAUGAGCUGGAGAAGUCCAAGCCGGG CCCUGGA	162	TCCAGGGCCCGCTT GGACTTCTCCAGCT CATGGAC	163	CAGTTACTGCCAGC AGCTGTGAAACTCT CACCTCCATTTC	164
CBFb-MYH11	***e5e8***	GAAAUGGAGGUGAGAGUUUCACAGCUGCUGGCAG UAACUGAAUGAAGUUGAGAGCGUCACAGGGAUGC UUAACGAGGCCG	165	CGGCCTCGTTAAGC ATCCCTGTGACGCT CTCAACTTCATT	166	CAGTTACTGCCAGC AGCTGTGAAACTCT CACCTCCATTTC	167
	e5e7	GAAAUGGAGGUGAGAGUUUCACAGCUGCUGGCAG UAACUGGCCAAGGCGAACCUAGACAAGAAUAAGC AGACGCU	168	AGCGTCTGCTTATT CTTGTCTAGGTTCG CCTTGGC	169	CAGTTACTGCCAGC AGCTGTGAAACTCT CACCTCCATTTC	170
E2A-PBX1	***e13-insertion-n27-e2***	ACCCUCCCUGACCUGUCUCGGCCUCCCCGACUCCCUA CAGUGAUGAAAGUGUUCGGUCACCUGGAACCUUU U	171	AAAAGGTTCCAGGT GACCGAACACTTTC ATCACTG	172	TAGGAGTCGGGAG GCCGAGACAGGTC AGGGAGGGT	173
	e13e2	AGCCAGCCAGGCACCCUCCCCUGACCUGUCUCGGCC UCCCGACUCCUACAGUGUUUUGAGUAUCCGAGGA	174	TCCTCGGATACTCA AAACACTGTAGGAG	175	GCCGAGACAGGTC AGGGAGGGTGCCT	176

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1713

MLL-AF4	*e8e7*	CAAAACCACUCCUAGUGAGCCCAAGAAAAAGCAG CCUCCACCACCAGAAUCAGAACAAUAUGAUACAU CU	177	AGATGTATCATATT GTTCTGATTCTGGT GGTGGAG	178	GCTGCTTTTTCTTG GGCTCACTAGGAGT GGTTTTG	179
	e9e5	AAAAAGUGGCUCCCCCGCCCAAGUAUCCCUGUAAA ACAAAAACCAAAAGAAAAGGAAAUGACCCAUUCA UG	180	CATGAATGGGTCAT TTCCTTTTCTTTTGG TTTTTG	181	TTTTACAGGGATAC TTGGGCGGGGAGC CACTTTTT	182
	e9e4	AAAAAGUGGCUCCCCCGCCCAAGUAUCCCUGUAAA ACAAAAACCAAAAGAAAAGCAGACCUACUCCCAAU GA	183	TCATTGGAGTAGGT CTGCTTTTCTTTTGG TTTTTG	184	TTTTACAGGGATAC TTGGGCGGGGAGC CACTTTTT	185
	e10e5	CUAAGCAAAAAAUUCCAGCAGAUGGAGUCCACAG GAUCAGAGUGGACUUUAAGGAAAUGACCCAUUCA UG	186	CATGAATGGGTCAT TTCCTTAAAGTCCA CTCTGAT	187	CCTGTGGACTCCAT CTGCTGGAATTTTT TGCTTAGC	188
	e10e4	CUAAGCAAAAAAUUCCAGCAGAUGGAGUCCACAG GAUCAGAGUGGACUUUAAGCAGACCUACUCCCAAU GA	189	TCATTGGAGTAGGT CTGCTTAAAGTCCA CTCTGAT	190	CCTGTGGACTCCAT CTGCTGGAATTTTT TGCTTAG	191
	e11e5	CUAUAACACCCAGGGUGGUUUGCUUUCUCUGUGC CAGUAGUGGGCAUGUAGAGGAAAUGACCCAUUCA UG	192	CATGAATGGGTCAT TTCCTΔTACATGCC CACTACT	193	GGCACAGAGAAAAG CAAACCACCCTGGG TGTTATAG	194
	e11e4	CUAUAACACCCAGGGUGGUUUGCUUUCUCUGUGC CAGUAGUGGGCAUGUAGAGCAGACCUACUCCCAAU GA	195	TCATTGGAGTAGGT CTGCTΔTACATGCC CACTACT	196	GGCACAGAGAAAAG CAAACCACCCTGGG TGTTATAG	197
PML-RARA	bcr1 (*e6e3*)	AUAGGAAGUGAGGUCUUCCUGCCCAACAGCAACC ACGUGGCCAGUGGCGCCGGGGAGGCAGCCAUUGA GACCCAGA	198	TCTGGGTCTCAATG GCTGΔCTCCCΔΔGC GCCACTG	199	GCCACGTGGTTGCT GTTGGGCAGGAAG ACCTCACTTCCTAT	200
	bcr3 (*e3e3*)	GGCUUCGACGAGUUCAAGGUGCGCCUGCAGGACC UCAGCUCUUGCAUCACCCAGGGGAAAGCCAUUGA GACCCAGA	201	TCTGGGTCTCAATG GCTTTCCΔCTGGGT GATGCAA	202	GAGCTGAGGTCCTG CAGGCGCACCTTGA ACTCGTCGAAGC	203
	bcr2 (*e6parti al_e3*)	CUCACCACCCCACCUGGAUGGACCGCCUAGCCCCA GGAGCCCCGUCAUAGGAACCAUUGAGACCCAGAG C	204	GCTCTGGGTCTCAA TGGTTCCTATGACG GGΔCTCC	205	TGGGGCTAGGCGGT CCATCCAGGTGGGG TGGTGAG	206
TEL-AML1	*e5e2*	GGUCUCUGUCUCCCCGCCUGAAGAGCACGCCAUG CCCAUUGGGAGAAUAGCAGGUGGUGGCCCUAGGG GA	207	TCCCCTAGGGCCAC CACCTGCTATTCTC ΔCAATGG	208	GCATGGCGTGCTCT TCAGGCGGGGAGA CAGAGACC	209
	e5e3	GGUCUCUGUCUCCCCGCCUGAAGAGCACGCCAUG CCCAUUGGGAGAAUAGCAGGGAAAAGCUUUCACUC UG	210	CAGAGTGAAGCTTT TCCCTGCTATTCTΔC CAATGG	211	GCATGGCGTGCTCT TCAGGCGGGGAGA CAGAGACC	212

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1714

nCounter® Analysis System overview

[85] The basis of the nCounter® Analysis system is the unique code assigned to each gene to be assayed (International Patent Application No. PCT/US2008/059959 and Geiss *et al.* Nature Biotechnology. 2008. 26(3): 317-325; the contents of which are each incorporated herein by reference in their entireties). The code is composed of an ordered series of colored fluorescent spots which create a unique barcode for each target to be assayed. A pair of probes is designed for each target, a biotinylated capture probe and a reporter probe carrying the fluorescent barcode.

[86] Specific reporter and capture probes are synthesized for each target. Briefly, sequence-specific DNA oligonucleotide probes are attached to code-specific reporter molecules. Capture probes are made by ligating a second sequence-specific DNA oligonucleotide for each target to a universal oligonucleotide containing biotin. Reporter and capture probes are all pooled into a single hybridization mixture, the "probe library".

[87] The expression levels of all targets are measured in a single multiplexed hybridization reaction. The sample is combined with the probe library, and specific hybridization of the reporter and the capture probes to their target molecules occurs in solution. After hybridization, the tripartite hybridized complexes are purified in a two-step procedure using magnetic beads linked to oligonucleotides complementary to universal sequences present on the capture and reporter probes. This dual purification process allows the hybridization reaction to be driven to completion with a large excess of target-specific probes, as they are ultimately removed, and, thus, do not interfere with binding and imaging of the sample. All post hybridization steps are handled robotically on a custom liquid-handling robot (Prep Station, NanoString Technologies).

[88] Purified reactions are deposited by the Prep Station into individual flow cells of a sample cartridge, bound to a streptavidin-coated surface via the capture probe, electrophoresed to elongate the reporter probes, and immobilized. After processing, the sample cartridge is transferred to a fully automated imaging and data collection device (Digital Analyzer, NanoString Technlogies). The expression level of a target is measured by imaging each sample and counting the number of times the code for that target is detected. For each sample, typically 600 fields-of-view (FOV) are imaged (1376 X 1024 pixels) representing approximately 10 mm^2 of the binding surface. Typical imaging density is 100-1200 counted reporters per field of view depending on the degree of multiplexing, the amount of RNA, and overall target expression

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1715

levels. Data is output in simple spreadsheet format listing the number of counts per target, per sample.

[89] In certain embodiments the analysis system used herein also used a reference sample as a control. The reference samples are known quantities of nucleic acid fragments with sequences that correspond to one or more fusion genes. For example, a reference sample with a sequence corresponding to a specific fusion gene is used with a nanoreporter with a target-specific probe that specifically binds to a sequence on the same fusion gene. When multiple nanoreporters are used for multiple fusion genes, the reference sample may contain multiple target nucleic acids which correspond to the sequences that the target specific probes of the nanoreporters specifically bind to.

Kits

[90] The present disclosure also describes kits useful for detecting and diagnosing leukemia. These kits comprise a set of probes described above. For example, the kit may include any number of probes described in Table 2, above. The kit may further comprise a computer readable medium.

[91] In another embodiment, the kit includes probes that may be provided in a lyophilized or reconstituted form, or may be provided as a set of nucleotide sequences. In one embodiment, the probes are provided in a microplate format, where each probe set occupies a well (or multiple wells, as in the case of replicates) in the microplate. The kit may further comprise reagents and instructions sufficient for the detection of the various target sequences described above.

EXAMPLE

[92] Example 1. Functional performance of fusion probes showing specificity for fusion gene isoforms on synthetic gene fragments.

[93] A multiplexed collection of nanoreporters containing probes to all the isoforms of BCR-ABL, E2A-PBX1, TEL-AML1 and PML-RARA listed in Table 1 was used to detect synthetic transcripts of sequences corresponding with each of these gene fusions. An nCounter® leukemia fusion gene expression assay was performed as described below, and some of the resulting counts for each probe are shown in log2 scale in Figure 1. Cross-hybridization for each individual probe to non-specific targets was below 1%.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1716

[94] The leukemia fusion gene expression assay was run on the nCounter® Analysis system (NanoString). Each sample was hybridized to the multiplexed nanoreporters in solution, followed by post-hybidization sample processing on the nCounter® Prep Station and data collection on the nCounter® Digital Analyzer.

[95] Hybridization Protocol

[96] Each hybridization reaction contained the following components: 10 μL Reporter CodeSet, 10 μL hybridization buffer, 5 μL synthetic RNA target to a final concentration of 1 pM, and 5 μL Capture ProbeSet.

[97] Aliquots of both the Reporter CodeSet and Capture ProbeSet reagent were removed from the freezer and thawed on ice. The aliquots were inverted several times to mix well. The reagent was briefly spun down at <1000 rpm.

[98] A master mix was created containing 130 μL of the Reporter CodeSet and 130 μL of hybridization buffer by adding the hybridization buffer to the tube containing the Reporter CodeSet. The master mix was inverted to mix and spun down, and 20 μL of master mix was added to each of 12 tubes.

[99] Samples of synthetic target transcripts were denatured 85°C for 5 minutes and quick-cooled on ice before being added to each tube and mixed. Targets were added to a final concentration of 1 pM.

[100] The thermocycler was pre-heated to 65°C. 5 μL of Capture ProbeSet were added to each tube and mixed well before placing immediately at 65°C. Hybridization assays were incubated at 65°C overnight.

[101] Hybridizations were left at 65°C until ready for processing. Once removed from 65°C, the assay proceeded immediately to post-hybridization processing on the nCounter® Prep Station.

[102] Post-Hybridization Processing and Data Collection

[103] After hybridization, an automated protocol on the nCounter® Prep Station was used to wash away excess probes and bind the captured probe/target complexes onto a cartridge, and then stretch and immobilize the captured nanoreporters in preparation for imaging. The purification procedure involved a two-step magnetic beadbased affinity purification. Magnetic beads derivatized with short nucleic acid sequences that were complementary to the Capture Probes and the Reporter Probes were used sequentially. First, the hybridization mixture

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1717

containing target/probe complexes was allowed to bind to magnetic beads complementary to sequences on the Capture Probe. Wash steps were performed to remove excess Reporter Probes. After washing, the Capture Probes and target/probe complexes were eluted off the beads and hybridized to magnetic beads complementary to sequences on the Reporter Probe. An additional wash was performed to remove excess Capture Probes. Finally, the purified Capture Probe/target/Reporter Probe complexes were eluted off the beads and the captured nanoreporters were bound, stretched and immobilized on a cartridge for data collection.

[104] Automated data collection was carried out in the nCounter® Digital Analyzer. Digital images of 600 fields of view of the bound nanoreporters were processed and the counts for each nanoreporter barcode were tabulated in a comma separated value (CSV) format.

[105] Example 2. Leukemia Fusion Gene Panel Tested on total RNA isolated from cell lines.

[106] Figures 2A-F show results from a Leukemia Fusion Gene Panel tested on total RNA isolated from 5 cell lines. The assay was performed as described in Example 1 with 100ng of input RNA. Probes included in the CodeSet identified their respective targets expressed in the different cell lines: the BCR-ABL e1a2 isoform expressed in cell line SUP B13 (A), the BCR-ABL b3a2 isoform expressed in cell line K562 (B), the PML-RARA bcr1 isoform expressed in cell line NB4 (C), the AML-ETO fusion gene transcript expressed in the Kasumi cell line (D), the BCR-ABL b2a2 isoform expressed in cell line KCL-22 (E) and the MLL-AF4 e9e5 isoform expressed in cell line MV4-11 (F).

OTHER EMBODIMENTS

[107] While the disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

[108] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1718

herein are hereby incorporated by reference.

[109] While this disclosure has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the disclosure encompassed by the appended claims.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1719

CLAIMS

What is claimed is:

1. A composition for the diagnosis of leukemia or the identification of a subtype of leukemia comprising one or more target-specific nucleic acid probes wherein each of the one or more target-specific probes comprises

(i) a target-specific region that specifically binds to one target nucleic acid analyte; and

(ii) a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe, said label having a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid,

wherein one or more targets of the one or more target-specific probes comprise a fusion gene or mRNA transcribed from said fusion gene and wherein the fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.

2. The composition of claim 1, wherein the fusion gene is selected from the group consisting of *BCR-ABL, AML-ETO*, *CBFB-MYH11*, *E2A-PBX1*, *MLL-AF4*, *PML-RARA*, or *TEL-AML1*.

3. The composition of claim 1, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.

4. The composition of claim 1, wherein at least one of the one or more target-specific probes has a target that is the sequence of the junction of the two genes that are fused.

5. The composition of claim 1, wherein at least one of the one or more target-specific probes comprises a target-specific region with one or more mismatches to the target of the target-specific region.

6. The composition of claim 1, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1720

7. The composition of claim 6, wherein each of said label monomers comprise a fluorescent moiety.

8. The composition of claim 1, wherein the unique label for each target-specific probe is composed of 4 label monomers.

9. The composition of claim 1, wherein the unique label for each target-specific probe is composed of 5 label monomers.

10. A composition for the diagnosis of leukemia comprising a probe pair comprising a first probe and a second probe, said first probe comprising a complex comprising:

(a) a first molecule, comprising:

(i) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal;

(ii) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and

(b) a first target-specific sequence attached to the first molecule,

said second probe comprising a second molecule, comprising

(i) a second target-specific sequence; and

(ii) an affinity tag

wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule, wherein the target molecule is a fusion gene or mRNA transcribed from said fusion gene, wherein the fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1721

11. The composition of claim 10, wherein the fusion gene comprises the fusion of at least a portion of a first gene and at least a portion of a second gene.

12. The composition of claim 11, wherein the first target-specific sequence hybridizes to a portion of the first gene and the second target-specific sequence hybridizes to a portion of the second gene.

13. The composition of claim 12, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

14. The composition of claim 11, wherein the first target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.

15. The composition of claim 14, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

16. The composition of claim 14, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.

17. The composition of claim 11, wherein the second target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.

18. The composition of claim 17, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

19. The composition of claim 17, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1722

20. The composition of claim 10, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.

21. The composition of claim 10, wherein the fusion gene is selected from the group consisting of *BCR-ABL, AML-ETO, CBFB-MYH11, E2A-PBX1, MLL-AF4, PML-RARA*, or *TEL-AML1*.

22. The composition of claim 10, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.

23. The composition of claim 22, wherein each of said label monomers comprise a fluorescent moiety.

24. The composition of claim 22, wherein the unique label for each target-specific probe is composed of 4 label monomers.

25. The composition of claim 22, wherein the unique label for each target-specific probe is composed of 5 label monomers.

26. A method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject comprising

(a) providing a sample from the subject; and

(b) contacting the sample with one or more target-specific probes wherein each of the one or more target-specific probes comprise

(i) a target-specific region that binds to one target nucleic acid analyte; and

(ii) a region to which is attached a plurality of label monomers that create a unique label for each target-specific probe, said label having a detectable signal that distinguishes one target-specific probe which binds to a first target nucleic acid from another target-specific probe that binds to a different second target nucleic acid,

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1723

wherein one or more targets of the one or more target-specific probes comprise a fusion gene or mRNA transcribed from said fusion gene that is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia and wherein when the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia is determined.

27. The method of claim 26, wherein fusion gene is selected from the group consisting of *BCR-ABL, AML-ETO, CBFB-MYH11, E2A-PBX1, MLL-AF4, PML-RARA*, or *TEL-AML1*.

28. The method of claim 26, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.

29. The method of claim 26, wherein at least one of the one or more target-specific probes has a target that is the sequence of the junction of the two genes that are fused.

30. The method of claim 26, wherein at least one of the one or more target-specific probes comprises a target-specific region with one or more mismatches to the target of the target-specific region.

31. The method of claim 26, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.

32. The method of claim 31, wherein each of said label monomers comprise a fluorescent moiety.

33. The method of claim 26, wherein the unique label for each target-specific probe is composed of 4 label monomers.

34. The method of claim 26, wherein the unique label for each target-specific probe is composed of 5 label monomers.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1724

35. A method for diagnosing leukemia, a specific subtype of leukemia or determining a genetic aberration underlying leukemia in a subject comprising:

(a) providing a sample from the subject; and

(b) contacting the sample with a probe pair comprising a first probe and a second probe, said first probe being a complex comprising:

(i) a first molecule, comprising:

(1) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal;

(2) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and

(ii) a first target-specific sequence attached to the first molecule,

said second probe comprising a second molecule, comprising

(1) a second target-specific sequence; and

(2) an affinity tag

wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule, wherein the target molecule is a fusion gene or mRNA transcribed from said fusion gene, wherein the fusion gene is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule and wherein when the fusion gene is detected the subject is diagnosed with leukemia, a specific subtype of leukemia or the genetic aberration underlying leukemia is determined.

36. The method of claim 35, wherein the fusion gene comprises the fusion of at least a portion of a first gene and at least a portion of a second gene.

37. The method of claim 35, wherein the first target-specific sequence hybridizes to a portion of the first gene and the second target-specific sequence hybridizes to a portion of the second gene.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1725

38. The method of claim 37, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

39. The composition of claim 35, wherein the first target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.

40. The method of claim 39, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

41. The method of claim 39, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.

42. The method of claim 35, wherein the second target-specific sequence hybridizes to a portion of the first gene and a portion of the second gene.

43. The method of claim 42, wherein the first target-specific sequence hybridizes to a sequence adjacent to a sequence that the second target-specific sequence hybridizes to on the fusion gene.

44. The method of claim 42, wherein at least one of the target-specific sequences comprises a region with one or more mismatches to the target of the target-specific sequence.

45. The method of claim 35, wherein the leukemia is selected from the group consisting of chronic myelogenous leukemia, acute myeloid leukemia and acute lymphoblastic leukemia.

46. The method of claim 35, wherein the fusion gene is selected from the group consisting of *BCR-ABL, AML-ETO, CBFB-MYH11, E2A-PBX1, MLL-AF4, PML-RARA*, or *TEL-AML1*.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1726

47. The method of claim 35, wherein each of said label monomers are selected from the group consisting of a fluorochrome moiety, a fluorescent moiety, a dye moiety or a chemiluminescent moiety.

48. The method of claim 47, wherein each of said label monomers comprise a fluorescent moiety.

49. The method of claim 35, wherein the unique label for each target-specific probe is composed of 4 label monomers.

50. The method of claim 35, wherein the unique label for each target-specific probe is composed of 5 label monomers.

51. A kit comprising one or more reporter probes and one or more capture probes, wherein the reporter probe and the capture probe each bind specifically to a fusion gene that is the result of the fusion of two or more genes in a patient at the onset of or during progression of leukemia.

52. The kit of claim 51, wherein the fusion gene is selected from the group consisting of *BCR-ABL, AML-ETO, CBFB-MYH11, E2A-PBX1, MLL-AF4, PML-RARA*, or *TEL-AML1*.

53. The kit of claim 51, comprising one or more reporter probes and one or more capture probes from Table 2.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1727

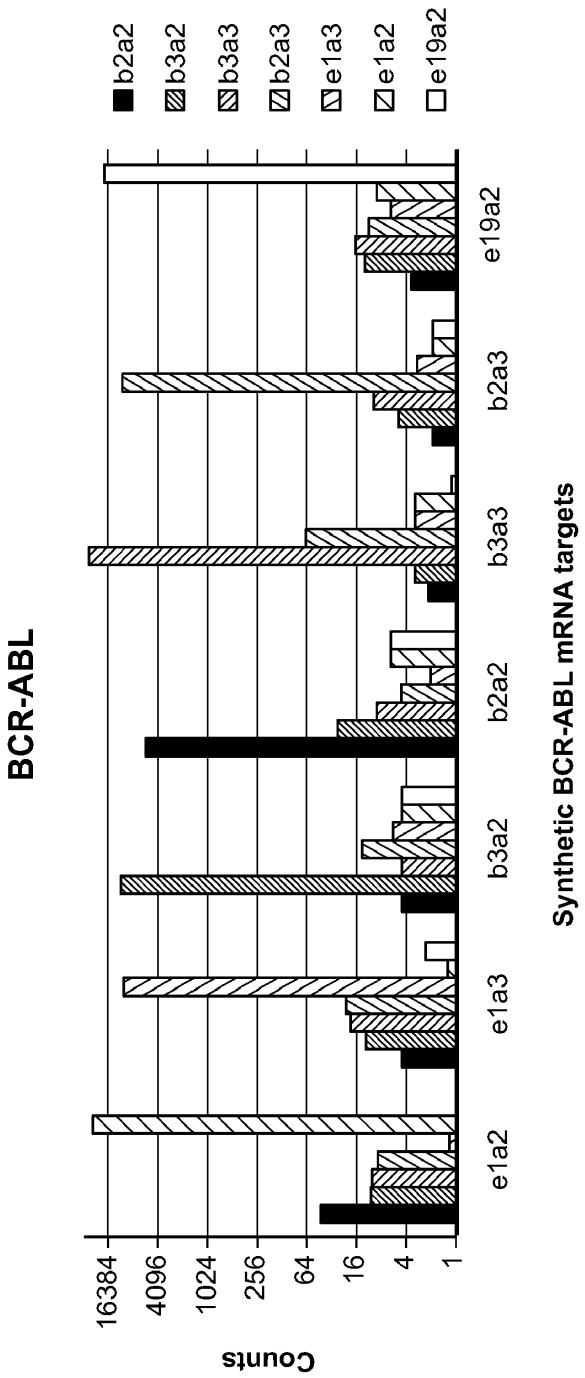


FIG. 1A

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1728

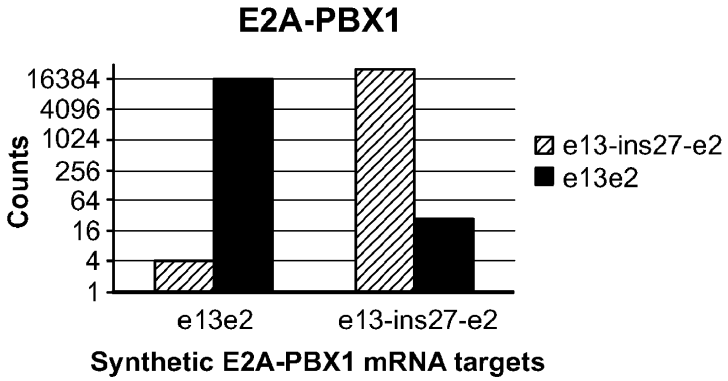


FIG. 1B

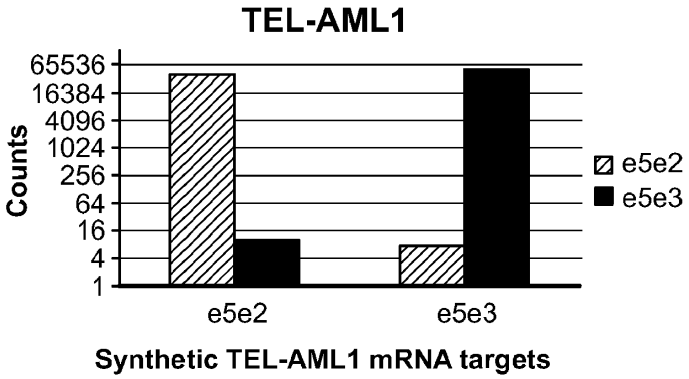


FIG. 1C

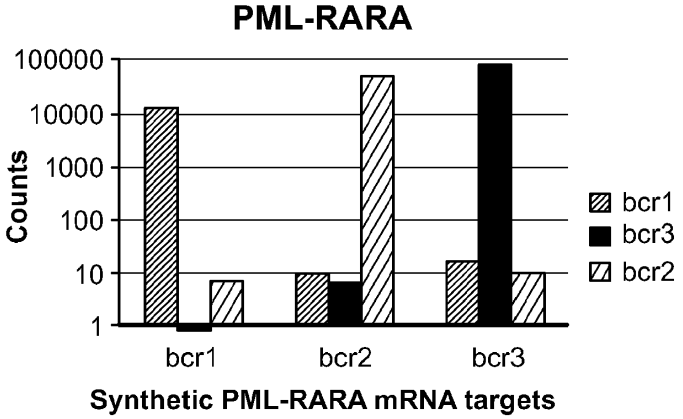


FIG. 1D

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1729

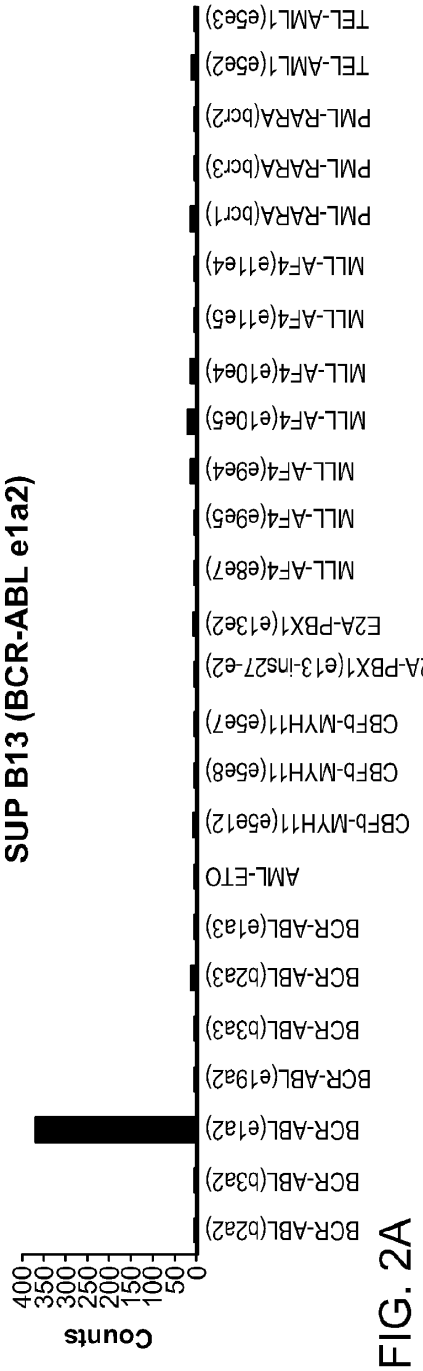


FIG. 2A

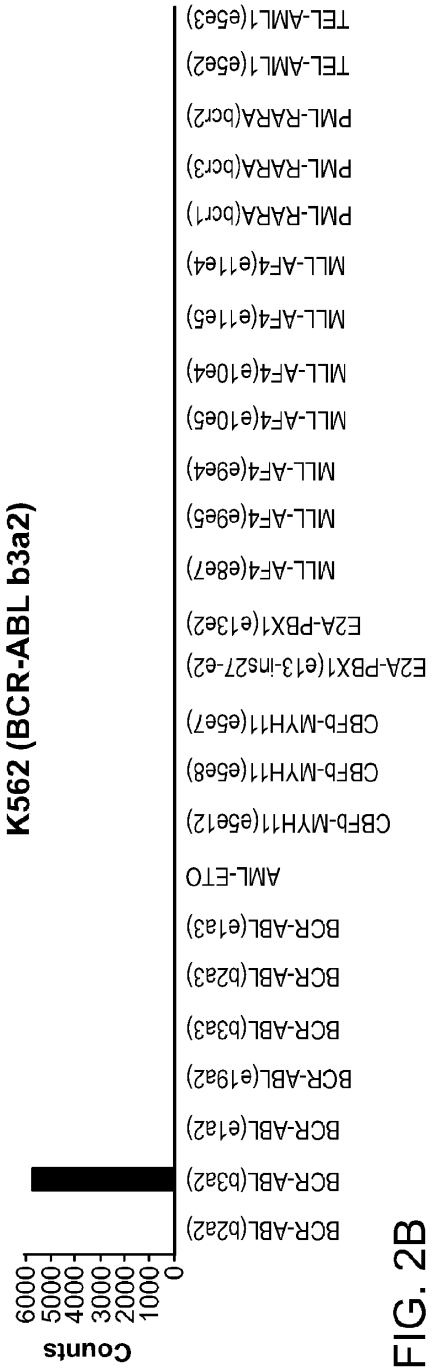


FIG. 2B

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1730

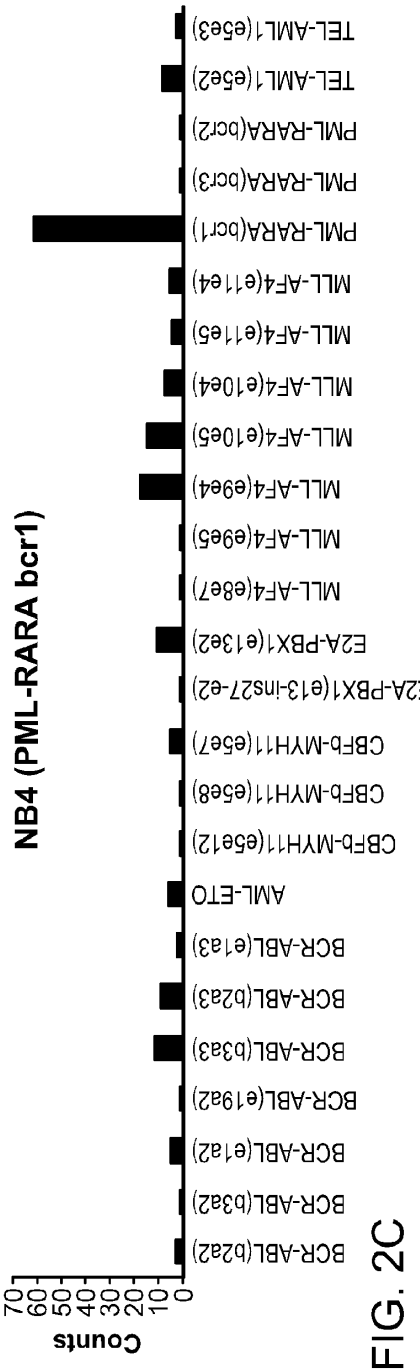


FIG. 2C

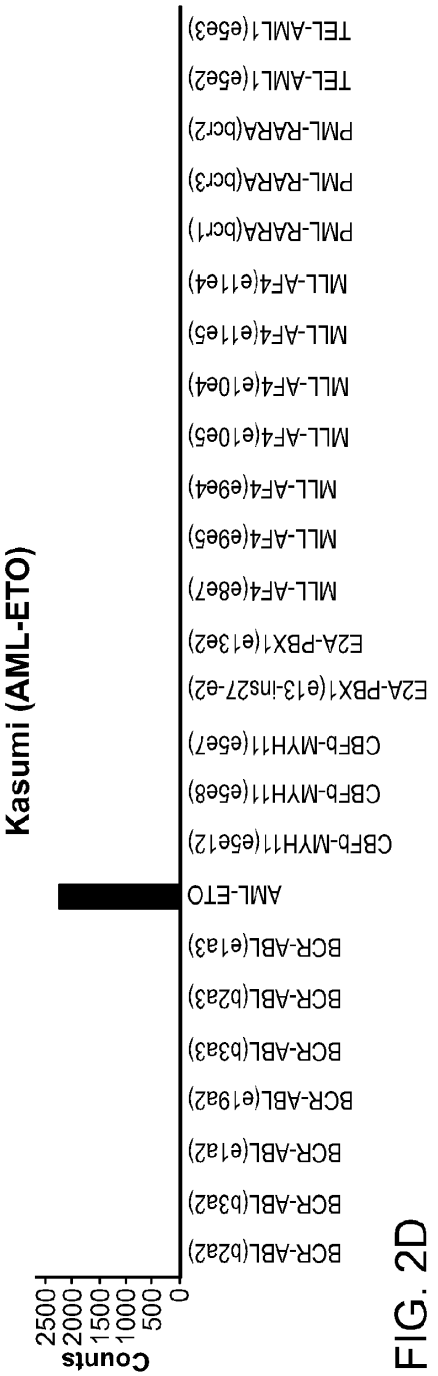


FIG. 2D

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1731

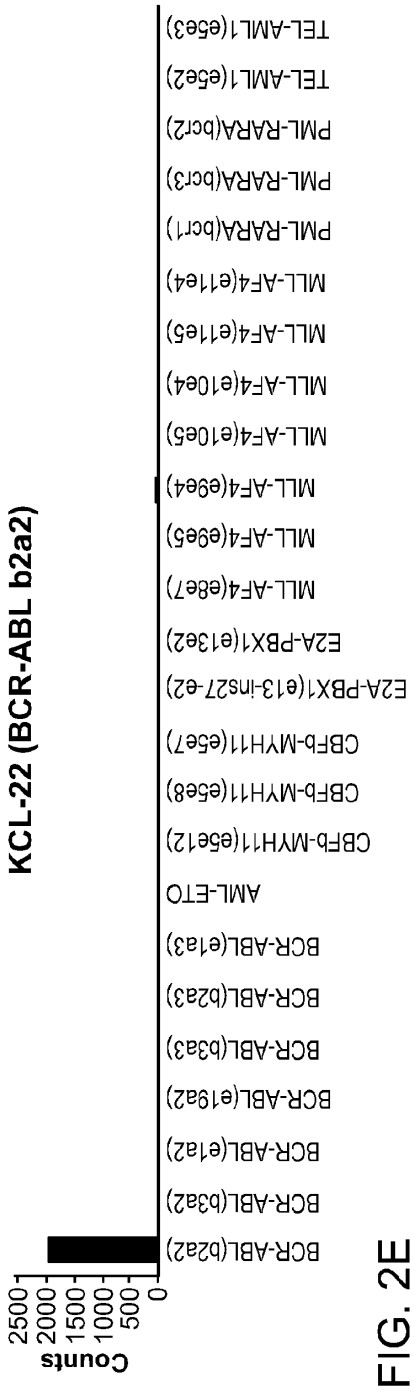


FIG. 2E

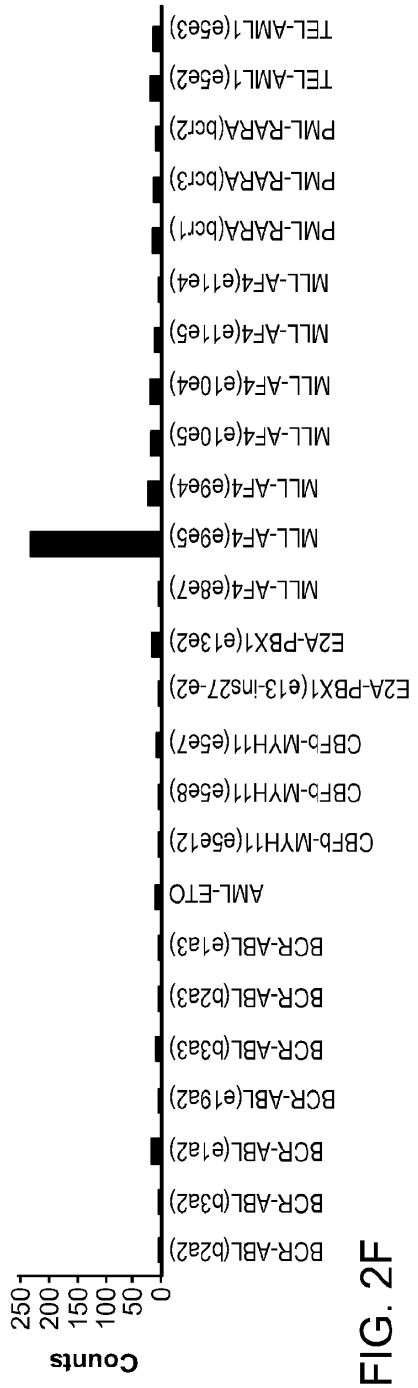


FIG. 2F

IPR2024-01177
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US 61/468,361 (CON)
Filed on 28 March 2011 (28.03.2011)

(71) Applicant *(for all designated States except US)*: **NANOSTRING TECHNOLOGIES, INC.** [US/US]; 530 Fairview Avenue North, Suite 2000, Seattle, WA 98109 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WEBSTER, Philippa Jane** [US/US]; 4317 Densmore Ave. North, Seattle, WA 98103 (US). **BOYKIN, Richard Kemble** [US/US]; 16741 10th Avenue NW, Shoreline, WA 98177 (US). **NUSSBAUM, Jeannette** [DE/US]; 4202 S. Genesee Street, Seattle, WA 98118 (US).

(74) Agents: **ELRIFI, Ivor, R.** et al.; Mintz Levin Cohn Ferris Glovsky And Popeo, P.C., One Financial Center, Boston, MA 02111 (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, QA, RO, RS, RU, RW, 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.

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR DIAGNOSING CANCER

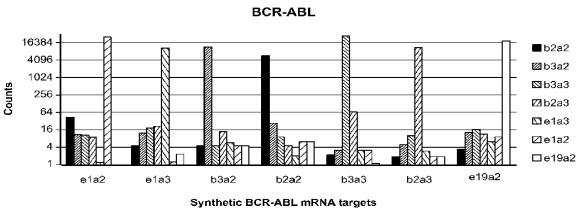


FIG. 1A

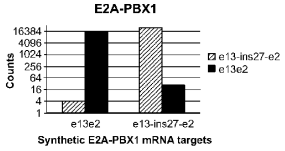


FIG. 1B

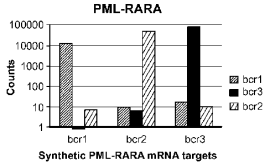


FIG. 1D

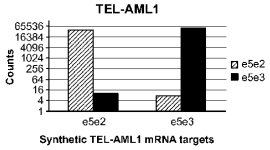


FIG. 1C

(57) Abstract: The application describes methods for diagnosing subjects with leukemia by detecting fusion genes associated with the onset of leukemia.

WO 2012/135340 A3

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, 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, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
20 December 2012

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/030940

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/53(2006.01)i, C12N 15/11(2006.01)i, C12Q 1/68(2006.01)i, G01N 33/52(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N 33/53; C07H 21/04; C07H 21/02; C40B 40/06; C12P 19/34; C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:leukemia, fusion gene, target-specific probe, plurality, label, nanoreporter.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2004/0235039 A1(GRAY, J.W. et al.) 25 Nov. 2004. See abstract, [0078], [0098], [0099], [0234]-[0240], [0333], [0356] and claim 127	1-25,51-53
Y	US 2010/0047924 A1(WEBSTER, P. J. et al.) 25 Feb. 2010. See the whole document, especially [0078] and figs. 1, 2	1-25,51-53
A	US 2003/0099987 A1 (WESTBROOK, C, A.) 29 May 2003 See [0036,[0038] and claims 1-3,5,14,21-23,29,30	1-25,51-53
A	US 2006/0134667 A1 (NARAHARA, M. et al.) 22 Jun 2006 See [0031],[0032],[0047],[0053] claims 1,9,10	1-25,51-53
A	US 6576421 B1 (WESTBROOK, C, A.) 10 Jun 2003 See column 7 lines 33-42 and claims 1,2,10,17,19,24,36	1-25,51-53
A	WO 2010/039275 A1 (OLIGONIX, INC.) 08 Apr 2010 See [00026] and claims 1,6,11,16	1-25,51-53

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 OCTOBER 2012 (09.10.2012)	**10 OCTOBER 2012 (10.10.2012)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer KIM, YUN-KYUNG Telephone No. 82-42-481-5605

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/030940

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 26-50
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 26-50 pertain to a methods of diagnosis of the human body, thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1736

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1737

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1738

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(54) Title: PRECISION FLUORESCENTLY DYED PARTICLES AND METHODS OF MAKING AND USING SAME

(57) Abstract

An improved method of making a series of bead or microsphere or particle populations characterized by subtle variation in a proportion or ratio of at least two fluorescent dyes distributed within a single bead of each population is provided. These beads, when excited by a single excitation light source are capable of giving off several fluorescent signals simultaneously. A set containing as many as 64 distinct populations of multicolored, fluorescent beads is provided and when combined with analytical reagents bound to the surface of such beads is extremely useful for multiplexed analysis of a plurality of analytes in a single sample. Thus, methods of staining polymeric particles, the particles themselves, and methods of using such particles are claimed.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1739

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Precision Fluorescently Dyed Particles and Methods of Making and Using Same

1. Field of the Invention

The invention generally relates to multicolored, fluorescently stained small particles of generally less than 100 μm in diameter. Disclosed are methods of dyeing or staining such particles or microspheres with at least two fluorescent dyes in such a manner that intra-sample variation of dye concentrations is substantially minimized. Specifically, the invention relates to microspheres stained with at least two fluorescent dyes and methods of using said microspheres for a simultaneous analysis of a plurality of analytes.

2. Background of the Invention

Fluorescent light emitting microparticles, microspheres, microbeads, beads, or particles are now quite common and are useful for a number of practical applications especially in combination with flow cytometry based methods. As used hereinafter the terms: microparticles, microspheres, microbeads, beads, or particles are used interchangeably and bear equivalent meanings. Often, these particles are labeled with just one fluorescent dye. In general, such particles are made by copolymerization process wherein monomers, e.g., unsaturated aldehyde or acrylate, are allowed to polymerize in the presence of a fluorescent dye, e.g., fluorescein isothiocynate (FITC), in the reaction mixture (see for example U.S. Pat. Nos. 4,267,234 issued to Rembaum; 4,267,235 Rembaum et al; 4,552,812, Margel et al.; 4,677,138, Margel).

One skilled in the art would recognize that two or more dyes of varying proportions could be used to increase the permutation number of unique combinations of dyes in a single particle. These unique characteristics, i.e., emission wavelengths and fluorescence intensities could be extremely useful for multiparameter analysis of a plurality of analytes in the same sample. Three means of making multicolored, fluorescent beads have been reported, including: (a) covalent attachment of dyes onto the surface of the particle, (b) internal incorporation of dyes during particle polymerization, and (c) dyeing after the particle has been already polymerized. All three methods have been disclosed in the prior art.

The examples of the first approach are in U.S. Pat. Nos. 5,194,300 Cheung; 4,774,189 Schwartz which disclose fluorescent microspheres that are coated by covalently attaching either one or a plurality of fluorescent dyes to their surface. As such these methods are unrelated to the instant invention dealing with incorporating dyes into particles internally.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1741

Second approach can be found in U.S. Pat. No. 5,073,498 to Schwartz, which discloses two or more fluorescent dyes added during polymerization process and randomly dispersed within the body of the particle. However, when such particles are exposed to a single excitation wavelength only one fluorescent signal is observed at a time and thus these particles are not useful for multiparameter analysis especially in a flow cytometry apparatus with a single excitation light source. The U.S. Pat. No. 4,717,655 issued to Fulwyler discloses two dyes mixed at five different ratios and copolymerized into a particle. Although five populations of beads were claimed as being obtainable the fluorescent properties of these beads were not provided, effectively preventing one skilled in the art to make and use such beads. Thus, Fulwyler method is only a conceptual method since it was not enabled. Furthermore, any of these two methods are unrelated to the instant invention dealing with incorporating fluorescent dyes into already polymerized particles.

The principle of the third method, i.e., internally embedding or diffusing a dye after a particle has been already polymerized was originally described by L.B.Bangs (Uniform Latex Particles; Seragen Diagnostics Inc. 1984, p. 40) and relates to the instant invention as it consists of adding an oil-soluble or hydrophobic dye to stirred microparticles and after incubation washing off the dye. The microspheres used in this method are hydrophobic by nature. This allows adopting the phenomenon of swelling of such particles in a hydrophobic solvent, which may also contain hydrophobic fluorescent dyes. Once swollen, such particles will absorb dyes present in the solvent mixture in a manner reminiscent to water absorption by a sponge. The level and extent of swelling is controlled by incubation time, the quantity of cross-linking agent preventing particle from disintegration, and the nature and amount of solvent(s). By varying these parameters one may diffuse a dye throughout particle or obtain fluorescent dye-containing layers or spherical zones of desired size and shape. Removing the solvent terminates the staining process. Microparticles stained in this manner will not "bleed" the dye in aqueous solutions or in the presence of water-based solvents or surfactants such as anionic, nonionic, cationic, amphoteric, and zwitterionic surfactants.

U.S. Pat. No. 5,723,218 to Haugland et al. discloses diffusely dyeing microparticles with one or more dipyrrometheneboron difluoride dyes by using a process, which is essentially similar to the Bangs method. However, when beads internally stained with two separate dipyrrometheneboron dyes, were excited at 490 nm wavelength, they exhibited overlapping

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1742

emission spectra, meaning that beads were monochromatic but not multicolored. U.S. Pat. Nos. 5,326,692 Brinkley et al; 5,716,855 Lerner et al; and 5,573,909 Singer et al. disclose fluorescent staining of microparticles with two or more fluorescent dyes. However, dyes used in their process had overlapping excitation and emission spectra allowing energy transfer from the first excited dye to the next dye and through a series of dyes resulting in emission of light from the last dye in the series. This process was intended to create an extended Stokes shift, i.e., a larger gap between excitation and emission spectra, but not the emission of fluorescence from each dye simultaneously. Thus, due to various reasons such as dye-dye interaction, overlapping spectra, non-Gaussian emission profiles and energy transfer between neighboring dyes the demand for multicolored beads simultaneously emitting fluorescence at distinct peaks was not satisfied. Zhang et al. (U.S. Pat. No. 5,786,219) devised microspheres with two-color fluorescent "rings" or microspheres containing a fluorescent spherical "disk" combined with a fluorescent ring. Nevertheless, such beads, designed for calibration purposes, cannot be used in multiparameter analysis since two dyes were mixed only at one fixed ratio. As mentioned above in regard to U.S. Pat. No. 4,717,655 issued to Fulwyler, the highest number of dyes ratios ever attempted with at least two dyes never exceeded five. Thus, until the reduction to practice of the present invention there were no reliable means of creating a series of microsphere populations or subsets in which at least two dyes were mixed at variable, precisely controlled ratios and were proven, upon exposure to a single excitation wavelength, to emit multiple fluorescent signals of variable intensity and at spaced, optically distant wavelengths.

In other words, the prior art failed to provide a reproducible method that would allow one skilled in the art to make a plurality of defined subsets of stained multicolored microparticles distinguishable by a subtle variation in fluorescence signal resulting from the combination of various dyes of distinct color and having variable intensity of color emission. As used hereinafter the term stained microspheres means that a plurality of dyes, which are used to stain a microsphere, are either uniformly diffused throughout the body of said microsphere or penetrated said microsphere in a manner that results in formation of fluorescent rings, disks, and other geometrically distinct patterns.

Clearly, it would be an important improvement to the art to have a means of precisely dyeing or staining a particle with two or more dyes premixed in a series of predetermined ratios and to have a collection of such dyed microspheres for use in multiparameter applications. This

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1743

precision in dyeing process is commonly expressed as the coefficient of variation, which is the ratio of the standard deviation to the mean intensity of the fluorescent particle population. By minimizing this value, more subsets or populations of non-overlapping, distinctly dyed particles can be obtained. It would be a further advance in the art if the methods were repeatable or reproducible to within a minimal variation, preferably no more than about a 20% intra-sample variation, more preferably no more than about a 15% variation, and most preferably no more than about a 8% variation.

3. Summary of the Invention

An improved method is described for incorporating two or more fluorescent dyes into already polymerized microspheres. The amount of each dye absorbed by the microsphere is precisely controlled so as to give rise to two or more reproducible fluorescent signals of precise intensities and emission peaks within a given population of particles. A series of such populations or subsets of beads are dyed in batches each one of them having predetermined ratio or proportion of two or more fluorescent dyes. Due to novel and improved method of staining, the particle-to-particle variation in the same batch is greatly reduced, which allows producing an unprecedented number of distinct populations of multicolored, fluorescent microspheres residing within optically uniform, tightly defined cluster.

Accordingly, a set containing optically distinct precision stained microspheres is also claimed which would be useful for simultaneous analysis of a plurality of analytes in a same sample. In other words, said beads will provide a lot more than the use of stained beads found in the prior art since the number of analytes that can be measured simultaneously in a single tube, using a single sample aliquot is drastically increased. The fluorescent microparticle obtained by the inventive staining method is characterized by having at least two fluorescent dyes mixed within the body of the particle and each one of them capable of giving off, simultaneously, multiple fluorescent emission lights of predetermined color and intensity. The combination of notions relating to the emission peak corresponding to a given color and intensity of the fluorescent color as expressed in fluorescence channel units is generally termed as the fluorescence signal. The specific ratio or proportion of dyes at which they are mixed within a population of particles will determine the location of said populations on a fluorescence map, which allocates these populations according to fluorescent color and brightness. By using as little

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1744

as two dyes, e.g., orange and red, as many as 64 populations of beads are made each one distinct from another by subtle variations in unique fluorescence characteristics recognized by a flow cytometry apparatus.

When each such population of beads, characterized by at least two fluorescent signals, is combined with an analytical reactant capable of binding a specific analyte in a clinical or test sample a powerful analytical tool is obtained, which can provide qualitative and quantitative assay results. The analytical method is also provided which is based on using multicolored fluorescent beads obtained by the instant invention. To achieve truly multiplexed analysis of a plurality of analytes in a sample, a third type of fluorescent signal, e.g., green fluorescent signal is provided, usually found in a label reagent, which is capable of binding the analyte of interest. Thus, methods of making multicolored beads, the beads themselves, multiple sets of such beads, and multiplexed methods of analyzing a plurality of analytes in a single sample are claimed by the instant invention.

A method of staining polymeric microspheres with two or more fluorescent dyes is disclosed, which method comprises: (a) combining at least two fluorescent dyes in a solvent mixture comprising at least one organic solvent in which the at least two fluorescent dyes are soluble and at least one alcoholic solvent in which the at least two fluorescent dyes are less soluble, to provide a solution of mixed dyes which is further characterized as having the capacity to swell at least partially but not dissolve a plurality of polymeric microspheres, which is brought into contact with the solution; (b) contacting a plurality of polymeric microspheres with the solution for a period of time sufficient to provide uniform staining of substantially all of the members of the plurality of polymeric microspheres with the at least two fluorescent dyes, the at least two fluorescent dyes being selected such that on isolation and excitation of the dyed plurality of polymeric microspheres, a distinct fluorescence signal is emitted from each dye, the intensity of which emitted signal is proportional to the amount of the dye in the dyed plurality of polymeric microspheres.

In a particular embodiment of the invention, the method further comprises dehydrating the plurality of polymeric microspheres. Such a dehydrating step is accomplished by washing the plurality of polymeric microspheres one or more times with an alcoholic solvent prior to contacting the microspheres with the solution of mixed dyes. In still a preferred method, the dehydrating step involves drying the washed microspheres or allowing the alcoholic solvent to

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1745

evaporate from the washed microspheres prior to contacting the microspheres with the solution of mixed dyes.

Typically, the dyed plurality of polymeric microspheres is isolated by any manner well known in the art, including but not limited to filtration or centrifugation. It has been found desirable to obtain dyed plurality of polymeric microspheres in which at least one of the fluorescent dyes is diffused throughout the interior of substantially all of the members of the dyed plurality of polymeric microspheres, or in which the at least two fluorescent dyes are diffused throughout the interior of substantially all of the members of the dyed plurality of polymeric microspheres. Still other advantages can be gained by providing dyed plurality of polymeric microspheres in which at least one of the fluorescent dyes is diffused through only a portion of the interior of substantially all of the members of the dyed plurality of polymeric microspheres.

In a specific method of the invention, the staining procedure further comprises preparing a series of the solutions having differing desired ratios of the at least two fluorescent dyes and further comprises contacting separate populations of a plurality of polymeric microspheres with the series of the solutions to provide multiple distinct populations or subsets of a plurality of polymeric microspheres, each distinct population or subset having a differing desired ratio of the at least two fluorescent dyes.

It has been observed that the distinct fluorescence signals emitted from the at least two fluorescent dyes differ in their respective wavelengths by at least about 10 nm, preferably by at least about 30 nm and most preferably by at least about 50 nm.

Hence, the present invention provides a population of polymeric microspheres substantially uniformly stained with at least two fluorescent dyes, each microsphere of the population upon excitation exhibiting at least two distinct fluorescence emission signals corresponding to the at least two fluorescent dyes, the intensity of each of the at least two emitted signals (i) being proportional to the amount of its corresponding dye in the microsphere, and (ii) exhibiting a coefficient of variation among all the members of the population, which is no greater than about 20 percent. In particular, preferred populations are those in which the intensity of each of the at least two emitted signals exhibits a coefficient of variation among all the members of the population, which is no greater than about 15 percent, more preferably no greater than about 10 percent and most preferably no greater than about 8 percent. In still other embodiments, the intensity of each of the at least two emitted signals exhibits a coefficient of variation among

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1746

all the members of the population, which is less than about 8 percent.

The present invention offers, thus, a collection of distinct populations of polymeric microspheres according to the specifications described above, each population exhibiting an emission spectrum in a Fluorescence Bead Map, which is unique to the population. In specific embodiments, the collection comprises eight or more distinct populations of polymeric microspheres, preferably sixteen or more distinct populations of polymeric microspheres, more preferably twenty-four or more distinct populations of polymeric microspheres, most preferably thirty-two or more distinct populations of polymeric microspheres and still most preferably sixty-four or more distinct populations of polymeric microspheres. Generally, the collection is further characterized in that there is substantially no overlap between any of the sixty-four or more emission spectra associated with the sixty-four or more distinct populations of polymeric microspheres.

Also contemplated by the invention, is a method of detecting simultaneously by flow cytometry a plurality of analytes in a sample, each of the analytes being recognized by a corresponding analytical reactant, comprising: (a) contacting the sample with a plurality of populations of uniformly stained microspheres, the microspheres having at least two fluorescent dyes uniformly mixed at a specific ratio within each microsphere of each the population, each population of the microspheres having a distinct analytical reactant bound to its surface, wherein, the reactant on each population of microspheres specifically interacts with one of the analytes in the sample; (b) providing a label reagent that specifically binds to the analyte and analyzing the microspheres to detect the label indicating binding of the analyte to the analytical reactant; and (c) determining the populations of microspheres having the fluorescent dyes mixed at the specific ratio within microspheres of each population to which the reactant is bound.

Other objects of the invention will become apparent from the further discussions and detailed descriptions provided herein.

Overview of the Invention and its Embodiments

Recent developments in instrumentation have necessitated the concurrent development of multiple and precisely dyed microspheres that can emit multiple fluorescent signals simultaneously. This invention describes techniques for absorbing at least two squaric acid-based fluorescent dyes into polymeric, i.e., polystyrene particles.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1747

The present invention describes techniques for precisely dyeing polystyrene microspheres of sizes ranging from approximately 10 nm to 100 μm in diameter. The size of particles is immaterial to this invention since the precision of the dyeing process is not affected. The only requirement is that particles are made of water-insoluble material but soluble in adequate solvents. The dyes employed are preferably squaric acid-based molecules that exhibit fluorescence extending into near infrared and/or infrared region, i.e., to ca. 1,000 nm. Use of other dyes may allow one to expand the range from the ultraviolet to infrared. This method allows for a highly reproducible process in which two or more dyes of independent concentration are absorbed uniformly into each microsphere, resulting in multiple fluorescent signals respective of the number of dyes present in the microsphere.

The technology is disclosed enabling one skilled in the art to make a series of multicolored, fluorescent particles with unique fluorescence characteristics and using such particles for multiparameter analysis of a plurality of analytes simultaneously.

4. Brief Description of Drawings

Fig. 1 showing flow chart with sequential steps of dyeing polymeric particles using the prior art technique.

Fig. 2 showing flow chart with sequential steps of dyeing polymeric particles using the instant invention.

Fig. 3 showing two-dimensional flow cytometry chart illustrating wide optical distribution of two-color-dyed microspheres using the prior art technique.

Fig. 4 showing two-dimensional flow cytometry chart illustrating tight clustering of two-color-dyed microspheres using the instant invention.

Fig. 5 showing that prior art method allows no more than 6 subsets of multicolored bead populations on a Fluorescence Bead Map.

Fig. 6 showing Fluorescence Bead Map for 64-Region Bead Set, indicating tight distribution of each bead subset fluorescence characteristics within boundaries prescribed by each region.

5. Detailed Description of Specific Embodiments

The invention provides novel polymeric beads or microspheres containing at least two fluorescent dyes. This invention further includes the improved method of making such beads by

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1748

mixing said beads with at least two fluorescent dyes combined at predetermined ratio so that optically distinct, multiple populations of multicolored beads are formed. These bead populations are easily discriminated as essentially non-overlapping clusters by visual detection methods such as microscopy or preferably by flow cytometry. The method of simultaneous, multiparameter analysis of a plurality of analytes is also provided whereby each distinct multicolored bead population would carry an additional analytical reactant, e.g., antibody, antigen, or nucleic acid probe, which would react with a specific analyte of interest in a sample containing the plurality of analytes.

Polymeric microspheres used in this invention are commercially available from a number of vendors and range in size from 0.01 to 100 micrometers (μm) in diameter. Even though the microparticle can be of any size, the preferred size is 0.1-50 μm, more preferably 1-20 μm, and even more preferably 3-9 μm. The sizes of beads in one set can be uniform or may differ in order to distinguish and classify them into further subsets according to their size. The size of the microparticle can be measured in practically any flow cytometry apparatus by so-called forward or small-angle scatter light. These subsets can be also further distinguished by different shape of microparticles. The shape of the particle can be also discriminated by flow cytometry, e.g., by high-resolution slit-scanning method.

The preferred make of microspheres is polystyrene or latex material. However, any type of polymeric make of microspheres is acceptable including but not limited to brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyacrylamide, polyacrolein, polybutadiene, polydimethylsiloxane, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polymethylmethacrylate, or combinations thereof.

The microspheres will also contain 1 to 30% of a cross-linking agent, such as divinyl benzene, ethylene glycol dimethacrylate, trimethylol propane trimethacrylate, or N,N'methylene-bis-acrylamide or other functionally equivalent agents known in the art. In preferred embodiment microspheres are made of polystyrene and contain 1 to 30% divinyl benzene.

The beads may or may not have additional surface functional groups, such as carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, or halides. The functionality of the microspheres' surface groups gives the microspheres their coupling capability allowing chemical binding of analytical reactants. In addition to functional

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1749

groups on microspheres the dyes themselves can also carry chemically reactive functional groups which in addition to groups listed above can also be carboxylic acid, carboxylic acid succinimidyl ester, carboxylic acid anhydride, sulfonyl chloride, sulfonyl fluoride, hydrazine derivatives, acyl azide, isocyanate, haloacetamide, phenols, thiols, and ketones. These functional groups are useful for attachment of analytical reactants, i.e., classical, commonly used reactants such as antibody, antigen (hapten), digoxigenin, or nucleic acid probe. These may also include reactants that can form specific, high-affinity conjugates such as avidin-biotin, receptor-ligand, ligand-ligate, enzyme-substrate, lectin-carbohydrate, protein A-immunoglobulin, etc. For flow cytometry analysis the analytical reactants are commonly labeled with fluorescent tags or labels such fluorescein (FITC) or rhodamine. These light-emitting conjugates of a dye and analytical reactant are termed as label reagents.

The analytical reactants can be also selected among fluorescent reporter molecules capable to react with a variety of analytes, e.g., O_2, CO_2, pH, Ca^{++}, Na^+, K^+, or Cl^- as disclosed for example in U.S. Pat. No. 5,747,349 issued to van den Engh et al.

Suitable solvents will be selected based on their ability to solubilize the particular class of hydrophobic dyes of interest. It is preferable that their solubility characteristics are substantially similar. The solvents can be acyl, aliphatic, cycloaliphatic, aromatic or heterocyclic hydrocarbons; the solvents may or may not have halogens, oxygen, sulfur, nitrogen, and/or phosphorous as either terminal groups or as integral parts of a ring or chain. Specifically, solvents such as toluene, xylene, hexane, pentane, acetone, DMSO, or methylene chloride can be used. In a preferred embodiment, chlorinated solvents, more preferably chloroform, are used to solubilize the squaric acid class of dyes, which are preferred dyes used in this invention.

In one embodiment two fluorescent squaraine dyes are used, e.g., red dye which is 1,3-bis [(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxy-cyclobutenediylium, bis(inner salt) and orange dye is 2-(3,5-dimethylpyrrol-2-yl) -4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one. The molar ratio between first and second dye, when present in a bead, will preferably be between about 0 and 10,000, more preferably between 0.00001 and 2,000. Both dyes would preferably be excited at the same absorption wavelength, e.g., ranging from ultraviolet to about 800 nm, and emit fluorescent light at two distinct, essentially non-overlapping wavelengths distant from each other by at least 10

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1750

nm, preferably 30 nm, and more preferably by at least 50 nm. For example, the emission peak of the dye #1 is at 585 nm, and the peak emission of dye #2 is at 630 nm.

The squaric acid based fluorescent dyes can be synthesized by methods described in the literature. See, for example, Sprenger et al. Angew. Chem., 79, 581 (1967); Angew. Chem., 80, 541 (1968); and Maaks et al., Angew Chem. Intern. Edit., 5, 888 (1966). Briefly, one equivalent of squaric acid (1,2-dihydroxycyclobutenedione) is condensed with two equivalents of an active compound, such as a pyrrole, indoline, or aniline, and refluxed in a mixture of an alcohol and an aromatic solvent (such as benzene) under conditions that allow removal of water from the reaction mixture. The resulting dye can be collected and purified by a number of standard methods, such as recrystallization, distillation, chromatography, etc. Additionally, unsymmetrically substituted squaric acid compounds can be synthesized by methods such as those described by Law et al., J. Org. Chem. 57, 3278, (1992). Specific methods of making some of such dyes are well known in the art and can be found for example in U.S. Pat. Nos. 5,795,981; 5,656,750; 5,492,795; 4,677,045; 5,237,498; and 5,354,873. Optionally such dyes will contain functional groups capable of forming a stable fluorescent product with functional groups typically found in biomolecules or polymers including activated esters, isothiocyanates, amines, hydrazines, halides, acids, azides, maleimides, alcohols, acrylamides, haloacetamides, phenols, thiols, acids, aldehydes and ketones.

In addition to specific squaric acid dyes are used in this preferred embodiment, related dyes can be further selected from cyclobutenedione derivatives, substituted cephalosporin compounds, fluorinated squaraine compositions, symmetrical and unsymmetrical squaraines, alkylalkoxy squaraines, or squarylium compounds. Some of these dyes can fluoresce at near infrared as well as at infrared wavelengths that would effectively expand the range of emission spectra up to about 1,000 nm.

In addition to squaraines, i.e., derived from squaric acid, hydrophobic dyes such as phthalocyanines and naphthalocyanines can be also selected as operating at longer wavelengths. Other classes of fluorochromes are equally suitable for use as dyes according to the present invention. Some of these dyes are listed herein: 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine, 5-Hydroxy Tryptamine (5-HT), Acid Fuhsin, Acridine Orange, Acridine Red, Acridine Yellow, Acriflavin, AFA (Acriflavin Feulgen SITSA), Alizarin Complexon, Alizarin Red, Allophycocyanin, ACMA, Aminoactinomycin D, Aminocoumarin, Anthroyl

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1751

Stearate, Aryl- or Heteroaryl-substituted Polyolefin, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9 (Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, BOBO 1, Blancophor FFG Solution, Blancophor SV, Bodipy Fl, BOPRO 1,Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbocyanine, Carbostyryl, Cascade Blue, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphtyl Sulphonic Acid), Dansyl NH-CH3, DAPI, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrrometheneboron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Eosin, Erythrosin ITC, Ethidium Bromide, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Hoechst 33258 (bound to DNA), Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nile Red, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oregon Green, Oxazine, Oxazole, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Propidium Iodide, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Rose Bengal, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS (Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulphO Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Texas Red, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, TOTO 1, TOTO 3, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, XRITC, YO PRO 1, or combinations thereof. One skilled in the art would certainly know which one to select among such dyes as long as desired

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1752

emission and absorption properties as well as their hydrophobic properties are appropriate. The spectral properties of the fluorescent dyes should be sufficiently similar in excitation wavelengths and intensity to fluorescein or rhodamine derivatives as to permit the use of the same flow cytometry equipment. It is preferable that the dyes, however, have higher solubility in organic solvents and have improved photostability and quantum yields. These dyes will be combined at predetermined ratio and embedded into a microsphere vehicle and total dye quantity will be between about 0.00001% and 15% by weight to particle weight. This limitation is however of little consequence to the present invention for as long as the particle impregnated with said dyes is stable and usable for its intended purpose.

Prior art method.

The prior art method teaches staining carrier particles with a single dye only (see Fig. 1). However, for the purpose of a meaningful comparison and in order to be consistent with the thrust of the instant invention, the said method was adapted to stain with two dyes simultaneously. The prior art of dyeing large polymer particles (>5 um) as stated in "Uniform Latex Particles" by Leigh B. Bangs was performed and the general outline of the procedure is shown in Fig. 1 and obtained results are shown in Fig. 3. Briefly, the process is started by placing 5 ml of undyed stock microspheres in an aqueous medium directly on a membrane covered fritted funnel. A vacuum pump pulled air through the microspheres plated onto the filter paper for one hour. Next, the dried microspheres were transferred to 50 ml of dye solution, covered, and stirred at room temperature over night. The next day the microspheres were separated by filtration from the dye solution and the dyed particles were placed in a vacuum dessicator for about four hours to remove residual solvent. Next is added 200 ml of Triton X-100 and water solution to the dried dyed microspheres in a 250 ml flask. The solution is stirred for three hours. The solution is filtered and the washing is repeated until no further dye is detected in the filtrate. The beads stained in this manner are tested for staining uniformity by flow cytometry (Fig. 3). It can be easily seen that, based on three separate experiments (tests A, B, and C), bead-to-bead variation at FL2 and FL3 parameters (CV or coefficient of variation) is rather high and inadequate (Table 1) to satisfy the increasing demand for precisely dyed multicolored microspheres.

General outline of the instant method.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1753

As the demand and applications for precisely dyed multicolored microspheres increases the development of alternate processes to the aforementioned method is warranted. As a result, a modification of the prior art method has been developed which has proven to be the most efficient method for precision dyeing of the microspheres (see Fig. 2). This method takes one tenth or even less of the time of the previously mentioned method and significantly enhances its precision. As before, it is critical to remove almost all traces of water from the microspheres. To accomplish this a volume of stock microspheres in an aqueous medium is pipetted onto a vacuum filter membrane, and the liquid is removed and discarded. Next, 100 ml of the rinse solvent (an aliphatic alcohol, such as propanol, methanol, ethanol, etc.) are added to the microspheres. The microspheres are resuspended by placing an ultrasonic probe directly into the solution and applying power for several seconds or as needed to affect resuspension. The suspension is filtered and previous step is repeated once more. Dyeing of the microspheres is accomplished by adding 50 ml of a dye solution (composed of one or more dyes in an organic solvent, as described below) to the filtering cup and resuspending as before. The suspension is allowed to sit for five minutes in the filtering cup. Next, 50 ml of rinse solvent is added to the dye suspension, sonicated and filtered. Another 100 ml of the rinse solvent is added, resuspended and filtered. The last step is repeated once more. In order to prepare the microspheres for storage, 100 ml of an aqueous medium is added to the microspheres, then sonicated and filtered. Finally, 50 ml of aqueous medium is added to the microspheres, sonicated and transferred to a storage container.

In a particular embodiment of the invention, two squaric acid-based dyes are mixed in a solvent suitable for the complete dissolution of both dyes, such as chloroform. Ethanol is added to the solution to increase wetting of the microspheres, and to create a process-dependent, final solvent density that is less than that of the microspheres. The concentrations of each dye are experimentally determined as a function of the target fluorescence intensity at each of the two center wavelengths. These concentrations maintain their relative intensity throughout this inventive process.

An important aspect of the present invention is the preparation of microspheres prior to the dyeing operation. Manufacturers often supply microspheres in an aqueous medium. It has been discovered that the surface of the microsphere that had been stored in aqueous medium must be treated to make it permeable to organic compounds. In a preferred embodiment, an amount of a polar organic solvent such as an alcohol is added to the microsphere solution to achieve

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1754

about a 50% mixture of the aqueous medium and the polar organic solvent. This ratio, however, may vary and adjusted at will according to particular needs that one may have or determined by chemical and physical properties of medium and solvent.

An equally efficient and precise technique involves "drying" the microspheres through a series of alcohol, e.g., methanol, ethanol, 2-propanol, rinses. The process begins by spinning down the aqueous suspension of microspheres, typically 10% solids in suspension. The aqueous medium is decanted, and the beads are re-suspended in methanol. The alcohol solution at ca. 5% solids is vortexed, sonicated and spun down. This step is performed once or twice more. The excess alcohol is decanted from the pellet, and residual solvent is evaporated under vacuum.

Test samples consisting of 0.05 gram of dried microspheres are used to help adjust the dye solution to its desired ratio. The dried 0.05 gram of microspheres are suspended in 0.5 ml of dye mixture containing two or more dyes of interest. The suspension of microspheres, now at 10% solids, is vortexed and sonicated into suspension. Once in suspension the mixture of microspheres and dyes is mixed for one hour. After that hour, the microspheres are spun down for a period of 1minute using a centrifuge. The dye solution is decanted back into the main flask, and the 0.05 g of microspheres are re-suspended in 1 ml of 90% of alcohol, e.g., methanol. The rinse step uses double the volume of the dye solution, thus maintaining a 5% solid solution. The sample is vortexed, sonicated and spun down. The methanol supernatant is decanted. The 90% methanol rinse step is repeated once more. Finally, the excess methanol is decanted from the pellet, and the microspheres are re-suspended in an aqueous medium. The resulting test samples are then tested to determine the fluorescence activity/intensity of the labeled beads.

When the test samples show that the dye solution, indeed, has the precise ratios of the desired dyes, a macro-scale batch is conducted. The principle of macro-scale work up is identical to that noted above. Briefly, 25 ml of the desired dye solution is transferred to a 50 ml vial, which contains 2.5 grams of dried microspheres. The microspheres, now at 10% solids, are vortexed and sonicated. Once the microspheres are completely in suspension, it is mixed for an hour. After that hour the microspheres are taken out of the dye solution by centrifugation. The dye solution is decanted back into the main flask, and the 2.5 grams of microspheres are re-suspended in 50 ml of 90% methanol. The rinse step uses double the volume of the dye solution, thus maintaining a mixture of 5% solids. The sample is vortexed, sonicated and spun down. The methanol supernatant is decanted. This step is repeated once more. After the final

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1755

methanol rinse is decanted, the microspheres are put through an aqueous rinse. The aqueous supernatant is decanted, and the beads are then re-suspended and stored in a fresh aqueous medium.

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These Examples are not intended in any way to otherwise limit the scope of the disclosure or the protection granted by Letters Patent hereon.

Example 1.

A single solution containing two different squaric acid dyes is prepared. One dye is a red fluorescent dye 1,3-bis [(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxy-cyclobutenediylium, bis(inner salt) and second dye is orange fluorescent dye can be 2-(3,5-dimethylpyrrol-2-yl)
-4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one. The peak emission of dye #1 is 585 nm, and the peak emission of dye #2 is 630 nm. These dyes are chosen because they fall in the center of two of the fluorescence channels of a Becton Dickinson FACScan flow cytometer, which is the measurement device used to determine the precision of prior art dyeing techniques compared with this innovative new technique. The choice of fluorescence channels is, however, relative and immaterial since another flow cytometry apparatus may have different settings.

Two samples of undyed microspheres are prepared. The first is dyed with the mixture of orange and red dyes using this innovative technique (shown Figs. 2 and 4), and the second is dyed using the prior art technique (Figs. 1 and 3). Samples are measured on the FACScan, and an X-Y plot is generated to show the relative homogeneity of each sample. X-axis represents brightness or fluorescence intensity of orange dye and Y-axis represents the same parameters of red dye. Mean intensities and coefficients of variation are also measured. It is clear that beads stained by the old method spread over much larger X-Y area, indicating that the ratio of orange and red dyes vary from particle-to-particle. In contrast, the coefficient of variation in the bead population dyed by the instant, improved method is much smaller. About 10,000 beads in each tests A, B, and C, were run in parallel with beads stained by Bangs method (Table 1).

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1756

Example 2

To make another population of beads with different fluorescent characteristics the ratio of red/orange dyes is altered by an adequate increment in proportion of dyes so that obtained population optically does not overlap with the former population. The prior art failed to provide multiple populations of multicolored beads due to inevitable intra-sample heterogeneity resulting from inadequate staining process resulting in poor dye distribution from particle-to-particle within given staining batch. Thus, upon excitation with a light source, stained beads containing more than one dye failed to emit uniform fluorescence signals of desired intensity. The instant invention overcomes this problem and achieves construction of as many as 64 subsets of optically distinct beads by varying the ratio of just 2 dyes. This example is not in any way a limiting one since one of ordinary skill may easily generate smaller or higher number of bead subsets by using the instant teaching. One skilled in the art may appreciate that nothing even close to this achievement has ever been enabled in the actual practice. Although such an eventuality was theoretically speculated as a possible one, the prior art failed to teach one of ordinary skill how to arrive at that.

The present inventors were able, for the first time, to reduce to practice the invention and representative experimental results of obtaining 64-bead population are shown in Fig. 6 and Table 2. The results illustrated in Fig. 5 show multicolored beads by using staining procedure of the prior art method. Due to imprecision in staining technique, which results in a wide dispersion of dyes ratio from bead-to-bead, no more than 6 subsets of multicolored bead populations can be fitted on a Fluorescence Bead Map. In contrast, Fig. 6 shows Fluorescence Bead Map containing 64 populations of beads, indicating tight distribution of each bead subset fluorescence characteristics within boundaries prescribed by each region. The cross-talk between various clusters is minimal. Most of the overlap is due to the presence of bead agglomerations which emit brighter signal but they are eliminated by size discrimination based on light scatter.

In general, as can be readily glanced from Table 2, there is an unequivocal relationship between two dye concentrations in a given population of beads and location of said populations on X-Y map. Each location is assigned in terms of red (FL3) or orange (FL2) dyes intensity as expressed in linear fluorescence channels units which fall in approximately 470, 580, 690, 750, 800, 900, and 990 series. For practical reasons, i.e., space limitation in the bead cluster, the last digit "0" is omitted. The first two digits in each bead population represents fluorescence intensity

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1757

of orange dye (FL2) and last two digits the intensity of the red dye (FL2). The fluorescent intensity increases as the numbers go higher. The beads with lowest intensity (4725) reside in lower left corner and brightest ones (9998) in upper right corner. As they move vertically up a column, both red and orange dye amounts in a bead must be increased. This is because there is a substantial amount of energy transfer from the orange dye to the red. When moving horizontally from left to right across a row, the red dye must be decreased in order to maintain a steady FL3 value. This is due to overlap of the orange dye spectrum into the red region, thus necessitating the increase in FL3 signal. In this manner multiple, non-overlapping populations of beads are constructed. Two parameters namely, a fluorescent color (red or orange) and color intensity or brightness (expressed in fluorescence channel units), are essential to classify obtained beads and are termed as a fluorescence signal.

Hence, particular populations of beads are provided whose fluorescence characteristics or signals fall within a prescribed region depicted in a Fluorescence Bead Map. Typically, about 80% or greater of the individual beads within a particular population of beads will exhibit fluorescence characteristics within the desired region, preferably about 90% or greater, more preferably about 97% or greater, most preferably about 99% or greater. For each set of beads, typically about 1% or less of the individual beads within a particular set of beads will exhibit fluorescence characteristics that fall within another, undesired region, preferably about 0.5% or less, more preferably about 0.3% or less, most preferably about 0.2% or less.

While theoretically any number of populations or subsets can be present in each Bead Map, due to the limitations in the prior art techniques it is not possible to obtain more than 6 subsets coexisting simultaneously. While theoretically it has been speculated that such subsets can be extremely valuable for multiplex analysis (see for example McHugh, "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes," in Methods in Cell Biology, 42, Part B, (Academic Press, 1994) so far there are no known examples in the art enabling and demonstrating the reduction to practice of tangible, multicolored beads. At best only 1 and perhaps a maximum of 5 population of beads containing various ratios of two dyes could have been possible. For example, U.S. Pat. No. 4,717,655 discloses such beads, however, the disclosure was not enabled and the method of incorporating dyes in these beads is by copolymerization process and as such it is unrelated to the instant invention. In contrast, due to a significant improvement over existing methodology it is now technically

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1758

possible to obtain 16-subset, 32-subset, 64-subset or even higher number of bead collections using the instant methodology.

As an example 64-subset bead collection or 64 populations of beads were constructed each population differing from another by a distinct location on the X-Y plot. These locations essentially do not overlap. As opposed to the prior art methods which result in up to 10-20% or even higher rate of dispersion the instant method allows to obtain essentially homogeneous populations of beads with only 0.2 -0.3 % dispersion. As used hereinafter the term essentially non-overlaping populations means that only about 0.2-0.3 % of beads in each population may display an optical pattern or fluorescent signal which can be ascribed to the neighboring cluster of beads having the same set of fluorescent dyes but mixed at different ratio. This is a significant improvement over the prior art.

Example 3

Although multiplexed analysis capability theoretically would provide enormous benefit in the art of flow cytometry, very little progress has been previously achieved due to technical limitations in obtaining sufficient variety of multicolored, non-overlaping subsets of fluorescent beads. A review of some of these prior art techniques is provided by McHugh (see above). These methods have been unsatisfactory as applied to provide fully multiplexed assay capable of analysis of more than a few different analytes. In the prior art when beads were incorporating a combination of 2 dyes only 5 subsets of beads were allegedly obtained (U.S. Pat. No. 4,717,655 issued to Fulwyler). A set with maximum of six subsets is obtained using Bangs method (see Example 1) which is still insufficient for the purposes of truly multiplexed assay.

A series of antibodies, antigens, or nucleic acid probes, collectively named hereinafter as analytical reactants, are attached to the beads by any of a number of conventional procedures such as by chemical or physical adsorption as described by Colvin et al., "The Covalent Binding of Enzymes and Immunoglobulins to Hydrophilic Microspheres" in Microspheres: Medical and Biological Applications, 1-13, CRC, Boca Raton, FL, 1988;

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1759

Cantarero et al., "The Adsorptive Characteristics of Proteins for Polystyrene and Their Significance in Solid-Phase Immunoassays," Anal Biochem, 105, 375-382 (1980); and Illum et al., "Attachment of Monoclonal Antibodies to Microspheres," Methods in Enzymol, 112, 67-84 (1985) 112, 67-84 (1985).

After attachment of a reactant to the beads' surface, aliquots from each subset are mixed to create a pool containing known amounts of beads within each subset. Preferably, the pooled set is prepared with equal volumes of beads from each subset, so that the set contains about the same number of beads from each subset or population. This pool is then be incubated with a fluid sample of interest, such as serum or plasma, to test for the presence of antibodies in the fluid that are reactive with antigens on the beads. Such incubation is generally performed under conditions of temperature, pH, ionic concentrations, and the like that facilitate specific reaction of antibodies in the fluid sample with antigen on the bead surface. After a sufficient period of time, the beads in the mixture are centrifuged, washed and incubated for another period of time with a "secondary" antibody such as, for example, fluorescein labeled goat anti human immunoglobulin. The secondary antibody or label reagent will bind to and fluorescently label antibodies bound to antigen on the beads. After washing (or without washing), the beads are processed by a flow cytometer and the four classification parameters forward light scatter, side light scatter, red fluorescence, and orange fluorescence are measured and used to identify the subset or population to which each bead belongs. A simultaneous measurement of green fluorescence (measurement parameter) for each bead allows one to determine whether the bead has antibody bound to it. Because the subset to which a bead belongs is correlated with the presence of a particular antigen, e.g., series of grass allergens, various substance abuse drugs, one may readily determine the specificity of the antibody bound to a bead as a function of the subset to which it belongs.

Displacement or competition assay

Assays for many substances in a clinical laboratory are based on the interference with specific ligand-ligate or antigen-antibody interactions. In these assays, one member of the ligand-ligate pair is labeled with the fluorophore or fluorochrome and one member is

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1760

immobilized on the beads. Soluble, unlabeled analyte, which may be ligand or ligate, is added to the reaction mixture to competitively inhibit interaction of the labeled component with the immobilized component. It is usually not important which member of the pair is labeled and which is immobilized; however, in certain assays, functional advantages may dictate the orientation of the assay. In an exemplary assay of this type, each bead subset is provided with an antigen. The antigen-coated beads are then reacted with labeled antibody specific for the antigen on the bead surface. Subsequent addition of a test fluid containing soluble analyte (inhibitor) will displace the labeled antibody from the beads in direct proportion to the concentration of the soluble analyte. A standard curve of known analyte concentrations is used to provide accurate quantification of analyte in the test sample.

Nucleic acid analysis

The power and sensitivity of PCR found its application to a wide variety of analytical problems in which detection of DNA or RNA oligonucleotide sequences is required. One major difficulty with the PCR technique is the cumbersome nature of the methods of measuring end-product, i.e., amplified DNA. A flow cytometric bead-based hybridization assay permits the extremely rapid and accurate detection of genetic sequences of interest. In a preferred embodiment of this invention, a bead to which a nucleic acid segment of interest has been coupled is provided. A PCR product of interest (or any other DNA or cDNA segment) is detected by virtue of its ability to competitively inhibit hybridization between the nucleic acid segment on the bead and a complementary fluorescent DNA probe. The method is sensitive and precise and allows the detection of single point mutations in the PCR product or DNA of interest. The multiplexed DNA analysis method can be applied to detect any PCR product or other DNA of interest for specific polymorphisms or mutations and one skilled in the art will recognize that numerous applications can be imagined such as presence of histocompatibility alleles associated with susceptibility to diseases, mutations associated with genetic diseases, autoimmune diseases, or mutations of oncogenes associated with neoplasia or risk of neoplasia. In a same way nucleic acid segments from pathogenic organisms such as bacterial, viral, fungal, mycoplasmal, rickettsial, chlamydial, or protozoan pathogens can be

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1761

detected simultaneously.

Enzyme assays

The invention is also useful for measurement of enzymes, enzyme inhibitors and other analytes. For example, bead subsets are generated with selected fluorescent substrates which are enzymatically cleaved from the bead, resulting in a loss of fluorescence. Enzymes that can be detected and measured using the invention include but are not restricted to, proteases, glycosidases, nucleotidases, and oxidoreductases. Any enzyme that results in selected bond cleavage can be measured. Alternatively, the action of the enzyme on the bead-bound substrate results in the formation or identification of a ligate for a fluorescent ligand present in the reaction mixture. The bead bearing the modified substrate then becomes fluorescent by virtue of binding of the fluorescent ligand to the newly formed ligate. Because each type of bead bearing the unique substrate can be distinguished, a mixture of bead subsets can be used to measure several enzyme activities simultaneously in the same reaction mixture.

Fluids or samples with analytes that can be analyzed using these techniques include plasma, serum, tears, mucus, saliva, urine, pleural fluid, spinal fluid and gastric fluid, sweat, semen, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses, and extracts of tissues including biopsies of normal, malignant, and suspect tissues.

The above examples can be used to perform most common immunodiagnostic and nucleic acid assays. Other applications such as high throughput screening of combinatorial chemistry libraries for discovering new drugs, environmental screening of pollutants, drug testing, food safety-related investigations, testing of multiple analytes for agricultural needs, etc, can be imagined.

It is to be understood that, while the foregoing invention has been described in detail by way of illustration and example of preferred embodiments, numerous modifications, substitutions, and alterations are possible without departing from the spirit and scope of the invention as described in the following claims.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1762

TABLE 1

Sample	FL2 (CV)	FL3 (CV)	Events
Old Method			
A	24.2	25.9	10000
B	27.7	25.5	10000
C	27.7	23.2	10000
Improved Method			
A	7.3	6.8	10000
B	7.3	6.7	10000
C	7.2	6.7	10000

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1763

TABLE 2

Region 47 series	FL3 Red Dye (mM)	FL2 Orange Dye (mM)
470/250	0.0495	0.00441
470/340	0.104	0.0041
470/415	0.0685	0.004
470/470	0.388	0.00576
470/525	0.458	0.0039
470/580	0.981	0.00662
470/635	0.132	0.00557
470/690	1.831	0.00637
470/745	3.84	0.01138
470/810	11.29	0.0278
Region 58-series	FL 3 Red Dye (mM)	FL2 Orange Dye (mM)
580/250	0.0201	0.00994
580/340	0.0582	0.00979
580/415	0.0763	0.00161
580/470	1.38	0.0256
580/525	0.42	0.0114
580/580	0.613	0.0162
580/635	1.15	0.0132
580/690	2.71	0.0267
580/745	0.643	0.0248
580/810	7.5	0.0489
580/870	9.71	0.008

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1764

TABLE 2 (Sheet 2 of 3)		
Region 69-series	FL3 Red Dye (mM)	FL3 Orange Dye (mM)
690/360	0.048	0.024
690/415	0.129	0.024
690/470	0.226	0.023
690/525	0.45	0.029
690/580	0.89	0.0459
690/635	1.11	0.0322
690/690	2.45	0.07
690/745	2.88	0.0624
690/810	5.54	0.185
690/870	5.68	0.145
750/940	10.52	1.07
Region 80-series	FL 3 Red Dye (mM)	FL2 Orange Dye (mM)
800/415	0.0465	0.15
800/470	0.123	0.131
800/525	0.226	0.061
800/580	0.504	0.0835
800/635	0.62	0.0805
800/690	1.18	0.118
800/745	1.7	0.113
800/800	3.06	0.218
800/870	5.7	0.31
800/940	7.54	0.806
950/940	7.93	0.736

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1765

TABLE 2 (Sheet 3 of 3)		
Region 90-series	FL 3 Red Dye (mM)	FL2 Orange Dye (mM)
900/510	0.084	0.0017
900/553	0.0666	0.0146
900/596	0.138	0.0913
900/645	1.29	0.253
900/690	0.861	0.298
900/734	0.699	0.229
900/779	0.984	0.214
900/823	1.13	0.435
900/867	2.61	0.381
900/912	3.41	0.579
900/953	4.88	0.861
Region 99-series	FL 3 Red Dye (mM)	FL 2 Orange Dye (mM)
990/600	------	0.274
990/640	0.125	0.21
990/680	0.21	0.268
990/720	0.472	0.216
990/760	0.712	0.275
990/800	0.82	0.278
990/840	1.12	0.446
990/880	1.87	0.431
990/920	3.24	0.477
990/980	2.93	0.763

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1766

What is claimed is:

1. A method of staining polymeric microspheres with two or more fluorescent dyes comprising:

(a) combining at least two fluorescent dyes in a solvent mixture comprising at least one organic solvent in which the at least two fluorescent dyes are soluble and at least one alcoholic solvent in which the at least two fluorescent dyes are less soluble, to provide a solution of mixed dyes which is further characterized as having the capacity to swell at least partially but not dissolve a plurality of polymeric microspheres, which is brought into contact with said solution;

(b) contacting a plurality of polymeric microspheres with said solution for a period of time sufficient to provide uniform staining of substantially all of the members of said plurality of polymeric microspheres with the at least two fluorescent dyes,

said at least two fluorescent dyes being selected such that on isolation and excitation of said dyed plurality of polymeric microspheres, a distinct fluorescence signal is emitted from each dye, the intensity of which emitted signal is proportional to the amount of said dye in said dyed plurality of polymeric microspheres.

2. The method of claim 1, which further comprises dehydrating said plurality of polymeric microspheres.

3. The method of claim 2 in which the dehydrating step comprises washing said plurality of polymeric microspheres one or more times with an alcoholic solvent prior to contacting same with said solution.

4. The method of claim 3, which further comprises drying said washed microspheres or allowing the alcoholic solvent to evaporate from said washed microspheres prior to contacting same with said solution.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1767

5. The method of claim 1, which further comprises isolating said dyed plurality of polymeric microspheres by filtration or centrifugation.

6. The method of claim 1 in which at least one of said fluorescent dyes is diffused throughout the interior of substantially all of the members of said dyed plurality of polymeric microspheres.

7. The method of claim 1 in which said at least two fluorescent dyes are diffused throughout the interior of substantially all of the members of said dyed plurality of polymeric microspheres.

8. The method of claim 1 in which at least one of said fluorescent dyes is diffused through only a portion of the interior of substantially all of the members of said dyed plurality of polymeric microspheres.

9. The method of claim 1, which further comprises preparing a series of said solutions having differing desired ratios of said at least two fluorescent dyes.

10. The method of claim 9, which further comprises contacting separate populations of a plurality of polymeric microspheres with said series of said solutions to provide multiple distinct populations or subsets of a plurality of polymeric microspheres, each distinct population or subset having a differing desired ratio of said at least two fluorescent dyes.

11. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes differ in their respective wavelengths by at least about 10 nm.

12. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes differ in their respective wavelengths by at least about 30 nm.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1768

13. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes differ in their respective wavelengths by at least about 50 nm.

14. The method of claim 1 in which at least one of said emitted signals is orange and at least another emitted signal is red.

15. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes exhibit wavelengths falling in a range between about 500 nm and about 1,000 nm.

16. The method of claim 1 in which said organic solvent comprises acyl group-containing, aliphatic, cycloaliphatic, aromatic, halogenated, or heterocyclic hydrocarbons.

17. The method of claim 16 in which said organic solvent is selected from the group consisting of chloroform, toluene, xylene, hexane, methylene chloride, pentane, acetone, or DMSO.

18. The method of claim 1 in which said at least two fluorescent dyes have substantially similar solubility characteristics in the organic solvent of said solvent mixture.

19. The method of claim 1 in which said alcoholic solvent is selected from the group consisting of 2-propanol, methanol, ethanol, isopropanol, butanol, or pentanol.

20. A population of polymeric microspheres substantially uniformly stained with at least two fluorescent dyes, each microsphere of said population upon excitation exhibiting at least two distinct fluorescence emission signals corresponding to the at least two fluorescent dyes, the intensity of each of said at least two emitted signals (i) being proportional to the amount of its corresponding dye in said microsphere, and (ii) exhibiting a coefficient of variation among all the members of said population, which is no greater than about 20 percent.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1769

21. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is no greater than about 15 percent.

22. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is no greater than about 10 percent.

23. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is no greater than about 8 percent.

24. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is less than about 8 percent.

25. The population of claim 20 in which the at least two fluorescent dyes are hydrophobic.

26. The population of claim 25 in which the at least two fluorescent dyes comprise squaric acid-based dyes.

27. The population of claim 26 in which said squaric acid-based dyes are selected from cyclobutenedione derivatives, symmetrical and unsymmetrical squaraines, substituted cephalosporin compounds, fluorinated squaraine compositions, alkylalkoxy squaraines, or squarylium compounds.

28. The population of claim 26 in which said squaric acid-based dyes are selected

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1770

from a red fluorescent dye and an orange fluorescent dye.

29. The population of claim 28 in which the red fluorescent dye comprises 1,3-bis[(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxycyclobutenediyliu m, bis(inner salt) and said orange fluorescent dye comprises 2-(3,5-dimethylpyrrol-2-yl)-4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one.

30. The population of claim 20 in which said microspheres comprise polystyrene, brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyacrylamide, polyacrolein, polydimethylsiloxane, polybutadiene, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polyglycidylmethacrylate, polymethylmethacrylate, or copolymers, blends, composites, or combination thereof.

31. The population of claim 20 in which said microspheres further comprise at least one analytical reactant bound covalently to functional groups present on the surface of said microspheres or passively adsorbed to the surface of said microspheres.

32. The population of claim 20 in which said microspheres have a diameter between about 10 nm and 100 μm.

33. A collection of distinct populations of polymeric microspheres according to claim 20, each population exhibiting an emission spectrum in a Fluorescence Bead Map, which is unique to said population.

34. The collection of claim 33 which comprises eight or more distinct populations of polymeric microspheres.

35. The collection of claim 33 which comprises sixteen or more distinct populations

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1771

of polymeric microspheres.

36. The collection of claim 33 which comprises twenty-four or more distinct populations of polymeric microspheres.

37. The collection of claim 33 which comprises thirty-two or more distinct populations of polymeric microspheres.

38. The collection of claim 33 which comprises sixty-four or more distinct populations of polymeric microspheres.

39. The collection of claim 38 in which there is substantially no overlap between any of the sixty-four or more emission spectra associated with said sixty-four or more distinct populations of polymeric microspheres.

40. A method of detecting a plurality of analytes in a sample, each of said analytes being recognized by a corresponding analytical reactant, comprising:

(a) contacting said sample with a plurality of populations of uniformly stained microspheres, said microspheres having at least two fluorescent dyes uniformly mixed at a specific ratio within each microsphere of each said population, each population of said microspheres having a distinct analytical reactant bound to its surface, wherein, said reactant on each population of microspheres specifically interacts with one of said analytes in said sample;

(b) providing a label reagent that specifically binds to the analyte and analyzing the microspheres to detect said label indicating binding of the analyte to the analytical reactant; and

(c) determining the populations of microspheres having said fluorescent dyes mixed at the specific ratio within microspheres of each population to which the reactant is bound.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1772

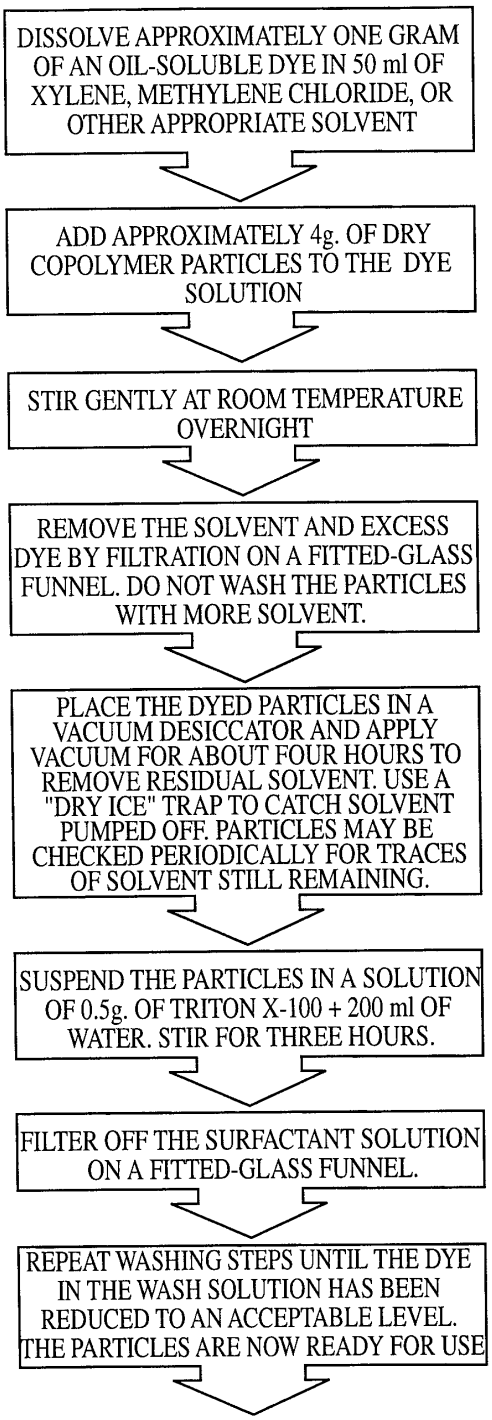


FIG. 1

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1773

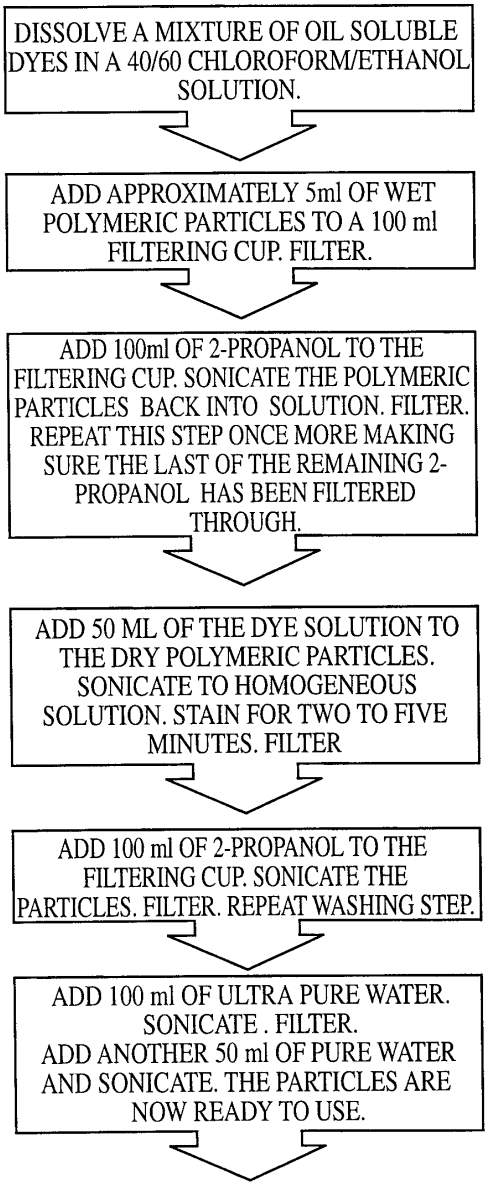


FIG. 2

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1774

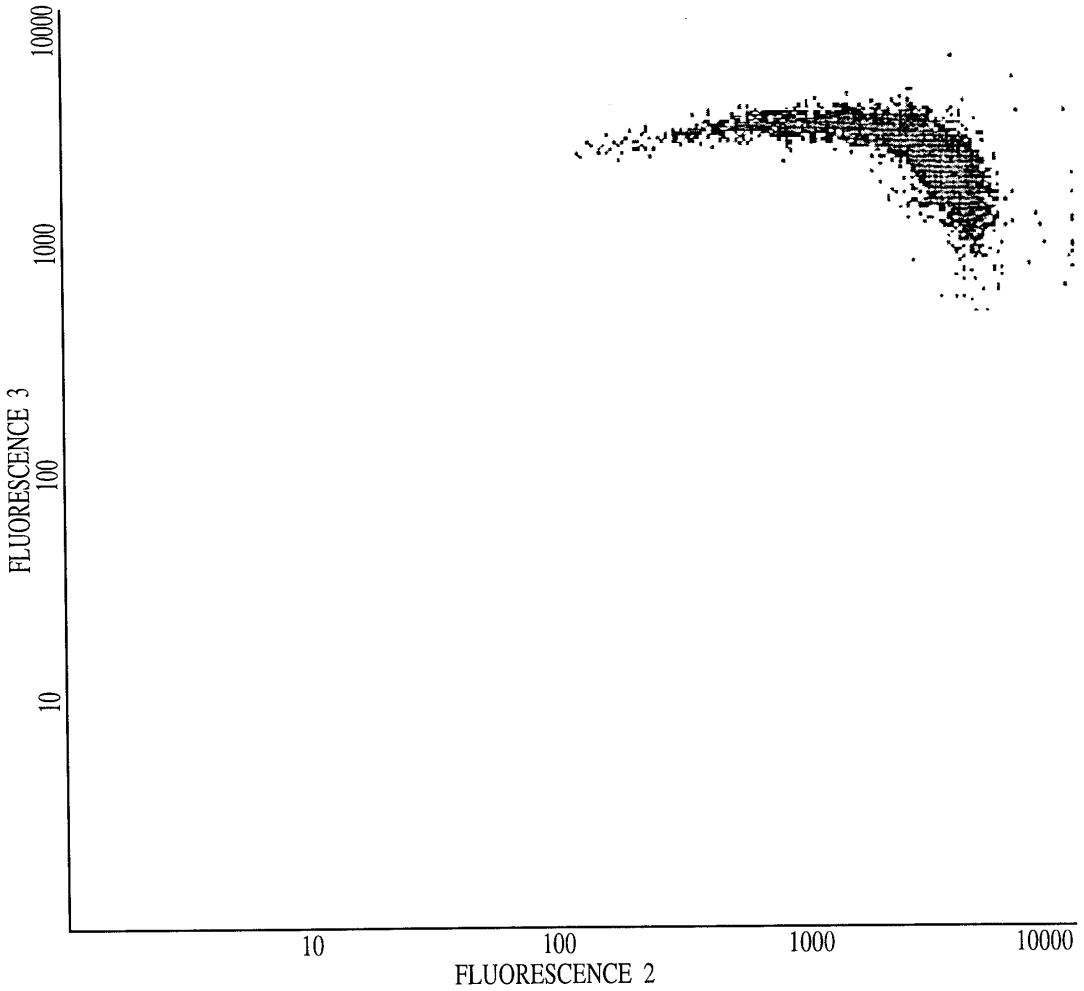


FIG. 3

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1775

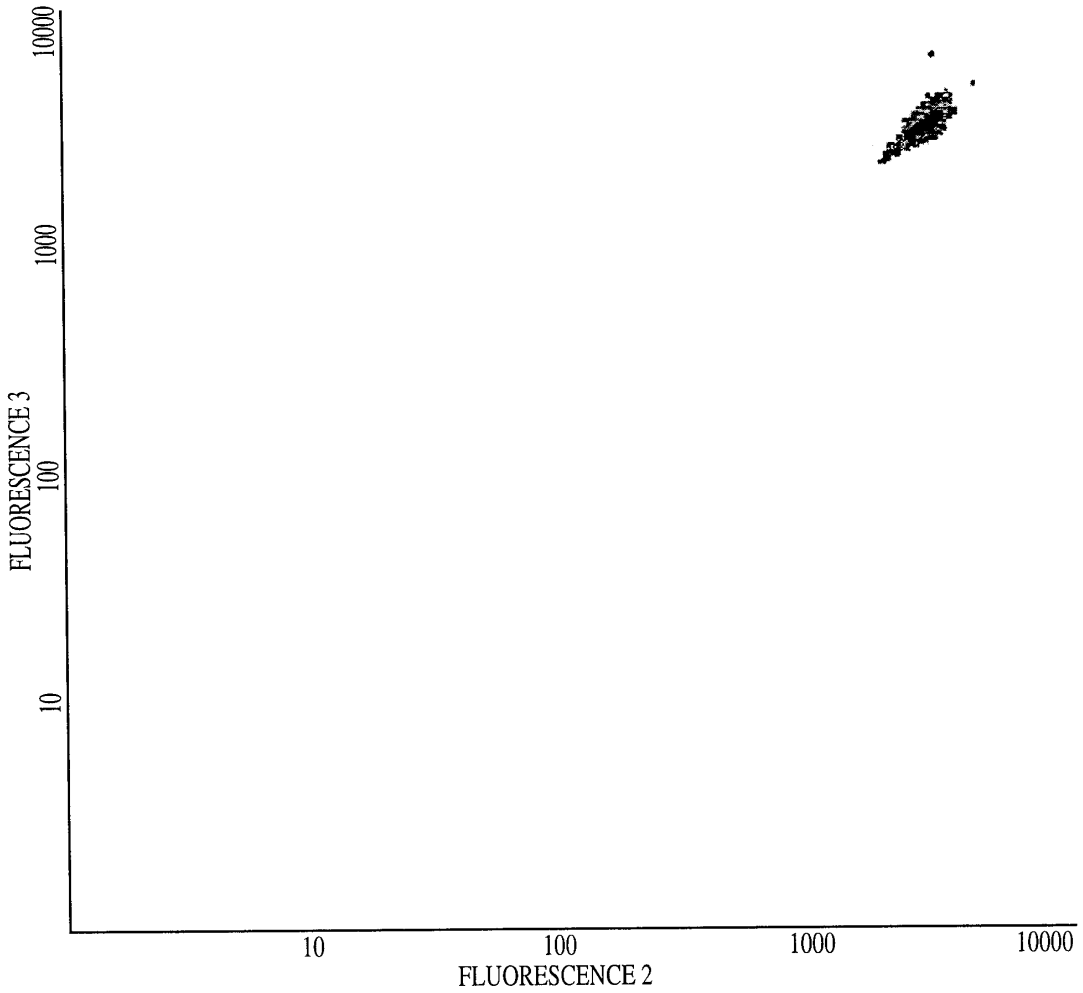


FIG. 4

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1776

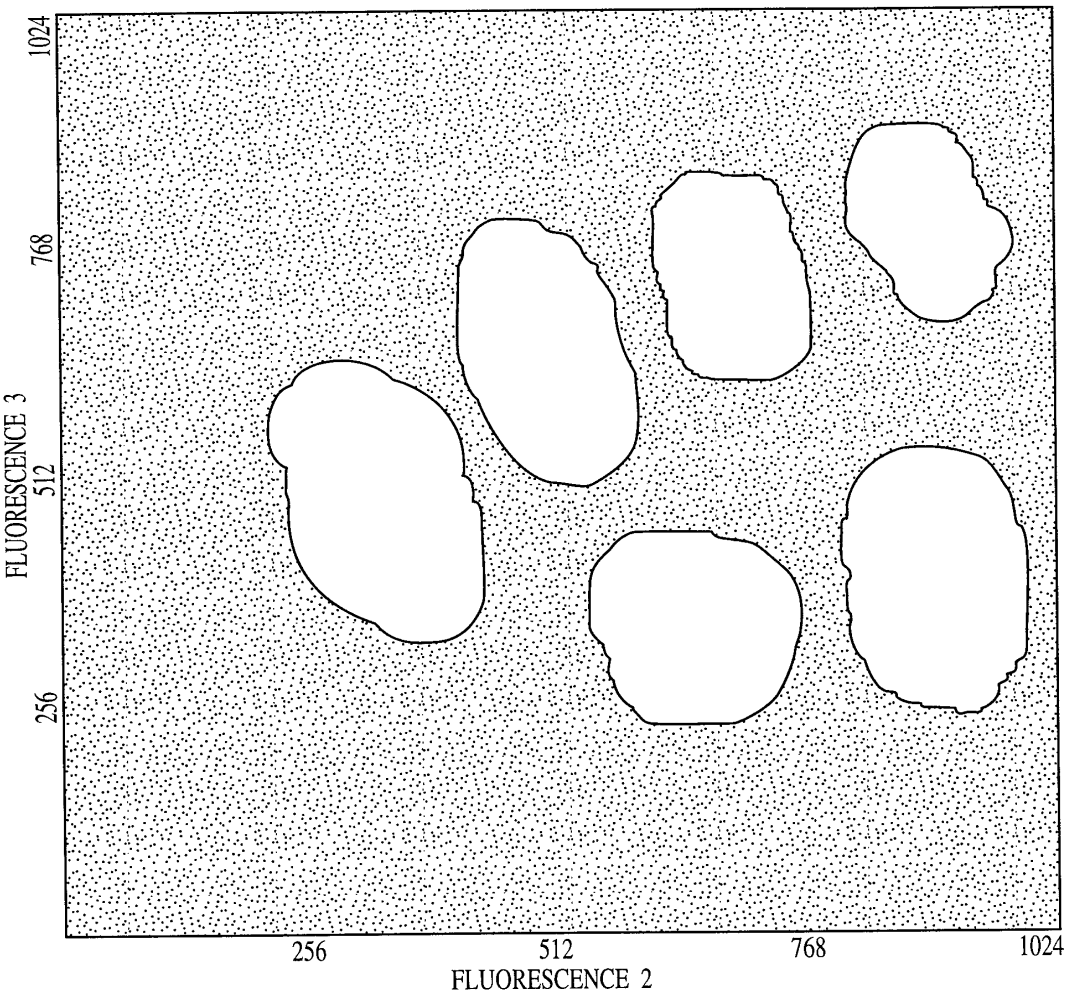


FIG. 5

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1777

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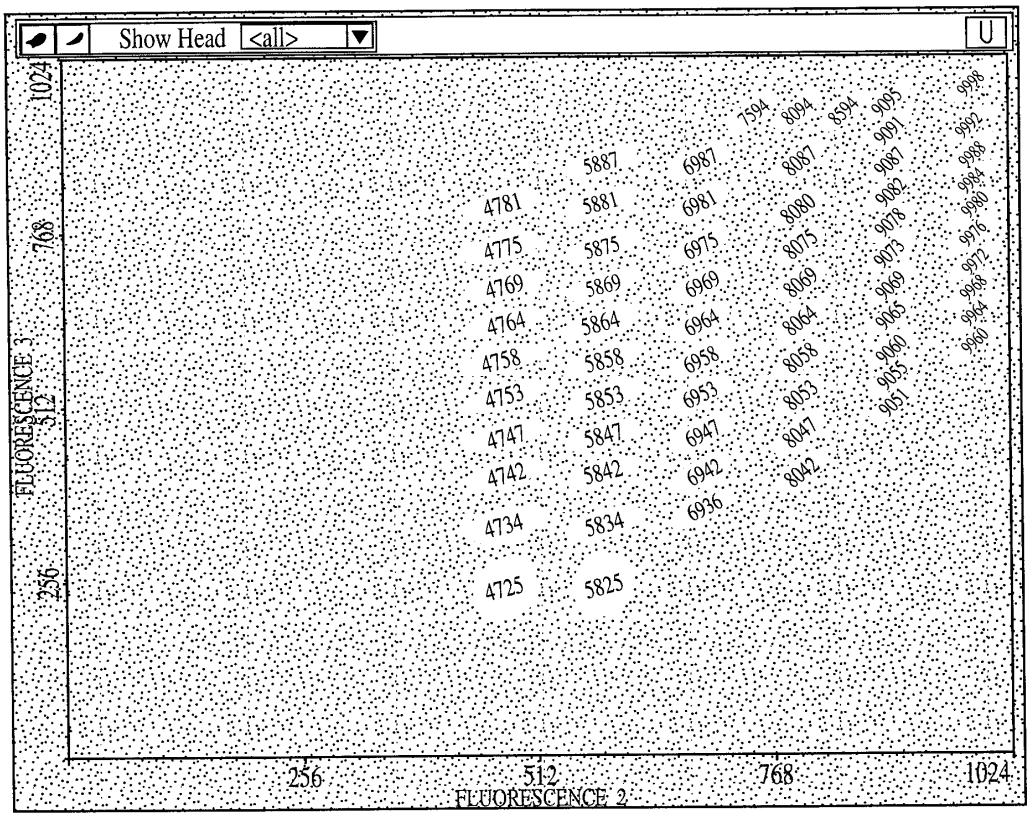


FIG. 6

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1778

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21562

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; D06P 1/90; B32B
US CL :435/6; 428/407; 8/506, 607, 611, 614, 616, 617, 638, 648
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 428/407; 8/506, 607, 611, 614, 616, 617, 638, 648

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS REG: SQUARIC ACID; SQUARAINE
APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,573,909 A (SINGER et al.) 12 November 1996 (12-11-96).	20-40
X	US 4,717,655 A (FULWYLER) 05 January 1988 (05-01-88), see examples and claims.	20-25, 30-40
A	US 3,790,492 A (FULWYLER) 05 February 1974) (05-02-74).	20-25, 30-40
P,X	US 5,723,218 A (HAUGLAND ET AL.) 03 March 1998 (03-03-98), see col 10, lines 37-62.	20-25
X	US 5,073,498 A (SCHWARTZ et al.) 17 December 1991 (17-12-91), col 4, lines 54-66 and examples.	20-25, 30-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search 14 DECEMBER 1998	Date of mailing of the international search report 20 JAN 1999
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21562

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,326,692 A (BRINKLEY et al.) 05 July 1994	all
$\overline{X}$	Example 6 in col 21 and 22	1-8,11-13 16-25 30-32

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★

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Published
With international search report.
With amended claims.

(54) Title: LIQUID LABELING WITH FLUORESCENT MICROPARTICLES

(57) Abstract

Methods of labeling liquids, solids, aerosols, and gases with fluorescent microparticles and/or with microparticles encoded with at least one other discriminator. Labeling protocols are described for such uses as adding at least one date code (for example, the expiration date of a perishable liquid), encoding routing information for one or more liquids in a pipeline having a plurality of branch points, tracing the flow of toxic or other substances into groundwater, tracing liquid flow from a point source into groundwater, tracing groundwater flow, adding a donor Social Security Number, adding a donor name, tracing flow of a liquid or a gas in a living system, adding unique identification to each of a plurality of liquid, solid or gaseous samples, encoding routing information to each of a plurality of liquid samples in a multiplexed analytical system, encoding routing information to each of a plurality of liquid reagents in a multiplexed analytical system, and combinations thereof. In one embodiment, two different fluorescent dye labels are bound to microparticles at eight independent, different concentrations each to create an 8 x 8 = 64 array of distinct microparticle sets suitable for labeling liquids, solids, and gases. When three discriminators are used at 8 levels each, an 8 x 8 x 8 = 512 array of distinct microparticle sets is created. Discriminators include such parameters as particle physical property, particle concentration, spectral property, dye type, dye concentration, label type, and combinations thereof; where particle physical property includes such attributes as particle size, particle shape, particle hydrophobicity, particle hydrophilicity, particle density, and combinations thereof; where spectral property includes such attributes as dye fluroescence absorption, dye fluorescence emission, dye absorption, dye fluorescence polarization, dye fluorescence lifetime, and combinations thereof; and where label type includes such attributes as magnetic property, reactive group, nuclear magnetic resonance, electron spin resonance, positron emission, radioactive property, fluorescence polarization, fluorescence lifetime, and combinations thereof. Kits are devised to permit users to create a large variety of sets of particles for identification purposes in liquids, solids, and gases, and microprocessor controlled, automatic pipetting of kit microparticles are also encompassed by the instant invention.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1781

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LIQUID LABELING WITH FLUORESCENT MICROPARTICLES

1.0 Cross Reference to Related Applications

This Application claims the priority date of Provisional U.S. Application Serial No. 60/081,487, filed April 13, 1998, the disclosure of which is herein incorporated by reference in its entirety.

2.0 Field of the Invention

The present invention relates generally to a method of labeling liquids. In particular, the present invention relates to a method of labeling liquids with particles, and to flow cytometric amenable methods of encoding such labels in liquids. Such particles and methods of their use also have applications in labeling solids and gases.

3.0 Background of the Invention

Knowing the correct identity of different liquids is critical in a variety of contexts. For example, the absolute identity of different grades of aviation fuel is required for optimum performance and safety in airplanes and other aircraft. Typically, different grades of aviation fuel are identified according to color in the visual spectrum, based on dyes added exogenously.

Fluids required by automobiles, other vehicles, motorized tools and equipment in general provide additional examples of the need for correct identification of liquids. For such motorized mechanical devices, fluids therefor are colored according to the mechanical compartment for which use is intended. Such color coding clearly is designed to prevent, or at least minimize the possibility of, damage or even total destruction of the mechanical device that would result from introduction of the incorrect fluid into a given mechanical compartment. Thus, the color of the antifreeze solution is different from that of the brake fluid, etc.

In addition, as a part of a quality assurance or quality control program, there often is a need to verify the identities of various biological and other fluids that are undergoing analysis. This is particularly true in situations where integrity and identity of a sample is critical, for example, in legal situations such as court ordered testing. Examples of biological fluids that could benefit from labeling include separated or unfiltered biological fluids such as urine, cerebrospinal fluid, blood, serum, plasma, lymph fluids, tissue homogenates, interstitial fluid, cell extracts, mucus, saliva, sputum, stool, semen, vaginal secretions, lacrimal fluid, physiological secretions, or other similar

fluids. Examples can also include samples obtained from environmental sources, such as soil, water, or air; or from industrial sources, such as those taken from a waste stream, a water source, a supply line, or a production lot. Industrial sources can include such fluids as fermentation media, for example, from a biological reactor or food fermentation process, or fluids generated from foodstuffs, such as from meat, grain, produce, or dairy products.

Liquids are notoriously difficult to label and thus identify. The use of visible spectrum color indicators for fluid identification suffer from at least two inherent disadvantages: 1) a limited range of separately identified liquids due to the limited range of colors that are readily distinct to the human eye, and 2) ease of alteration and/or sabotage of the liquid for which the easy, visual based identification is sought. In addition, a significant portion of the human population suffers from color blindness, thereby increasing problems of identification related to the use of visible spectrum color indicators. This difficulty is further compounded because clinically distinct forms of color blindness have different wavelength ranges over which color perception is absent or impaired.

In addition to the perception of color, viscosity, odor, taste, and "feel" can also provide useful identification parameters. However, not only are these alternate sensory modes of identification typically impractical, or even dangerous to perform, they generally give ambiguous and inconclusive results.

A need therefore exists for a means to label liquids which is precise, tamper proof, reproducible, and easily read.

Fluorescent dyes and lasers have been used for a number of years to label and detect cells, biochemical interactions, and uniform latex particles. For such uses, laser techniques often are utilized to determine the presence or absence of a dyed constituent, and/or concentrations thereof. Fluorescent dyes have been applied to such uses as: (1) detecting the presence (and quantity) or absence of cells and/or specific types of cells in a fluid sample; (2) gauging cell vitality; (3) estimating pH and/or changes in pH in cellular compartments; (4) determining intracellular pH measurement in general; (5) identifying acidic vesicles in cells; (6) identifying DNA; (7) identifying "GC" rich or "AT" rich regions of DNA; (8) identifying cellular surface markers; (9) quantifying analytes by ELISA plate assays; (10) estimating blood flow; (11) labeling proteins for gel electrophoresis analysis; (12) labeling cellular cytoskeleton or other intracellular proteins; (13) identifying hydrophobic and hydrophilic sites on proteins and membranes; (14) measuring total amounts of protein in a sample; (15) measuring intra-molecular distances by energy transfer; (16) performing optical sectioning with confocal microscopy; (17) measuring and studying various molecular and physical chemical properties and biochemical parameters, including enzyme-substrate interactions, as well as interactions between enzymes and various modulators (activators, inhibitors,

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1784

cofactors, etc.) or other molecules, including interactions with homologous and heterologous protein subunits, and with antibodies; and (18) detecting the presence of, absence of, and/or number of, uniform dyed particles in a liquid sample.

According to the practice of earlier technologies, particles often have been labeled with a single fluorescent dye. In general, these particles are made by a copolymerization process, wherein monomers, such as unsaturated aldehyde or acrylate, are allowed to polymerize in the presence of a fluorescent dye, such as fluorescein isothiocyanate or "FITC," in the reaction mixture. For example, see U.S. Patent Nos. 4,267,234 to Rembaum, 4,267,235 to Rembaum, et al., 4,552,812 to Margel, et al. and 4,677,138 to Margel.

The use of two or more dyes of varying proportions would increase the permutation number of unique combinations of dyes in or on a single particle. Particles having varying combinations of unique characteristics, such as differences in emission wavelengths and/or fluorescence intensities, could be extremely useful for labeling liquids.

However, several problems arise when more than one dye is absorbed into or onto a particle. First, the close proximity of embedded dye molecules gives rise to significant amounts of fluorescent energy transfer (for example, Stokes shifts). This energy transfer leads to fluorescent emissions that are inconsistent with relative dye concentrations and their original emission patterns.

Second, difficulties arise when the dyes have differing solubilities in the solvent used to incorporate the dye in or on the particles. Typically, all dyes must be absorbed simultaneously; otherwise, in practice, possible dye ratios are restricted by solvent properties.

A third problem that has been encountered when multiple dyes are embedded in particles is the change in dye spectra. Specifically, it has been noted that, when the particle is composed of crosslinked polystyrene, a significant broadening of the fluorescent emission peak occurs. This can result in an overlapping of the emission spectra of neighboring dyes.

A need therefore exists for multicolored, fluorescent particles comprising a plurality of defined sets of stained multicolored microparticles, and/or a plurality of defined sets of dyed nanoparticles attached to a carrier microparticle, which can be used for labeling liquids. It would also be desirable to use such microparticles or carrier microparticles in conjunction with other labeling elements ("discriminators"), such as radioactive property, nuclear magnetic resonance, electron spin resonance, chemical reactive group, dye fluorescence absorption, dye fluorescence emission, fluorescence polarization, fluorescence lifetime, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, particle density, particle size, particle shape, dye concentration in or on particles, and dye type. Applications of this invention include, but are not limited to: (1) adding date codes to perishable or other liquids; (2) adding routing

information to liquids in a pipeline; (3) tracing toxic substances in groundwater; (4) adding donor's Social Security Number to blood units; (5) tracing absorption and/or secretion and excretion patterns of fluids into, out of, or within plant and animal tissues; (6) adding patient identification to clinical samples; (7) adding identifiers to clinical reagents; and (8) adding routing information to liquids in a process stream, for example, a plurality of liquid samples from one or more patients or other sources of samples, where the liquid samples are each routed to an analytical machine for a specific assay, or to an analytical machine for a panel of assays.

4.0 Summary of the Invention

It is therefore an object of the present invention to provide methods, instrumentation, and products for precisely and unambiguously labeling fluids (liquids, aerosols, and gases), and solids.

It is a further object of the present invention to provide methods, instrumentation, and products for precisely and unambiguously reading the labels within the fluids (liquids, aerosols, and gases), and solids.

The present invention uses precisely dyed microparticles and/or nanoparticles attached to carrier microparticles, and also may utilize a variety of other discriminators. In a preferred embodiment, the particles are made of polystyrene, crosslinked with 5 percent divinyl-benzene, and have a diameter of about 5.5 microns. The particles are impregnated with three different fluorescent dyes in eight different concentrations of each of the dyes, so as to create 512 subsets of labeled particles. The subset classification of each particle is identified by fluorescence measurement using appropriate instrumentation, such as a flow cytometer. Depending upon the needs of a particular application, the discriminators can be combined in a variety of ways to achieve the required number of unique labels. A first embodiment uses precisely dyed microparticles (core microparticles). In an alternative embodiment, precisely dyed nanoparticles are attached to carrier microparticles. Other embodiments incorporate such discriminators as radioactive property, nuclear magnetic resonance, electron spin resonance, chemical reactive group, dye fluorescence absorption, dye fluorescence emission, fluorescence polarization, fluorescence lifetime, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, particle density, particle size, particle shape, dye concentration in or on particles, and dye type. Thus, use of microparticles according to the present invention functions as an identifying means analogous to a bar code.

Multiplexed diagnostic instruments, such as those in the current art (including flow cytometers), can be programmed to recognize each specific permutation or particle set, look up the parameters in a database, and identify the sample's origin/destination and/or perform the appropriate analysis or routing function.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1786

The present invention provides a method of labeling a gaseous, liquid, or solid material, including combinations thereof, to permit the subsequent identification of the labeled gaseous, liquid, or solid material, which comprises adding to a gaseous, liquid, or solid material, whose subsequent identification is desired, a sufficient quantity of particles belonging to one or more distinguishable sets of particles, to provide a labeled gaseous, liquid, or solid material that subsequently can be identified. In a particular embodiment of the invention, the method makes use of particles in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set. In particular, the one or more discriminators is selected from a physical, chemical, biological, magnetic, or spectral property of the particle or combinations thereof. Specific properties are described further in the detailed description of the preferred embodiments of the invention and others may be apparent to those of ordinary skill in the art.

In the present method, for example, a sufficient quantity of a combination of particles belonging to two or more distinguishable sets of particles is added to the gaseous, liquid, or solid material, whose subsequent identification is desired.

The present invention also provides a method of identifying a gaseous, liquid, or solid material, including combinations thereof, which has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles. This method comprises: (a) obtaining a sample comprising a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles; (b) analyzing the particles found in the sample to determine which of one or more distinguishable sets of particles such particles belong to; and (c) comparing the results of the analyzing step with a key which establishes the identity of the gaseous, liquid, or solid material from which the sample was taken.

In various embodiments, the present invention makes possible the manufacture, use and sale of labeled materials which comprise (i) a gaseous, liquid, or solid material, including combinations thereof, and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles. In a preferred embodiment the labeled gaseous, liquid or solid material makes use of particles in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set. Kits for labeling liquids, solids, aerosols, and gases comprise particles from a plurality of distinct particle sets, and a dispenser.

6.0 Description of the Specific Embodiments

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1787

One method of practice of the present invention involves the use of precisely dyed core microparticles. An alternative method of practice entails the use of carrier microparticles to which nanoparticles comprising a plurality of dyes with spectrally distinct fluorescence emission spectra are attached, preferably covalently. The following summarizes exemplary but non-limiting embodiments for the production of such labeled particles suitable for use in practice of the present invention, as well as how to use such particles to label liquids, and subsequently identify such labels, each of which may be used to uniquely identify a specific liquid. As used herein: (1) the terms nanospheres and nanoparticles have equivalent meanings; (2) in the context of incorporating labels (fluorescent and otherwise) into or onto particles, the terms attach, attachment, bound, coupled, etc., are used broadly to include covalent and non-covalent incorporation of chemicals and dyes, particles, etc., as well as entrapment of such entities within the matrix of particles ("matrix entrapment"), for example, subsequent to the swelling of a particle; (3) the terms microparticles, microspheres, or microbeads bear equivalent meanings; (4) particles refers to either microparticles or nanoparticles; (5) where nanoparticles are attached to microparticles, the microparticles are referred to as carrier microparticles; and (6) where microparticles are precisely dyed, the microparticles are referred to as core microparticles.

6.1 Particles

In one embodiment, nanoparticles are coupled to carrier microparticles to form sets of distinct particles. Basically, fluorescently distinguishable particle sets are obtained through variation of the amount or type of dye in or on each nanoparticle. Then, the desired number of dye categories of nanoparticles are attached to the surface of each carrier microparticle. Ratios of differently dyed nanoparticles attached to the surfaces of the carrier microparticles are selected according to the particular need, and in accord with the encoding or identification criteria developed therefor. These ratios reflect differences in the dyes used, and in their respective quantities attached to each particle.

Similarly, and in an alternative embodiment, fluorescently distinguishable microparticle sets are obtained through variation of the amount or type of dye in or on each microparticle, and no nanoparticles are utilized. The encoding or identification criteria can be identical to those used with nanoparticles.

Nanoparticles used in this invention are commercially available in sizes ranging from about 5 to 10 nanometers (nm) to more than 1,000 nm in diameter. Optimal diameters typically will fall within the range from about 10 to about 1,000 nm, preferably from about 200 to about 500 nm. Nanoparticles as small as about 2-3 nm can also be prepared.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1788

Microparticles that are used as carrier particles, to which the nanoparticles are attached, typically range in diameter from about 0.1 to about 100 micrometers (μm). Even though a microparticle for practicing the present invention can be of any size, the preferred diameter usually will range from about 0.1 to about 50 μm, more preferably from about 1 to about 20 μm, and even more preferably from about 2 to about 12 μm. The particles preferably will exhibit a high degree of uniformity with respect to size, or to a set of discretely different size classes such that 1) the differences can be determined by size-dependent properties, such as light scattering, and 2) the high degree of uniformity within each class size is sufficient to permit the use of particle size as a discriminator.

Microparticles used in this invention as core particles have a diameter of less than one millimeter, and preferably in the range of about 0.1 to 1,000 micrometers (μm). Even though the microparticle can be of any size, the preferred size is 1-100 μm, more preferably 2-50 μm, more preferably 3-25 μm, and even more preferably about 5-12 μm.

Particles are made of any approximately regularly shaped material. Often, the preferred shape will be approximately spherical; however, particles of any other shape can be employed since, for most uses, this parameter will be immaterial to practice of the instant invention. However, the shape of a class of particles also can serve as an additional discriminator, which can be detected by a flow cytometric method, for example, by a high-resolution slit-scanning method that measures forward and side light scattering parameters.

Usually the nanoparticles and the microparticles are made of the same material, such as polystyrene or latex. However, other polymeric materials are acceptable, including polymers selected from chemical groups such as carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers, block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched polymers, cyclo-polymers, polyaldehydes, and mixtures thereof. More specifically, brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyamide, polyacrylamide, polyacrolein, polybutadiene, polycaprolactone, polyester, polyethylene, polyethylene terephthalate, polydimethylsiloxane, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polymethylmethacrylate, polylactide, polyglycolide, poly(lactide-co-glycolide), polyanhydride, polyorthoester, polyphosphazene, or combinations thereof, are preferable. Representative combination polymers of which the polymeric particles can be composed include, for example, poly-(styrene-co-vinylbenzyl chloride-co-acrylic acid) (in approximately an 85:10:5 molar ratio), poly(styrene-co-acrylic acid) (in approximately a 99:1 molar ratio),

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1789

poly(styrene-co-methacrylic acid) (in approximately a 90:10 molar ratio), poly(styrene-co-acrylic acid-co-m&p-divinyl-benzene) (in approximately an 89:10:1 molar ratio), poly-(styrene-co-2-carboxyethyl acrylate) (in approximately a 90:10 molar ratio), poly(methyl methacrylate-co-acrylic acid) (in approximately a 70:30 molar ratio) and poly(styrene-co-butyl acrylate-co-methacrylic acid)(in approximately a 45:45:10 weight ratio). Other exemplary polymers that can be used for the formation of microparticles include homopolymers and copolymers of lactic acid and glycolic acid (PLGA) (for example, as described in U.S. Patent Nos. 5,213,812 to Ruiz; 5,417,986 to Reid, et al.; 4,530,840, 4,897,268, 5,075,109, and 5,360,610 to Tice, et al.; 5,102,872 to Singh, et al.; 5,384,133 to Boyes, et al.; and European Patent Application Publication Number 248,531 to Southern Research Institute); block copolymers, such as tetronic 908 and poloxamer 407 (for example, as described in U.S. Patent No. 4,904,479 to Illum); and polyphosphazenes (for example, as described in U.S. Patent No. 5,149,543 to Cohen, et al.). Particles formed from synthetic polymers (such as polystyrene, polyacrylamide, polyacrylate, or latex) are commercially available from numerous sources, such as Bio-Rad Laboratories (Richmond, Calif.) and LKB Produkter (Stockholm, Sweden). Particles formed from natural macromolecules and particles, such as agarose, crosslinked agarose, globulin, deoxyribose nucleic acid, and liposomes, are commercially available from a variety of sources, such as Bio-Rad Laboratories, Pharmacia (Piscataway, N.J.), and IBF (France). Particles formed from copolymers of polyacrylamide and agarose are commercially available from various sources, including IBF and Pharmacia. Magnetic particles are commercially available from such sources as Dynal Inc. (Great Neck, N.Y.). Other materials, such as carbohydrates (e.g., carboxymethyl cellulose, and hydroxyethyl cellulose), proteinaceous polymers, polypeptides, eukaryotic and procaryotic cells, viruses, lipids, metals, resins, rubbers, silicas, and silicones (e.g., polydimethyldiphenyl siloxane, glass, ceramic, and the like), also can be utilized, depending on the specific use therefor.

Nanoparticles are preferably made of the same material as the microparticles. However, if required or advantageous, they can be made of different material.

The particles typically will also contain approximately 1% to approximately 50% by weight of one or more cross-linking agents, such as divinyl benzene, ethylene glycol dimethacrylate, trimethylol propane trimethacrylate, or N,N'methylene-bis-acrylamide, or other functionally equivalent agent(s) known in the art. Crosslinking of a carbohydrate polymer, such as hydroxypropyl cellulose, can be achieved with such agents as adipic acid, sebacic acid, succinic acid, citric acid, 1,2,3,4-butanetetracarboxylic acid, or 1,10-decanedicarboxylic acid. In a preferred embodiment, microparticles and nanoparticles are made of polystyrene, and contain about 1% to about 50% by weight of divinyl benzene.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1790

The particles also can have additional surface functional groups to facilitate attachment to each other or to yet another entity, examples including carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, and halides. For many practical applications, it is critical that the particles have surface carboxyl groups available for attachment of a reactive amine- or sulfhydryl-containing compound, for example. Such groups are preferably added to the particles by incorporating monomers containing such groups into the polymers. These monomers can include, but are not limited to, acrylic acid, methacrylic acid, itaconic acid, and the like. Alternatively, they can be added to the particles by further chemical reaction of a polymer having other precursor reactive groups, which then can be converted to carboxyl groups, for example, by hydrolysis of anhydrides, such as maleic anhydride, or by oxidation of surface methylol or aldehyde end groups. Other compounds, such as diamines, dihydrazides, mercaptoalkylamines and dimercaptans, can be attached to particles as linking moieties for later attachment of drugs, enzymes or other reactive species, including nanoparticles. Although the preferred attaching method is by covalent linkage, non-covalent binding methods, such as adsorption, can also be used.

6.2 Dyes

A dye is selected to give a detectable response that is different from that of other dyes and /or discriminators. Any fluorescence detection system (including visual inspection) can be used to detect differences in spectral properties between dyes, with differing levels of sensitivity. Such differences include, but are not limited to, differences in excitation maxima, emission maxima, fluorescence lifetimes, fluorescence emission intensity at the same excitation wavelength or at a different wavelength, absorptivity, fluorescence polarization, or combinations thereof. Thus, the detectably different dye has different spectral properties and/or different selectivity.

In one aspect of the invention, the two or more dyes forming the dye-microparticle complex have the same or overlapping excitation spectra, but possess distinctly different emission spectra, generally having emission maxima separated by greater than 10 nm, preferably greater than 20 nm, and more preferably greater than 50 nm. Simultaneous excitation of all fluorescent reagents may require excitation of the sample at a wavelength that is suboptimal for each reagent individually, but optimal for the combination of reagents. Alternatively, the additional reagent(s) can be simultaneously or sequentially excited at a wavelength that is different from that used to excite the subject dye.

A preferred category of fluorescent dyes used in this invention are of the general class known as polymethine cyanine dyes, with typical emission wavelengths falling between 550 nm and 900 nm. Spectral properties, including emission wavelengths, are strongly influenced by the number of

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1791

methine groups. Thus, the monomethine dyes that are pyridines typically have blue to blue-green fluorescence emissions, whereas those that are quinolines typically have green to yellow-green fluorescence emissions. On the other hand, the fluorescence emissions of the trimethine dye analogs are substantially shifted toward red wavelengths, and those of pentamethine dyes are shifted even further, often exhibiting infrared fluorescence emissions (for example, see U. S. Patent No. 5,760,201). Although polymethine cyanine dyes constitute a preferred category of dyes for practice of the present invention, any dye that is soluble in an organic solvent (i.e., hydrophobic dyes) can be used when organic liquids are to be labeled.

On the other hand, when predominantly aqueous liquids are to be labeled, the above-indicated hydrophobic dyes, including the hydrophobic particles to which they are attached, may be used by subsequently or previously attaching hydrophilic groups to increase the solubility in aqueous solutions. In addition, ranges of hydrophilicity and hydrophobicity for liquids (i.e., different proportional mixtures thereof) are contemplated for mixed solvents, such as oil-in-water, water-in-oil, micelles, mixed micelles, detergent solutions, etc.

Squaric acid based fluorescent dyes can be synthesized by methods described in the literature; for example, see Sprenger, et al. *Angew. Chem.*, 79, 581 (1967); *Angew. Chem.*, 80, 541 (1968); and Maaks, et al. *Angew. Chem. Int'l. Ed.*, 5, 888 (1966). Briefly, one equivalent of squaric acid (1,2-dihydroxycyclobutenedione) is condensed with two equivalents of an active compound, such as a pyrrole, indoline, or aniline, and refluxed in a mixture of an alcohol and an aromatic solvent (such as benzene) under conditions that allow removal of water from the reaction mixture. The resulting dye can be collected and purified by any of such standard methods as recrystallization, distillation, chromatography, etc. Additionally, unsymmetrically substituted squaric acid compounds can be synthesized by methods such as those described by Law, et al., *J. Org. Chem.*, 57, 3278, (1992). Specific methods of making such dyes are well known in the art, and can be found, for example, in U.S. Patent Nos. 5,795,981; 5,656,750; 5,492,795; 4,677,045; 5,237,498; and 5,354,873. Some practical uses of such fluorescent dyes, for example, phthalocyanines, 2,3-naphthalocyanines, squaraines and croconic acid derivatives, are disclosed in U.S. Patent No. 5,525,516 to Krutak, et al.

Optionally, such dyes will contain functional groups capable of forming a stable fluorescent product with functional groups typically found in biomolecules or polymers including, for example, activated esters, isothiocyanates, amines, hydrazines, halides, acids, azides, maleimides, alcohols, acrylamides, haloacetamides, phenols, thiols, acids, aldehydes and ketones.

In addition to the specific squaric acid fluorescent dyes that are used in this preferred embodiment, related dyes can be further selected from such classes of compounds as cyclobutenedione derivatives, substituted cephalosporin compounds, fluorinated squaraine

compositions, symmetrical and unsymmetrical squaraines, alkylalkoxy squaraines, and squarylium compounds. Some of these dyes can fluoresce at near infrared as well as at infrared wavelengths -- effectively expanding the range of emission spectra up to about 1,000 nm. In addition to squaraines (i.e., derivatives of squaric acid), hydrophobic dyes, such as phthalocyanines and naphthalocyanines, also can be selected when operation at longer wavelengths is advantageous. Other classes of fluorochromes are equally suitable for use as dyes according to the present invention. Non-limiting examples of these dyes include 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine (5-HT), Acid Fuchsin, Acridine Orange, Acridine Red, Acridine Yellow, Acriflavin, AFA (Acriflavin Feulgen SITSA), Alizarin Complexon, Alizarin Red, Allophycocyanin, ACMA, Aminoactinomycin D, Aminocoumarin, Anthroyl Stearate, Aryl- or Heteroaryl-substituted Polyolefin, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9 (Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, BOBO 1, Blancophor FFG Solution, Blancophor SV, Bodipy Fl, BOPRO 1, Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbocyanine, Carbostyryl, Cascade Blue, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphthyl Sulphonic Acid), Dansyl NH-CH3, DAPI, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrrometheneboron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Eosin, Erythrosin ITC, Ethidium Bromide, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Hoechst 33258, Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nile Red, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oregon Green, Oxazine, Oxazole, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Propidium Iodide, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Rose Bengal, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS

(Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulphO Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Texas Red, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, TOTO 1, TOTO 3, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, XRITC, YO PRO 1, or combinations thereof. One skilled in the art will readily know which one to select among such dyes, in part based on the emission, absorption, and hydrophobic/hydrophilic properties required for a particular application. The spectral properties of the fluorescent dyes often will be sufficiently similar in excitation wavelengths and intensity to fluorescein or rhodamine derivatives to permit the use of the same flow cytometry equipment. However, generally it is preferable that a dye have high solubility in the particular liquid that is to be "marked," and improved photostability and quantum yields also are advantageous.

More preferably, the multiple dyes used in a single application or in a related series of applications will have the same or overlapping excitation spectra, but possess distinguishable emission spectra ("spectrally distinct"). Any detection system can be used to detect differences in spectral characteristics between two or more dyes, including a solid state detector, photomultiplier tube, photographic film, or eye -- any of which can be used in conjunction with additional instrumentation or methodology, such as a spectrometer, luminometer microscope, plate reader, fluorescence scanner, flow cytometer, confocal microscope, scanning microscope, epifluoresis detector, digital (CCD) camera, video camera, photographic film, visual inspection, photodiode, quantum counter, photomultiplier tube, capillary electrophoresis detector, or any combination thereof, to complete a detection system. Preferably, dyes will have emission maxima separated by greater than 10 nm, more preferably greater than 25 nm, and even more preferably greater than 50 nm. When differentiation between two or more dyes is accomplished by visual inspection, the two or more dyes preferably have emission wavelengths of perceptibly different colors to enhance visual discrimination. When it is desirable to differentiate between two or more dyes using instrumental methods, a variety of filters and diffraction gratings are commercially available to allow the respective emission maxima to be independently detected. When two or more dyes are selected that possess relatively small differences in emission maxima, instrumental discrimination can be enhanced by ensuring that the emission spectra of the two or more dyes have similar integrated amplitudes and similar emission peak widths, and that the instrumental system's optical throughput will be equivalent across the emission peak widths of the respective two dyes. Instrumental discrimination can also be enhanced by selecting dyes with narrow emission peak widths; however, such dyes must necessarily possess high amplitude emissions, or be present in sufficient concentration that the loss of integrated signal strength is not detrimental to signal detection/discrimination, for example, as measured by the signal-to-noise ratio.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1794

6.3 Staining Process

The present invention describes techniques for precisely dyeing particles of varying sizes. The size of particles is immaterial to this invention since the precision of the dyeing process is not affected, except that (1) a given set of particles must be of uniform size within tight tolerances, for example, about ±10%, more preferably about ±5%, even more preferably about ±3%, and most preferably about ±2%, and (2) a particular application often will require a specific particle size, or range of sizes or size tolerance. Typically, particles are made of water-insoluble material, but may be made miscible or soluble in aqueous solvents by the addition of groups to increase hydrophilicity. The dyes employed are preferably squaric acid-based molecules that exhibit fluorescence extending into near infrared and/or infrared region, i.e., to about 1,000 nm. Use of other dyes may allow one to expand the range from the infrared to ultraviolet. The present method allows for a highly reproducible process in which two or more dyes of independent concentration are absorbed uniformly into or onto each particle, resulting in multiple spectrally distinct fluorescent signals corresponding to the number of different dyes present in or on the particle.

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These Examples are not intended in any way to limit the scope of the disclosure or the protection granted by Letters Patent hereon.

6.3.1 Example 1: General Outline of the Fluorescent Staining of Particles

A 5-10 gram (dry weight) quantity of stock particles in an aqueous medium is pipetted onto a vacuum filter membrane, and the liquid is removed and discarded. Next, a quantity of the rinse solvent (for example, 100 ml of an aliphatic alcohol, such as propanol, methanol, ethanol, etc.) is added to the particles. The particles are resuspended by placing an ultrasonic probe directly into the solution and applying power for several seconds, or as needed to effect resuspension. The suspension then is filtered, and the previous step is repeated once more. Dyeing of the particles is accomplished by adding 50 ml of a dye solution (composed of one or more dyes in an organic solvent) to the filtering cup and resuspending as before. The suspension then is allowed to sit for five minutes in the filtering cup. Next, 50 ml of rinse solvent is added to the dye suspension, sonicated and filtered. Another 100 ml of the rinse solvent is added, resuspended, and filtered. The last step is repeated once more. In order to prepare the particles for storage, 100 ml of an aqueous medium is added to the particles, the solution of which is then sonicated and filtered. Finally, 50 ml of aqueous medium is added to the particles, followed by sonication and transfer to a storage container.

In a particular embodiment of the invention, one or more dyes are mixed in a solvent suitable for the complete dissolution of the dyes, such as chloroform. Ethanol is added to the solution to increase wetting of the particles, and to create a process-dependent, final solvent density that is less than that of the particles. The concentration of each of the one or more dyes is experimentally determined as a function of the target fluorescence intensity at each of two center wavelengths of two fluorescence channels of the measuring instrument. These concentrations maintain their relative intensity throughout this inventive process.

An important aspect of the present invention is the preparation of particles prior to the dyeing operation. Manufacturers often supply particles in an aqueous medium. It has been discovered that the surfaces of particles that have been stored in an aqueous medium must be treated to make them permeable to organic compounds. In a preferred embodiment, a quantity of a polar organic solvent, such as an alcohol, is added to the particle solution to achieve about a 50-50 mixture of the aqueous medium and the polar organic solvent. This ratio, however, can be varied and adjusted at will according to particular needs that one may have, or determined by chemical and physical properties of the medium and the solvent.

An equally efficient and precise alternate technique entails "drying" the particles through a series of alcohol rinses, e.g., with methanol, ethanol, 2-propanol, rinses. The process is begun by spinning down the aqueous suspension of particles, typically about 10% solids (v/v) in suspension. The aqueous medium is decanted, and the particles are re-suspended in methanol. The alcohol solution with about 5% solids is vortexed, sonicated, and spun down. This step is performed once or twice more. Excess alcohol is decanted from the pellet, and residual solvent is evaporated under vacuum.

Test samples consisting of 0.05 gram quantities of dried particles are used to help adjust the dye solution to its desired ratio. Each dried 0.05 gram quantity of particles is suspended in 0.5 ml of a dye mixture containing two or more dyes of interest. The suspension of particles, now at 10% solids (w/v), is vortexed, and sonicated into suspension. Once in suspension, the mixture of particles and dyes is mixed for one hour. After that hour, the particles are spun down for a period of one minute using a centrifuge. The dye solution is then decanted, and the 0.05 g quantity of particles is re-suspended in 1 ml of 90% alcohol, e.g., methanol. The rinse step uses double the volume of the dye solution, thus maintaining a 5% solid solution. The sample is vortexed, sonicated, and spun down. The methanol supernatant is decanted. The 90% methanol rinse step is repeated once more. Finally, the excess methanol is decanted from the pellet, and the particles are re-suspended in an aqueous medium. Each test sample is then tested to determine the fluorescence activity of the labeled particles.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1796

When the test samples show that the dye solution has the precise ratios of the desired dyes, a macro-scale batch is conducted. The principle of macro-scale work up is identical to that noted above. Briefly, (1) 25 ml of the desired dye solution is transferred to a 50 ml vial, which contains a 2.5 gram quantity of dried particles. (2) The particles, now at 10% solids (w/v), are vortexed and sonicated. (3) Once the particles are completely in suspension, the solution is mixed for an hour. After that hour, (4) the particles are separated from the dye solution by centrifugation, followed by decanting. (5) The 2.5 gram quantity of particles is then re-suspended in 50 ml of 90% methanol. The rinse step uses double the volume of the dye solution, thus maintaining a mixture of 5% solids. (6) The sample then is vortexed, sonicated, and spun down, and the methanol supernatant is decanted. (7) Step 6 is repeated once more. (8) After the final methanol rinse is decanted, the particles are put through an aqueous rinse. (9) The aqueous supernatant is decanted, and the beads are then re-suspended and stored in a fresh aqueous medium.

6.3.2 Example 2: Dyeing of 300 nm Amino Functionalized Polystyrene Particles

A 1% (w/w) organic solvent solution of particles (300 nm diameter polystyrene, amino functionalized) is stirred in a round bottom flask. To this is added a solution of the desired dye(s) in an organic solvent, such as chloroform. When the dye solution is no longer absorbed by the particles (determined empirically by prior experiments), addition of the dye is halted, and the solvent is quickly removed under reduced pressure. For some reaction conditions, the dyeing reaction can be halted within about ten minutes; however, this time depends upon a variety of reaction parameters. Alternative embodiments therefore include times shorter than and times longer than ten minutes.

6.3.3 Example 3: Use of Two (Red and Orange) Squaraine Dyes

In another embodiment, two fluorescent squaraine dyes are used, for example, a red fluorescent dye, such as 1,3-bis[(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxy-cyclobutenediylium, bis(inner salt), and an orange fluorescent dye, such as 2-(3,5-dimethylpyrrol-2-yl)-4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one. These dyes are used to stain two separate populations of particles. Preferably, both dyes are excitable at approximately the same absorption wavelength, for example, ranging from an ultraviolet wavelength to about 800 nm, and emit fluorescent light at two distinct, essentially non-overlapping wavelengths that are separated from each other by at least 10 nm, preferably by at least 30 nm, and more preferably by at least 50 nm. For example, the emission peak of the first dye above is 585 nm, and the emission peak of the second dye is 630 nm. These dyes are chosen because they fall in the center of two of the fluorescence channels of a Becton Dickinson FACScan flow cytometer, which is

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1797

the measurement device used in a preferred embodiment of the present invention. However, the choice of fluorescence channels is relative and immaterial since different flow cytometry apparatuses can have different settings.

Once these dyes are embedded into or onto a particle, the total dye quantity will be between about 0.00001% and about 15% (w/w) of the final, total particle weight. However, this limitation is of little consequence to the present invention as long as the particles impregnated with said dyes are stable and usable for the intended purpose(s).

Optimal staining with a particular dye is dependent upon the physical and chemical parameters of the individual dye(s), the polymeric substrate(s), and the dye medium or solvent. Incubation times can vary widely depending on the desired results, the concentration of the dye(s), the chemical compositions and physical properties of the particles, and the reaction conditions. The optimal time is usually the minimum time required for the dye, in the concentration being used, to achieve the highest specific signal, while avoiding degradation of the dye and/or the particle over time, and minimizing all other undesirable fluorescent signals due to the dye(s).

In a preferred embodiment, chlorinated solvents such as chloroform are used to solubilize the dye(s). In alternative embodiments, suitable solvents are selected based on their ability to solubilize the particular class of hydrophobic dyes of interest. For example, the solvents can be acyl, aliphatic, cycloaliphatic, aromatic, or heterocyclic hydrocarbons. In further embodiments the solvents can have halogens, oxygen, sulfur, nitrogen, and/or phosphorus as either terminal groups or as internal/integral parts of a ring or chain. Specifically, solvents such as any of the alcohols, ethyl acetate, toluene, xylene, hexane, pentane, benzene, ether, acetone, various oils, carbon tetrachloride, carbon disulfide, DMSO, or methylene chloride can be used. In addition, other solvents known in the art can be used, such as the solvents listed in the *Merck Index* (Eleventh Edition; see sections MISC-63-68).

6.3.4 Example 4: General Procedure Used to Produce the X-Y Plot of Figure 1

A 5 ml quantity of undyed stock particles is placed in an aqueous medium directly on a membrane covered fritted funnel. A vacuum pump pulls air through the particles plated onto the filter paper for about one hour. Next, the dried particles are transferred to 50 ml of dye solution (having at least two dyes with spectrally distinct emission spectra), covered, and stirred at room temperature for about an hour. The particles then are separated by filtration from the dye solution, and the dyed particles are placed in a vacuum dessicator for about four hours to remove residual solvent. Next, a 200 ml quantity of Triton X-100 and water is added to the dried dyed particles in a

250 ml flask. The solution then is stirred for about three hours, followed by filtering and washing, which are repeated until no further dye is detected in the filtrate.

This procedure prepares a particular population of particles whose fluorescence emission spectra fall within a prescribed region or cluster depicted in a Fluorescence Particle Map, such as one of the ovals of Fig. 1. Typically, about 80% or greater of the individual particles within a particular set exhibit fluorescence characteristics within the desired region, preferably about 90% or greater, more preferably about 97% or greater, and most preferably about 99% or greater. When dyeing conditions are tightly controlled, a particular set of particles typically will exhibit about 1% or less of the individual particles having fluorescence characteristics that fall outside the desired region ("dispersion"), preferably about 0.5% or less, more preferably about 0.3% or less, and most preferably about 0.2% or less.

As an example, Figure 1 illustrates a 64-set particle collection, wherein each population or set differs from another by a distinct location or cluster on the X-Y plot. These locations essentially do not overlap. As opposed to the prior art methods that typically result in up to 10-20% or even higher rates of dispersion, the instant method allows production of essentially homogeneous populations or sets of particles having only about a 0.2 -0.3 % dispersion, which constitutes a large and significant improvement over the prior art.

Where nanoparticles have been dyed, the resulting dyed nanoparticles can then be linked to microparticles by any of the various well-known coupling reactions, such as carbodiimide coupling. Other methods of coupling can use such groups as carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, or halides.

6.4 Discriminators and Discrimination Power

Figure 1 illustrates eight levels of each of two dyes that are attached to particles according to practice of the present invention, which permits the creation of an 8 x 8 = 64 array of categories of fluorescently distinguishable particle sets. Fluorescently distinguishable discriminators thus provide the basis for creating arrays of categories of particle sets.

Additional discriminators operate similarly. For example, addition of a third dye at eight different levels requires 3-dimensional representation of an 8 x 8 x 8 = 512 array of categories of fluorescently distinguishable particle sets.

Other practical discriminators include size of particle, shape of particle, magnetic particle or particle with other magnetic property, dye type, radioactive property, chemical reactive group, nuclear magnetic resonance, dye fluorescence absorption, dye fluorescence emission , dye

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1799

absorption, fluorescence polarization, fluorescence lifetime, electron spin resonance, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, and particle density. For example, if two sizes of particles and two categories of magnetism (with or without magnetism) are added to the above 3-dimensional array, the array becomes 8 x 8 x 8 x 2 x 2 = 2,048 categories of particle subsets. Sizes and shapes of particles can be determined by light scattering measurements of forward and side scatter. The size of the particle can be measured in most flow cytometry apparatuses by forward or small-angle scatter light. Particle sets can also be further distinguished by differentiating between shapes of the particles, for example, using a high-resolution slit-scanning method. Magnetic particles incorporate magnetic or magnetically responsive metal oxides (for example, superparamagnetic, paramagnetic, or ferromagnetic oxides) into the polymer or polymer matrix.

Another category of discriminators is that of reactive groups that are attached to the particles, where each reactive group is capable of reaction with a specific compound. In some cases, the use of such discriminators will require two or more additional steps (for example, addition of a compound, followed by an incubation period, and measurement of an electromagnetic parameter, such as absorption, fluorescence emission, or magnetic resonance) to determine the reactive group and, hence, the category of the particle. In some cases, chromophores can be attached to the particles so that just a single optical or other measurement is required to determine the presence or absence of the chromophore on a given particle. Such discriminators provide powerful additional discrimination power. For example, as but one conservative calculation, use of 20 such discriminators in a labeling operation increases the above discussed array to 8 x 8 x 8 x 2 x 2 x 20 = 40,960 categories of particle sets.

An additional embodiment is that of inclusion of quantum dots within, on, or attached to particles. Quantum dots are fluorescing crystals which are excitable by a large range of wavelengths of light, but which have emission wavelengths that vary according to the size of the crystal -- with short wavelengths of emission occurring with small particles, and long wavelengths occurring with large particles. Thus, a large range of sizes of the crystals is used to create a large number of discrete emission wavelengths, creating a powerful expansion of discrimination power. Many dozens of classes of particles therefore are created according to the number of size classes of the quantum dot crystals. The size classes of the crystals are created either 1) by tight control of crystal formation parameters to create each desired size class of particle, or 2) by creation of batches of crystals under loosely controlled crystal formation parameters, followed by sorting according to desired size and/or emission wavelengths. Use of quantum dots for labeling particles, in the context of the present invention, is new, but is old in the art of semiconductors. Two examples of earlier references in

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1800

which quantum dots are embedded within intrinsic silicon epitaxial layers of semiconductor light emitting/detecting devices are United States Patent Nos. 5,293,050 and 5,354,707 to Chapple-Sokol, et al.

6.5 Methods of Labeling Liquids and Uses Therefor

The present invention basically entails the addition of particles, such as core microparticles and/or carrier microparticles having attached nanoparticles, to liquids as a labeling means.

Once a plurality of particle sets is produced, labeling a liquid simply requires adding the correct category of particle set(s) to the liquid that is to be so labeled according to that particular category of particle set(s). In a preferred embodiment, the particles are impregnated with three different fluorescent dyes in eight different concentrations or levels of each of the dyes so as to create 512 subsets of particles. Each particle is later classified into its member subset using a measurement instrument. These 512 subsets of particles comprise 512 labeling elements or discriminators. Depending on the needs of the user, these discriminators can be combined with other discriminators to increase the number of unique labels, as discussed in section 6.4. For example, the presence or absence of a labeling element yields certain permutations. Using eight levels of a discriminator at a time, the presence or absence of a discriminator gives (512!/8!(512-8)!), or approximately 10^{17} possible unique labels. In addition, varying the concentration of one or more uniquely labeled particle subsets expands the possibilities further, particularly when a uniform distribution of particles is achieved in the liquid. Also, a check digit can be added which has half or other fraction of the number of particles as the identifying particles.

In one embodiment, a signature can be created to meet the needs of a particular application by using unique particle sets. For example, a donor "Social Security Number" can be created by 1) assigning nine different order discriminators to nine different particle sets, where each particle set is respectively assigned to one of the nine ordered digit positions that make up a Social Security Number (i.e., ones, tens, hundreds, thousands, . . . to hundred millions), and 2) further categorizing each of the order class particle sets into one of ten numerical classes of digits zero through nine by assigning each of ten different levels of each of the nine order class discriminators to correspond to a selected digit that is different from the remaining nine digits. For example, order discriminators are assigned based on relative ratios of red to orange fluorescing dyes in or on particles as follows. A digit in the one's position is encoded by a first relative concentration ratio of red to orange fluorescing dyes, a digit in the ten's position is encoded by a second relative concentration ratio of red to orange fluorescing dyes, a digit in the hundred's position is encoded by a third relative concentration ratio of red to orange fluorescing dyes, etc. In a first embodiment, specific digits zero

through nine (i.e., the ten numerical classes) are then encoded according to the absolute amounts of the two dyes ("levels of the two dyes") in or on each particle. Alternatively, in a second embodiment, ten different concentrations of the order discrimination particles (or ten different total numbers of particles/sample) may be used to represent the ten digits. In this way, a single discriminator can serve the dual role of providing discrimination with respect to two different encoding aspects. In particular, in the second embodiment, only nine sets of beads are needed to encode any Social Security Number. Of course, other discriminators can be used with equal efficiency and practicality. Obviously, variations in level of a discriminator need only be such as to permit ready discrimination by the instrumentation to be used to read the Social Security Number. Furthermore, the progression of the ten digits of the ten numerical classes need not be represented by a monotonically increasing level of a discriminator(s) for the progression; rather, a monotonically decreasing level of the discriminator(s) may be employed, or any other pattern that achieves the goal of unique classification of each of the ten numerical classes. Also, separate order and numerical discriminators can be used (a 10 x 9 = 90 array of particles); however, use of such an array to encode a Social Security Number(s) usually is not as simple and not as economical as an encoding scheme that utilizes just one category of discriminator, or a combination of discriminators, to define the nine ordered digit positions, with attendant encoding of digits zero through nine by use of levels of the selected discriminator(s). However, a second discriminator (for example, particle density) in some applications may be used advantageously to provide numerical class encoding, for example, ten different densities of particles to represent digits zero through nine within each of the nine order class particle sets; alternatively, a range of nine different particle densities can serve as the nine order discriminators. In such an application, particles with the lowest density usually will be required to have a density greater than the liquid(s) they encode.

Analogous to the coding for a patient's Social Security Number, a similar scheme can be used to encode desired alphanumeric information of a patient, such as birth date, date of sample collection, name of patient, etc. To provide the most parsimonious and efficient use of particles, order discriminators are utilized according to the number of desired alphanumeric characters for the encoding. Then, as in one encoding embodiment for encoding a Social Security Number, concentrations of particles and/or other discriminators are used. For example, if 20 character positions are required for the encoding, and only alpha characters are to be used, 26 concentrations of particles would be required; alternatively, 10 concentrations of particles x 3 levels of a second disriminator to create 30 possible alpha characters for each of the 20 character positions; or any other such combination of discriminators to create the 26 required alpha characters. Also, characters such

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1802

as "space" and apostrophe, etc., can be incorporated. In addition, if liquid labeling identifies the patient sample, virtually all laboratory errors can be detected, including inter-sample contamination.

In another embodiment, a date code is created for labeling a liquid, for example, with respect to the date that a sample is taken from the liquid, an expiration date, a birth date, an expiration date for a perishable liquid, etc. For example, one of an array of 12 particle sets is assigned to the month January, a second to February, etc.; one of an array of 31 particle sets is assigned to the date 01, a second to 02, and so on through the date 31; and one of an array of five or more particle sets is assigned to a first year of interest, for example, 1999, a second to 2000, etc., for five consecutive years. This yields over 1800 exact dates by using only distinct 48 particle sets (more if greater than 5 years are used).

An alternative embodiment for encoding dates uses the concept of a series of small arrays. For example, four different "month" particles (having $2^4 = 16$ possible combinations) can be used to encode the 12 months, leaving 4 unused combinations; five different "day" particles are then assigned to encode the 31 days ($2^5 = 32$ possible combinations, leaving one unused combination); and five different "year" particles are then used to encode 32 years. Thus, only 14 different particles need be used to encode all the days over a span of 32 years. Furthermore, use of the unused combinations would further expand this span of years. For example, simple direct use (i.e., one particle combination per year) of the five unused particle combinations could be employed to add another 5 years of encoding coverage. Alternatively, use of the 4 unused month particle combinations, together with the presence or absence of the one unused day particle, would permit encoding of an additional 8 years, or 40 years total.

The concept of use of a series of small arrays of different particles can readily be applied to other encoding situations, including for routing, alphanumeric representation, Social Security Number, etc. In addition, the concept of "present" versus "absent" for different particles, that underlies calculation of numbers of possible permutations, may be applied in the form of "high" and "low," where high and low refer, for example, to high and low concentrations of particles in a sample, to high and low absolute numbers of particles in a sample, to high and low concentrations or levels of fluorescent dyes in or on the particles, to high and low levels of any other discriminator, etc.

Another embodiment for liquid labeling of the present invention is that of labeling solids, which later are solubilized, liquified, or extracted to recover particles contained therein for purposes of encoding. As but one illustrative example, microparticles are incorporated into sodium chloride crystals for such purposes as identification of manufacturer, lot number, production date, expiration date, degree of purity, etc. Such uses also include identification and/or tracing of pirated materials and products. Applications wherein solids are benficially labeled with microparticles are essentially

limitless, examples including glues and adhesives (for example, use on postage stamps, envelopes, memo pages that adhere to a wall, page, or other surface, etc.), ink, paint, explosives, plastics and plastic products, explosives, chemicals in general where minute quantities of an appropriate kind of microparticles do not interfere with the end use of the chemical(s), tracing dissolution attributes and flow patterns *in vivo* of pharmaceutical tablets, etc.

In yet another embodiment, microparticles are used to label gases. Typically, such a use will require microparticles of low density, unless the gas or gases are flowing sufficiently (and possibly including sufficient turbulent flow) to maintain the microparticles in suspension therein. As with applications in solids, uses of microparticles in gases have an essentially unlimited range of applications, for example, including for purposes of tracing and/or identifying pirated gases, tracing the flow of gases in an animal, and encoding of manufacturer, lot number, production date, physical properties, degree of purity, etc.

In an embodiment for encoding to route liquids through a complex of pipelines having a plurality of branch points, different particle sets are created to uniquely represent each desired destination for a plurality of liquids coursing through the complex of pipelines, and particles from a particular particle set are added in an appropriate amount (determined by experimentation) to the liquid to be encoded by that particle set. Discriminators and physical properties are selected to be consonant with the physical properties of the liquid to which the particles are to be added. For example, hydrophobic particles typically will be used in hydrophobic liquids unless the hydrophobic liquids also contain detergents or amphiphiles sufficient to evenly disperse the particles in the liquid. Similarly, the particles typically are selected to have a greater density than the liquid to promote even dispersion. Selection of the final concentration of particles in the liquid that is so encoded will depend on such factors as cost/benefit, and sensitivity of the instrumentation to perform the analyses on the particles. This embodiment functions by providing analysis at an appropriate point in the pipeline to which the particles are first added, for example, at or near and upstream from a branchpoint or decision point in a pipeline. Switches and valves are then connected to the instrumentation, and control the final routing per the desired destination, based on the one or more discriminators of the particular particle set.

Similar protocols may also be used in a clinical setting to direct one or multiple body fluid samples from one or more patients to the appropriate instrument to perform the desired clinical chemistry (multiplexed clinical assays). In such a clinical chemical setting, a plurality of body fluid samples can include the addition of a quantity of particles into each body fluid sample to correspond to the desired clinical chemical analysis and/or machine. In some cases, for example, it is desirable to route a body fluid sample to a machine that performs a panel of analyses. Typically, a flow

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1804

cytometer will be in-line with the tubing carrying the body fluid samples to detect routing particles and to transmit data on same to the appropriate switch(es) and valve(s) to direct the body fluid samples to the desired analytical site. Flow cytometers are the preferred instrument for such use, though any instrument that is capable of detecting the particular discriminator may be used.

Historically, the use of precision dyed particles in multiplexed clinical assays is provided, for example, by WO Patent No. 97/14028 and U.S. Patent No. 5,736,330, disclosures of which are herein incorporated by reference in their respective entireties. When these earlier disclosures are combined with the current invention, it is possible to greatly improve quality assurance in the clinical laboratory. For example, by selecting a unique permutation of particle sets, and permanently assigning that permutation to a particular assay, a fail-safe assay is created. A particle set also can be coupled to an associated reagent in a GMP (General Manufacturing Practice) environment, and combined into a single reagent vial. In addition, current multiplexed diagnostic instruments can be programmed to recognize a specific permutation, look up the parameters of that assay in a database, perform that assay, and record the results, thus eliminating the possibility that incorrect reagents will be added to a patient sample.

In embodiments for tracing toxic substances in groundwater, selection of the kinds of particles and their respective concentrations will include consideration of such factors as density and hydrophobicity of the toxic liquid(s), and size of the faults and/or densities of the soil(s) connecting a point toxic liquid pollution source with one or more aquifers of interest, as well as prospective or known dilution factors from underground water sources and/or above ground water sources, for example, recent rain or generally high rain fall. In addition, the sensitivity of the instrumentation to be used to decode or read the particles taken from a site distant from the point pollution source must be considered.

In embodiments for tracing absorption patterns of fluids in plants and animals, generally smaller particles will be advantageous; however, a large variation in particle size will be required for a wide range of tissue types, body cavities, etc. Factors similar to those of the preceding examples also require consideration in plants and animals. For example, particles that would be used in studies relating to bile flow would benefit from amphiphilic particles due to the presence of amphiphilic bile salts in bile. Of course, instrumentation sensitivity and dilution during use in an experiment must also be considered.

In general, a label is constructed by selecting one or more unique particle sets according to the particular application, adding them to the liquid, and mixing. The concentration of the particle volume with respect to the liquid volume also is a variable that is application dependent, for example,

reflecting the priority of cost versus speed: as the concentration of particles is increased, so, too, is the cost increased, whereas the reading time typically will be decreased.

In general and ideally, the particles are inert in the liquid being labeled. Furthermore, criteria for matching particle types to liquids include such parameters as stability of the particle in the particular liquid, stability of the dye attachment to the particle, and non-interference of the particle(s) with the use for which the liquid is intended. Thus, hydrophobic particle types generally will be used only with hydrophobic liquids, unless the hydrophobic liquid also contains detergents, amphiphiles, and/or hydrophiles. Particles that are used in organic solvents require that the dye or other discriminator be encapsulated in, or matrix entrapped by, a solvent stable polymer, or covalently attached to the particle.

Kits can also be produced to allow a user to mix particles in predetermined proportions and thereby appropriately label a liquid according to user defined or elected parameters. For example, a kit can feature 512 distinct particle sets based on eight levels of each of three fluorescent dyes. Alternatively, or in addition, other discriminators can be featured to create the number of distinct sets of particles necessary for use in a particular liquid labeling application, which also will take into account such attributes as hydrophobicity and density of the liquid to be labeled, as well as parameters such as stability of the fluorescent dye, or stability of matrix entrapment of the fluorescent dye, in the particular liquid. Specifically, a kit features particle sets for each desired discriminator, and each particle set comprises particle subsets which represent an appropriate range of values for that particular discriminator so as to create a functional range of subsets thereof. Thus, the user simply dispenses an appropriate quantity of each of the one or more sets/subsets of particles into the liquid selected to be labeled by that particular set(s)/subset(s) of particles. Furthermore, multiple classifications can be applied concurrently, for example, including one or more sets of particles to encode the patient's name and/or Social Security Number, and a second one or more sets of particles to encode routing for test procedures, storage, etc.

Another embodiment that can be coupled to a kit entails the use of a microprocessor controlled automatic pipetting system. In this embodiment, the user types into the system, using a standard keyboard, the desired numbers or other indicators (for example, for routing, indication of test to be performed, birth date of patient, Social Security Number, etc.). The user similarly supplies the desired microparticle type that is to correspond to a selected indicator; i.e., consideration is given to the physical properties or other bases for best matching particles with the liquids at hand. A simple software program allows the user to input the desired discriminators and the alphanumeric or other symbols to which the discriminators respectively correspond. Such programs are well known in the art, and may, for example, include lookup tables for the correspondence between

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1806

discriminators and symbols. Output signals from the microprocessor then operate to control automatic pipetting of the selected microparticles into one or more liquid or gaseous samples that are positioned in receiving modes relative to the pipetting system. Indicators indicate the purpose(s) for which the liquid or gas samples are labeled with particles.

The discriminators are used to distinguish particles based on differences in particle concentration, particle physical property, dye type, dye concentration, spectral property, label type, and combinations thereof. These broad categories of discriminators can be further categorized into other discriminators as follows. The particle physical property can include such attributes as particle size, particle shape, particle hydrophobicity, particle hydrophilicity, particle density, and combinations thereof. The spectral property can include such attributes as dye fluorescence absorption, dye fluorescence emission, dye absorption, dye fluorescence polarization, dye fluorescence lifetime, and combinations thereof. Label type refers to physicalchemical properties of chemical groups added to particles, and can include such attributes as magnetic property, (chemical) reactive group, nuclear magnetic resonance, electron spin resonance, positron emission, radioactive property, fluorescence polarization, fluorescence lifetime, and combinations thereof. Dye type refers to the chemical composition and structure of fluorescent dye molecules. Dye concentration refers to the concentration of the dye(s) on or in a particle. Particle concentration refers to the concentration of particles in the liquid being labeled.

When a liquid is ready for analysis or decoding of its label, the liquid is thoroughly mixed, and a sample is withdrawn. Next, a centrifugation, filtering, magnetic, or other separation means is used to separate the particles from the liquid thereby so encoded. The particles are then analyzed.

When encoding is achieved by the use of a series of spectrally distinct dyes, various commercially available instruments can be used to read the liquid labels. For example, a fluorescence microscope can be used on a sample of the liquid to locate and characterize the particles in the sample. In a preferred embodiment, a flow cytometer is used according to procedures known in the art, such as is disclosed in U.S. Patent No. 4,665,024 to Mansour, et al. Flow cytometry is an optical technique that analyzes particles in a fluid mixture based on the particles' optical characteristics using a flow cytometer. Background information on flow cytometry is set forth in Shapiro, Practical Flow Cytometry, Third Ed. (Alan R. Liss, Inc. 1995), incorporated herein by reference in its entirety. A flow cytometer can automatically display the clusters formed by the fluorescent emissions of the particle sets, for example, as shown herein in Fig. 1. In a preferred embodiment, the host computer that controls the flow cytometer is programmed to automatically detect the clusters and convert the particle set information into its ordinal information, such as date, "Social Security Number," patient's name, patient's birth date, or other identifying parameter.

Other means for detection and analysis can also be utilized, including, but not limited to, visual inspection, digital (CCD) cameras, video cameras, photographic film, or instrumentation such as laser scanning devices, fluorometers, photometers, luminometers, photodiodes, quantum counters, plate readers, epifluorescence microscopes, scanning microscopes, confocal microscopes, capillary electrophoresis detectors, or any other means that provides amplification of an electromagnetic signal, such as a photomultiplier tube or other light detector capable of detecting the presence, location, and intensity of excitation and emission spectra, fluorescence polarization, fluorescence lifetime, and other physical properties of a fluorescent or other electromagnetic signal.

When particles are processed by a flow cytometer, measurement of four exemplary classification parameters [forward light scatter, side light scatter, a first fluorescence emission peak (for example, red fluorescence), and a second fluorescence emission peak (for example, orange fluorescence)] can be used to identify the set, population, or cluster to which each particle belongs. Furthermore, additional chromophores, reactive groups, and other discriminators can be measured. Although methods and products of the present invention have been described in detail for purposes of general illustration, it is understood that such detail is solely for that purpose, and that variations can be made therein and thereto by those skilled in the art without departing from the scope of the invention, which is defined by the following claims. For example, the use of sets of small arrays of particles to encode respective portions of an alphanumeric string can be applied to virtually any encoding application.

WHAT IS CLAIMED IS:

1. A method of labeling a gaseous, liquid, or solid material to permit the subsequent identification of the labeled gaseous, liquid, or solid material, which comprises adding to a gaseous, liquid, or solid material, whose subsequent identification is desired, a sufficient quantity of particles belonging to one or more distinguishable sets of particles, to provide a labeled gaseous, liquid, or solid material that subsequently can be identified.

2. The method of claim 1 in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set.

3. The method of claim 2 in which a sufficient quantity of a combination of particles belonging to two or more distinguishable sets of particles is added to the gaseous, liquid, or solid material, whose subsequent identification is desired.

4. The method of claim 2 in which said one or more discriminators is selected from a physical, chemical, biological, magnetic, or spectral property of the particle or combinations thereof.

5. A method of identifying a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles, which comprises:

(a) obtaining a sample comprising a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles;

(b) analyzing the particles found in the sample to determine which of one or more distinguishable sets of particles such particles belong to; and

(c) comparing the results of said analyzing step with a key which establishes the identity of the gaseous, liquid, or solid material from which the sample was taken.

6. A labeled material which comprises (i) a gas, liquid, solid, or combinations thereof and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles.

7. The labeled material of claim 6 in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set.

8. The method of claim 1 in which said material comprises a gas.

9. The method of claim 1 in which said material comprises a liquid.

10. The method of claim 1 in which said material comprises a solid.

11. The method of claim 5 in which said material comprises a gas.

12. The method of claim 5 in which said material comprises a liquid.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1809

13. The method of claim 5 in which said material comprises a solid.

14. The labeled material of claim 6 which comprises a gas.

15. The labeled material of claim 6 which comprises a liquid.

16. The labeled material of claim 6 which comprises a solid.

17. A kit for labeling liquids, solids, and gases comprising particles from a plurality of distinct particle sets for labeling liquids, solids, and gases, and a dispenser.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1810

AMENDED CLAIMS

[received by the International Bureau on 18 August 1999(18.08.99);
original claim 6 amended; remaining claims unchanged (1 page)]

6. A labeled material whose subsequent identification is desired, which comprises (i) a gas, liquid, solid, or combination thereof and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1811

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07993

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B32B 5/16
US CL :425/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 264/ 5,6,7; 425/5,6; 428/402,403,407; 436/518,525,529,533

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSTIC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,723,218 A (HAUGLAND et al) 03 March 1998, see entire document.	1-17
Y	US 4,981,625 A (RHIM et al) 01 January 1991, column 3, line 63 - column 11, line 63.	1-17
A	US 5,736,330 A (FULTON) 07 April 1998, column 2 line 16 - column 7, line 12.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 MAY 1999	18 June 1999 (18.06.99)

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer
GAILENE R. GABEL

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

CIT / 38075-716.601
WN/2012/ADCO/RBA/DALT/SPEL

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
ZAKI MEGEED
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 12 APR 2013
Applicant's or agent's file reference 38075-716601	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 13/24509	International filing date *(day/month/year)* 01 February 2013 (01.02.2013)
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. Following the expiration of 30 months from the priority date, these comments will also be made available to the public.

   Shortly after the expiration of **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer Lee W. Young PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2010)

WSGR PATENT DOCKET
U.S. 201 IPS FOREIGN: X
DOCKETED: 4-15-13 BY: KDJ
ACTION: Amend Claims 10-2-13
DUE DATES: Written Opinion 10-3-13
ATTY ___ C/M # ___

RECEIVED
APR 15 2013
WILSON SONSINI, GOODRICH & ROSATI

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 38075-716601	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US 13/24509	International filing date *(day/month/year)* 01 February 2013 (01.02.2013)	(Earliest) Priority Date *(day/month/year)* 03 February 2012 (03.02.2012)
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 7 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

   c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

   a. the figure of the **drawings** to be published with the abstract is Figure No. _____

      ☐ as suggested by the applicant.

      ☐ as selected by this Authority, because the applicant failed to suggest a figure.

      ☐ as selected by this Authority, because this figure better characterizes the invention.

   b. ☒ none of the figures is to be published with the abstract.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1814

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/24509

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC 435/6.1
IPC C12Q 1/68 (2013.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 435/6.11; 435/6.19 (Text search - see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar Search Terms: signal, encoding, decoding, multiplexed, biochemical, assays

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2010/0267064 A1 (KARTALOV et al) 21 October 2010 (21.10.2010) entire document	1-74
A	US 2005/0260640 A1 (ANDERSEN et al) 24 November 2005 (24.11.2005) entire document	1-74
A	US 2007/0231824 A1 (CHEE et al) 04 October 2007 (04.10.2007) entire document	1-74
A	US 2002/0022273 A1 (EMPEDOCLES et al) 21 February 2002 (21.02.2002) entire document	1-74
A	US 2010/0210472 A1 (EMPEDOCLES et al) 19 August 2010 (19.08.2010) entire document	1-74
A	US 2008/0069733 A1 (MALTEZOS et al) 20 March 2008 (20.03.2008) entire document	1-74
A	US 2009/0062129 A1 (McKERNAN et al) 05 March 2009 (05.03.2009) entire document	1-74
A	US 2011/0237459 A1 (NOVA et al) 29 September 2011 (29.09.2011) entire document	1-74
A	US 2005/0214753 A1 (SCHULTZ et al) 29 September 2005 (29.09.2005) entire document	1-74
A	US 2005/0164264 A1 (SHIPWASH) 28 July 2005 (28.07.2005) entire document	1-74

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2013 (22.03.2013)	12 APR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: ZAKI MEGEED
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	12 APR 2013

Applicant's or agent's file reference	FOR FURTHER ACTION
38075-716601	See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 13/24509	01 February 2013 (01.02.2013)	03 February 2012 (03.02.2012)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.1

Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1 *bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Fom PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer:
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	22 March 2013 (22.03.2013)	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 13/24509

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

      ☐ on paper

      ☐ in electronic form

   b. (time)

      ☐ in the international application as filed

      ☐ together with the international application in electronic form

      ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1817

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 13/24509

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-74	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	1-74	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	1-74	YES
	Claims	NONE	NO

2. Citations and explanations:

Claims 1-48 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest an assay that is capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve.

Regarding claim 1-48, all comprising similar subject matter, there is no prior art of record that discloses an assay that is capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve. The closest prior art, US 2010/0267064 A1 to Kartalov et al. (hereinafter "Kartalov"), discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the assay as described above.

Claims 49-54 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; providing a sample comprising, or potentially comprising, at least one of said analytes; contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

Regarding claims 49-54, all comprising similar subject matter, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; providing a sample comprising, or potentially comprising, at least one of said analytes; contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme. The closest prior art, Kartalov, discloses a reporting scheme and intensity and/or spectrum of an optical signal (para. [0080]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the method as described above.

---------- CONTINUED IN SUPPLEMENTAL BOX ----------

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1818

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US 13/24509

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of:
-Box V-

Claims 55-60 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; providing a sample comprising, or potentially comprising, at least one of said analytes; contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

Regarding claims 55-60, all comprising similar subject matter, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; providing a sample comprising, or potentially comprising, at least one of said analytes; contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value. The closest prior art, Kartalov, discloses a reporting scheme, tuning and detuning (para. [0077]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the method as described above.

Claims 61-64 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a non-degenerate coding scheme capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, or melting curve analysis.

Regarding claims 61-64, all comprising similar subject matter, there is no prior art of record that discloses a non-degenerate coding scheme capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, or melting curve analysis. The closest prior art, Kartalov, discloses a reporting scheme, tuning and detuning (para. [0077]). There is nothing of record to lead one of ordinary skill in the art to modify the reporting scheme as taught by Kartalov to arrive at the non-degenerate coding scheme as described above.

Claim 65 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a system for detecting the presence or absence of each analyte of a plurality of analytes, compnsmg:
a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;
b. providing a sample comprising, or potentially comprising, at least one of said analytes;
c. contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;
d. cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

Regarding claim 65, there is no prior art of record that discloses a system for detecting the presence or absence of each analyte of a plurality of analytes, compnsmg:
a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;
b. providing a sample comprising, or potentially comprising, at least one of said analytes;
c. contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;
d. cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the system as described above.

-Continued on following Supplemental Box-

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1819

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of:
-Continued from previous Supplemental Box-

Claim 66 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a system for detecting the presence or absence of each analyte of a plurality of analytes, compnsing:
a. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;
b. providing a sample comprising, or potentially comprising, at least one of said analytes;
c. contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present;
d. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and
e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme,
wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

Regarding claim 66, there is no prior art of record that discloses a system for detecting the presence or absence of each analyte of a plurality of analytes, compnsing:
a. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;
b. providing a sample comprising, or potentially comprising, at least one of said analytes;
c. contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present;
d. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and
e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme,
wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the system as described above.

-Continued in the following Supplemental Box-

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1820

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:
-Continued from previous Supplemental Box-

Claim 67 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes,
compnsmg:
a. obtaining the identity of each of said analytes from a party;
b. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;
c. providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party:
i. contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and
ii. cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and
d. obtaining said cumulative measurement from said party;
e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and
f. providing said party with information about the presence or absence of each of said analytes.

Regarding claim 67, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes,
compnsmg:
a. obtaining the identity of each of said analytes from a party;
b. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;
c. providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party:
i. contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and
ii. cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and
d. obtaining said cumulative measurement from said party;
e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and
f. providing said party with information about the presence or absence of each of said analytes. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the system as described above.

-Continued in following Supplemental Box-

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1821

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of:
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Claim 68 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method of detecting the presence or absence of each analyte of a plurality of analytes,
compnsmg:
g. obtaining the identity of each of said analytes from a party;
h. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;
1. providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party:
i. contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and
ii. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and
j. obtaining said cumulative measurement from said party;
k. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and
1. providing said party with information about the presence or absence of each of said analytes.

Regarding claim 68, there is no prior art of record that discloses a method of detecting the presence or absence of each analyte of a plurality of analytes,
compnsmg:
g. obtaining the identity of each of said analytes from a party;
h. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;
1. providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party:
i. contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and
ii. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and
j. obtaining said cumulative measurement from said party;
k. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and
1. providing said party with information about the presence or absence of each of said analytes. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the method as described above.

-Continued in following Supplemental Box-

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1822

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of:
-Continued from previous Supplemental Box-

Claim 69 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.

Regarding claim 69, there is no prior art of record that discloses a composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov,to arrive at the composition as described above.

Claim 70 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

Regarding claim 70, there is no prior art of record that discloses a composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the composition as described above.

Claim 71 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.

Regarding claim 71, there is no prior art of record that discloses a kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

Claim 72 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

Regarding claim 72, there is no prior art of record that discloses a kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

-Continued in following Supplemental Box-

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1823

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 13/24509

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:
-Continued from previous Supplemental Box-

Claim 73 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.

Regarding claim 73, there is no prior art of record that discloses a kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

Claim 74 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a kit for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

Regarding claim 74, there is no prior art of record that discloses a kit for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value. The closest prior art, Kartalov, discloses five analytes (para. [0158]), 6 samples and combinations thereof (para. [0092) and immobilization (para. [0112]). There is nothing of record to lead one of ordinary skill in the art to modify the method as taught by Kartalov to arrive at the kit as described above.

Claims 1-74 have industrial applicability as defined by PCT Article 33(4) because the subject matter may be made or used in industry.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1824

SEARCH HISTORY

Application Number	PCT/US 13/24509
Search Conducted By	JCG
Search Approved By	SV

US/IPC Classifications Searched	435/6.1; C12Q 1/68 (2013.01)
Date Conducted	[22 March 2013 (22.03.2013)]

Documentation Searched	435/6.1,6.11,6.19
Search Terms Used	signal, encoding, decoding, multiplexed, biochemical, assays
Date Conducted	[22 March 2013 (22.03.2013)]

Electronic Database Searched	PatBase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	[22 March 2013 (22.03.2013)]
Search Logic:	

1) UC=(435/6.1 or 435/6.1 or 435/6.11 or 435/6.19) (15067)
2) IC=(C12Q1/68) (90142)
3) PA=(california institute of technology) (2284)
4) 3 and FT=(multiplex#) (363)

5) 4 and FT=(analyte? or detect or presence or absence not immobilize# not separate# not "mass spectometry" not "melting curve") (19)

6) (1 or 2) and 5 (1)
7) (1 or 2) and FT=(multiplex#) (11237)
8) 7 and FT=(intensity# or luminosity# or bright# or fluorescence?) (6909)
9) 7 and SP=((three or "3") and (intensity# or luminosity# or bright# or fluorescence?)) (3983)
10) 7 and SP=((three or "3" OR=3:100) and (intensity# or luminosity# or bright# or fluorescence?)) (5457)
11) (8 or 9 or 10) and FT=(analyte?) (1543)
12) 10 and FT=(analyte?) (1301)
13) (11 or 12) and FT=(code# or coding) (1032)
14) 12 and FT=(code# or coding) (886)
15) (13 or 14) and FT=(wavelength# or wavelength?) (717)
16) 14 and FT=(wavelength# or wavelength?) (624)
17) 15 and sp=((("5" or "6" or "7" or "8" or "9" or "10") or (five or six or seven or eight or nine or ten)) and (wavelength?)) (292)
18) 16 and sp=((("5" or "6" or "7" or "8" or "9" or "10") or (five or six or seven or eight or nine or ten)) and (wavelength?)) (272)
19) 17 and FT=(electromagnet#) (18)
20) 18 and SP=(signal? and (fluorescence? or fluorescent)) (157)
21) 18 and DSC=(fluorescence? or fluorescent) (266)
22) 17 and FT=(lyophilize# or lyophilized) (51)
23) 21 and FT=(lyophilize# or lyophilized) (46)
24) 21 and FT=(hybridize# and probe?) (206)
25) 24 and SP=(complement# and primer? and probe?) (32)
26) 24 and FT=(PCR or "polymerase chain reaction") (190)
27) 26 and SP=((PCR or "polymerase chain reaction") and ("end?point" or "real?time" or "RT?PCR" or "digital")) (120)
28) 27 and FT=(chromatogram?) (10)
29) 26 and FT=(chromatogram?) (16)
30) 29 and SP=(chromatogram? and positive and control?) (0)
31) 27 and FT=(polynucleotide?) (70)
32) 26 and FT=(polynucleotide?) (112)
33) (31 or 32) and SP=((polynucleotide?) and (animal or plant or bacteria or fungus or parasite or virus)) (26)
34) 31 and ft=("hiv" or "human immunodeficiency virus" or "herpes simplex virus" or "human papilloma virus" or "hpv") (41)
35) 32 and ft=("hiv" or "human immunodeficiency virus" or "herpes simplex virus" or "human papilloma virus" or "hpv") (68)
36) 33 and ft=("hiv" or "human immunodeficiency virus" or "herpes simplex virus" or "human papilloma virus" or "hpv") (13)
37) 34 and ft=(poly?protease or "p17" or "e6" or "e7") (11)
38) 13 and FT=(non?degenerate#) (8)
39) 13 and FT=(coding NOT degenerate NOT degeneracy) (466)
40) 39 and 37 (4)
41) PN=(WO2005094532) (1)
42) PN=(US8137626) (1)
43) CTF 42 (20)
44) 43 and FT=(multiplex) (2)
45) CTB 42 (50)
46) 45 and FT=(multiplex) (11)

47) PN=(US8187541) (1)
48) PN=(EP2267451) (1)
49) FT=(multiplex and intensities and wavelengths) (2190)
50) 49 and SP=(intensities and 3:100) (897)
51) 50 and SP=(wavelengths and 5:100) (447)
52) 51 and SP=((code# or coding) and wavelengths and intensities) (37)

Electronic Database Searched	Google Scholar

Files Searched	
Date Conducted	[22 March 2013 (22.03.2013)]
Search Logic:	
SIGNAL ENCODING AND DECODING MULTIPLEXED BIOCHEMICAL ASSAYS "California institute of technology" coding scheme (three and intensities) and (five and wavelengths)	

Electronic Database Searched	**THOMSON INNOVATION**
Files Searched	
Date Conducted	29 March 2013 (29.03.2013)
Search Logic:	

9	9	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	((((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample))) AND (ALL=(without ADJ immobilization))) AND (ALL=(encoding));
8	9	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App,	(((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or

		JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample))) AND (ALL=(without ADJ immobilization));
7	0	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	(((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample))) AND (ALL=(without ADJ melting ADJ curve ADJ analysis));
6	0	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	(((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample))) AND (ALL=(without ADJ mass ADJ spectrometry));
5	204	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App,	((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ

		Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(single ADJ sample));
4	0	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	(((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis) same assay))) AND (ALL=(single ADJ sample ADJ volume));
3	296	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	((ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)))) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis) same assay));
2	1300	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App,	(ALL=(assay and (immobilization or separation

		DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume)) AND (ALL=(without near5 (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)));
1	17526	US Grant, CA Grant, US App, CA App, WO App, FR App, EP Grant, DE Util, EP App, DE Grant, GB App, DE App, JP App, KR Grant, KR App, Other, DWPI, JP Util, KR Util , CN Util, JP Grant, CN Grant, CN App, VN Grant, MY Grant, TH Grant, VN App	ALL=(assay and (immobilization or separation or mass ADJ spectrometry or melting ADJ curve ADJ analysis)) AND ALL=(sample ADJ volume);

Electronic Acknowledgement Receipt	
EFS ID:	20837771
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Vernon A. Norviel/April Choi (RBAJ)
Filer Authorized By:	Vernon A. Norviel
Attorney Docket Number:	38075-716.301
Receipt Date:	02-DEC-2014
Filing Date:	05-AUG-2014
Time Stamp:	11:38:32
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		IDS38075-716-301-12-02-14.pdf	255792 dc3578a5b6b7a0c4fc43b0a576627372847 4bac3	yes	13

	Multipart Description/PDF files in .zip description				
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	Transmittal Letter		1	4	
	Information Disclosure Statement (IDS) Form (SB08)		5	13	
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2	Foreign Reference	EP1448581B1.PDF	2392841 c093832c5fc2fc4720759709767 72ee79e1dcd03	no	32
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3	Foreign Reference	EP1963531B1.PDF	7177618 71ae7bd00119a55255126c0427b861ee501f54b6	no	100
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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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor: Emil KARTALOV, et al.	Group Art Unit: 1631
Serial Number: 14/451,876	Examiner: Unassigned
Filing or 371(c) Date: August 5, 2014	**CONFIRMATION NO: 1065**
Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

FILED ELECTRONICALLY ON: December 2, 2014

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97

Madam:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§ 1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

IPR2024-01177
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A. ☒ *37 CFR § 1.97(b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53(d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in §1.491 in an international application;

-- OR --

☒ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☐ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution on the merits, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in § 1.97(e) provided concurrently herewith;

-- OR --

☐ a fee of $180.00 as set forth in § 1.17(p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97(d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113 or (2) a notice of allowance under § 1.311, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97(e);

-- AND --

ii. a fee of $180.00 as set forth in § 1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR § 1.97(e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97(c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97(d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e)(1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. § 1.704(d).* Each item of information contained in the information disclosure statement was first cited in a communication from a foreign patent office in a counterpart application that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1843

requirements of 37 C.F.R. § 1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR § 1.98(a)(2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are NOT enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR § 1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR § 1.98(a)(2)(iii).

G. ☐ *37 CFR § 1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR § 1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR § 1.98(a)(3)(ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR § 1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR § 1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR § 1.98.

IPR2024-01177
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I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 23-2415 (Docket No. 38075-716.301).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: December 2, 2014

By: /Reena Bajpai/
Reena Bajpai Ph.D., Patent Agent
Reg. No. 69,996

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(650) 493-9300
Customer No. 021971

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1845

Electronic Acknowledgement Receipt	
EFS ID:	20838071
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Vernon A. Norviel/April Choi (RBAJ)
Filer Authorized By:	Vernon A. Norviel
Attorney Docket Number:	38075-716.301
Receipt Date:	02-DEC-2014
Filing Date:	05-AUG-2014
Time Stamp:	11:53:57
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.)
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		Total Files Size (in bytes):	59810639		

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/451,876	08/05/2014	Emil Kartalov	38075-716.301

CONFIRMATION NO. 1065

21971
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PUBLICATION NOTICE

OC000000073628782

Title:SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Publication No.US-2015-0057178-A1
Publication Date:02/26/2015

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.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.	Document Number / 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
		None			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Patent Document / Country Code[3]-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]
	001	CN-101570782-A (in Chinese with English abstract)	11-04-2009	CHUNHUA YANG [CN]		x

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[2]

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Sheet 2 of 2

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

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

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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	/Reena Bajpai/	Date (YYYY-MM-DD)	2016-01-25
Name/Print	Reena Bajpai	Registration Number	69,996

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EFS ID:	24719381
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Reena Bajpai/Frank Chen (RBAJ)
Filer Authorized By:	Reena Bajpai
Attorney Docket Number:	38075-716.301
Receipt Date:	25-JAN-2016
Filing Date:	05-AUG-2014
Time Stamp:	21:08:19
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Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	CN101570782A-w-Eng-abst.pdf	1068628 17269c53d31a53c139a6612d32fd447f0f529d6b	no	23
Warnings:					
Information:					

2		IDS38075-716-301-1-25-2016.pdf	241226 3218f9ec58c7ebb2e0d19439d70f344175d0cff1	yes	7

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	Transmittal Letter	1	4
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Espacenet

Bibliographic data: CN101570782 (A) — 2009-11-04

Detection kit and detection method for 8 species of pathogenic bacteria in dairy products

Inventor(s): CHUNHUA YANG [CN] ± (YANG CHUNHUA, ; CAO JIJUAN, ; WANG SHUO)

Applicant(s): CHUNHUA YANG [CN] ± (YANG CHUNHUA, ; XU JUNYI, ; CAO JIJUAN, ; WANG SHUO)

Classification: **- international:** ***C12Q1/68; G01N30/02;*** *C12R1/01; C12R1/22; C12R1/37; C12R1/385; C12R1/425*

- cooperative:

Application number: CN2009110803 20090320

Priority number (s): CN2009110803 20090320

Also published as: CN101570782 (B)

Abstract of CN101570782 (A)

The invention discloses a detection kit and a detection method for 8 species of pathogenic bacteria in dairy products, which are used for food microbial contamination monitoring and detection. The kit comprises a detection solution A and a detection solution B, wherein the detection solution A and the detection solution B both contain 10 millimols of Tris.HCl, 50 millimols of KCl, 25 millimols of MgCl2, 2.5 millimols of dNTP and Taq DNA polymerase with a concentration of 5U/mu L and contain specific primer pairs of respective 4 species of target bacteria in an amount of 10 mu M each.; The detection kit and the detection method of the invention can detect the 8 species of pathogenic bacteria in the diary products with high sensitivity, is short in detection time, simple and quick in operation, and can save a large amount of labor and financial resources and meet requirements for quick detection.

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[19] 中华人民共和国国家知识产权局

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[51] Int. Cl.
C12Q 1/68 (2006.01)
G01N 30/02 (2006.01)
C12R 1/425 (2006.01)
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C12R 1/385 (2006.01)

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代理人 刘晓琴
权利要求书 2 页 说明书 17 页 附图 2 页

[54] 发明名称

乳制品中 8 种致病菌检测试剂盒及其检测方法

[57] 摘要

用于食品微生物污染监控及检测的乳制品中 8 种致病菌检测试剂盒及其检测方法。所述的试剂盒包括检测溶液 A 和检测溶液 B；检测溶液 A 和 B 中均含 10mM Tris·Cl、50mM KCl、25mM $MgCl_2$、dNTP 各 2.5mM 及 Taq DNA 聚合酶 5U/μL，还分别各自含 4 种目标菌的特异性引物对各 10μM。使用本发明的检测试剂盒及检测方法，可以高灵敏度地检测乳制品中 8 种致病菌，并且检测耗时短，操作简捷，可以节省大量的劳力和财力，适合快速检测的要求。

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1858

1、乳制品中 8 种致病菌检测试剂盒，其特征在于包括检测溶液 A 和检测溶液 B；

其中，检测溶液 A 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 各 2.5 mM、Taq DNA 聚合酶 5U/μL 及阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的引物对各 10 μM；

检测溶液 B 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 各 2.5 mM、Taq DNA 聚合酶 5U/μL 及致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌的引物对各 10 μM；

其中，所述 8 种致病菌的特异性引物序列如下：

菌株	上、下游引物序列（5′-3′）
阪崎肠杆菌	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG
粘质沙雷氏菌	GGAAGCGACTGCATTATTACCAT TGGTGGTGGCGATCACTT
绿脓杆菌（ETA 基因）	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT
普通变形杆菌	GATGGCAAGTACAAGTAAG GACGCTGAGATTGACCTA
奇异变形杆菌	CAACGTGAGATTAGTGGTGA CTGCTTTAAGTTCACAAATTAAGTG
肺炎克雷伯氏菌	TGGCCCGCGCCCAGGGTTCGAAA GATGTCGTCATCGTTGATGCCGAG
气味沙雷氏菌	TCGAGCGGTAGCACAGGA CAACGTATTAAGCTATTG
致病性嗜水气单胞菌（aerA 基因）	CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG

2、乳制品中 8 种致病菌的检测方法，其特征在于使用权利要求 1 所述的试剂盒，包括如下步骤：

① 取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 A 和 14ul 灭菌超纯水，

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1859

总体积 25ul；另取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 B 和 14ul 灭菌超纯水，总体积 25ul；两个反应体系分别在 5000 r/min 离心 10s，然后按下列参数分别进行 PCR 扩增：

预变性：94℃，3 min；

进入循环：94℃变性 60s，56℃退火 60s，72℃延伸 60s，35 次循环；

终止延伸：72℃，7 min；

② 将 PCR 扩增产物进行 DHPLC 分析：

色谱柱：PS-DVB & C18 DNASep 色谱柱，4.6 mm×50 mm，粒度 3 μm；

柱温：50℃；

流动相（体积比）：0.0 min，51.0% 缓冲溶液 A，49.0% 缓冲溶液 B；

0.5 min，45.7% 缓冲溶液 A，54.3% 缓冲溶液 B；

2.7 min，41.3% 缓冲溶液 A，58.7% 缓冲溶液 B；

4.9 min，38.7% 缓冲溶液 A，61.3% 缓冲溶液 B；

7.1 min，37.1% 缓冲溶液 A，62.9% 缓冲溶液 B；

9.3 min，35.9% 缓冲溶液 A，64.1% 缓冲溶液 B；

其中，缓冲溶液 A 是浓度 0.1mM 的 TEAA 水溶液；缓冲溶液 B 是浓度为 0.1M TEAA 水溶液与乙腈按体积比 3:1 的混合溶液；

流速：0.9 mL/min；

检测器：荧光检测器，光源：150W Xenon 灯；激发谱带宽：15 nm；发射谱带宽：15.3 nm；检测灵敏度：在波长 350 nm 积分 2 s；

上样量：PCR 产物 5μL。

IPR2024-01177
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乳制品中8种致病菌检测试剂盒及其检测方法

技术领域

本发明涉及乳制品中 8 种致病菌检测试剂盒及其检测方法，尤其涉及利用 mPCR-DHPLC 技术进行检测的检测方法及所使用的试剂。。

背景技术

2004 年，安徽阜阳劣质奶粉哺喂的“大头娃娃”事件曝光后，奶粉安全已成为全社会的焦点，2008 年因婴幼儿配方奶粉中三聚氰胺问题而引发的婴幼儿疾病的事件，更使全世界的目光都聚焦在奶粉等的食用安全方面。针对国内外不同品牌奶粉和进口原料奶粉中对婴幼儿有害病原菌的污染情况进行调研，发现奶粉中污染坂崎肠杆菌、肺炎克雷伯氏菌(*Klebsiella Pnaumoniae*)、变形杆菌(*Proteuse*)、金黄色葡萄球菌、大肠杆菌、绿脓杆菌(*Pseudomonas aeruginosa*)、溶血性链球菌(*Streptococcus*)、粘质沙雷氏菌(*serratia macescens*)、气味沙雷氏菌(*serratia odorifera*)等的污染几率较高。奶粉是母乳的替代品，婴幼儿的主要食物来源，奶粉安全意义重大。

阪崎肠杆菌(*Enterobacter sakazakii*)是人和动物肠道内寄生的一种革兰阴性无芽孢杆菌，属肠杆菌科的一种。该菌在一定条件下可引起人和动物致病，因此被称为条件致病菌。1961 年，英国首次报道 2 例由阪崎肠杆菌引起的脑膜炎病例，以后相继在美国、希腊、荷兰、冰岛、加拿大、比利时等国家报道了新生儿阪崎肠杆菌感染事件。具不完全资料报道，阪崎肠杆菌能引起严重的新生儿脑膜炎、小肠结肠炎和菌血症，并且可能引起神经系统后遗症和死亡，死亡率高达 50%以上。2001 年 4 月美国田纳西州发生阪崎肠杆菌感染事件后，医务人员与疾病控制中心协同对感染源进行了调查，结果从 10 例婴儿体内分离到的阪崎肠杆菌，与从婴儿所食用的已开罐和未开罐的婴儿配方奶粉中分离到的指纹图谱一致。由此导致国际间第一次因阪崎肠杆菌污染而致商业婴儿配方粉的广泛召回。目前，虽然还不能确定阪崎肠杆菌的宿主和转播途径，但多起新生儿阪崎肠杆菌感染事件基本证实了婴儿配方奶粉是主要感染源。

变形杆菌属有 4 个种：普通变形杆菌、奇异变形杆菌、产粘变形杆菌和潘

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1861

氏变形杆菌，其中以普通变形杆菌和奇异变形杆菌与临床关系较密切，特别是奇异变形杆菌可引起败血症，病死率较高。近年来，有关变形杆菌引起食物中毒的事件有所增加。

绿脓杆菌又称铜绿假单胞菌，属于假单胞菌属，在自然界中分布广泛，多见于水、空气、土壤中和物体表面上，也可见于正常人体表及人和动物的肠道中，人、畜肠道是绿脓杆菌的繁殖场所，为环境的主要污染源之一，也是一种重要的条件致病菌，常引起人皮肤化脓感染，特别是烧伤、烫伤、眼部疾病患者被感染后，常使病情恶化，并可引起败血症。2004 年也见从卫生巾中分离出绿脓杆菌的报道。国家质量监督检验检疫总局、卫生部等部门有明确规定，在食品、化妆品、外用药品以及絮用纤维制品、妇女卫生用品等物品中不得检出绿脓杆菌。传统的检测方法主要依靠绿脓杆菌的生物学特征，通过分离培养结合生化鉴定等方法进行检测，不仅操作繁琐、耗时长，且容易延误诊断。

目前国内外对微生物的检测方法还多停留在检测效率低、目标单一的水平上，还主要依靠传统的增菌、分离、生化鉴定方法，对于产芽孢菌的检测则更为落后。一般说来鉴定一种细菌需要 7～10 天，一些生化特性复杂的细菌（如致病性嗜水气单胞菌）最长的检测周期可达 20 天之久，这就影响了检测鉴定的周期。该现状显然不能满足目前食品安全迅速发展的需求。即便现在已经发展起来了 PCR 和实时 PCR 技术，也仅可达到对已知的常规微生物进行检测。而对于混合微生物样本、不常见的、苛刻的、发生变异的细菌则缺乏稳定而可靠的鉴定手段。致病性嗜水气单胞菌等对培养条件要求较高，生长缓慢，检测和鉴定周期长，而且传统培养生化检测方法检出率不高。因此，建立针对乳制品中多种微生物的高效、灵敏、便捷的检测方法很有必要。

发明内容

本发明的目的在于提供用于乳制品中 8 种致病菌的检测试剂盒及其 mPCR-DHPLC 检测方法，这几种乳制品中的常见致病菌分别是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌、普通变形杆菌、奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌、致病性嗜水气单胞菌。

首先，本发明所述的乳制品中 8 种致病菌检测试剂盒，该试剂盒包括检测溶液 A 和检测溶液 B；

其中，检测溶液 A 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1862

混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的引物对各 10 μM；

检测溶液 B 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌的引物对各 10 μM；

其中，所述 8 种致病菌的特异性引物序列如下：

菌株	上、下游引物序列（5′-3′）	扩增片段
阪崎肠杆菌	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG	228bp
粘质沙雷氏菌	GGAAGCGACTGCATTATTACCAT TGGTGGTGGCGATCACTT	332bp
绿脓杆菌（ETA 基因）	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396 bp
普通变形杆菌	GATGGCAAGTACAAGTAAG GACGCTGAGATTGACCTA	413 bp
奇异变形杆菌	CAACGTGAGATTAGTGGTGA CTGCTTTAAGTTCACAAATTAAGTG	241 bp
肺炎克雷伯氏菌	TGGCCCGCGCCCAGGGTTCGAAA GATGTCGTCATCGTTGATGCCGAG	368bp
气味沙雷氏菌	TCGAGCGGTAGCACAGGA CAACGTATTAAGCTATTG	412bp
致病性嗜水气单胞菌（aerA 基因）	CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG	209 bp

本发明还提供了利用上述试剂盒检测乳制品中 8 种致病菌的检测方法，包括如下步骤：

① 取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 A 和 14ul 灭菌超纯水，总体积 25ul；另取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 B 和 14ul 灭菌超纯水，总体积 25ul；两个反应体系分别在 5000 r/min 离心 10s，然后按下列参数分别进行 PCR 扩增：

预变性：94℃，3 min；

进入循环：94℃变性 60s，56℃退火 60s，72℃延伸 60s，35 次循环；

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1863

终止延伸：72℃，7 min；

② 将 PCR 扩增产物进行 DHPLC 分析：

色谱柱：PS-DVB & C18 DNASep 色谱柱，4.6 mm×50 mm，粒度 3 μm；

柱温：50℃；

流动相（体积比）：0.0 min，51.0% 缓冲溶液 A，49.0% 缓冲溶液 B；

0.5 min，45.7% 缓冲溶液 A，54.3% 缓冲溶液 B；

2.7 min，41.3% 缓冲溶液 A，58.7% 缓冲溶液 B；

4.9 min，38.7% 缓冲溶液 A，61.3% 缓冲溶液 B；

7.1 min，37.1% 缓冲溶液 A，62.9% 缓冲溶液 B；

9.3 min，35.9% 缓冲溶液 A，64.1% 缓冲溶液 B；

其中，缓冲溶液 A 是浓度 0.1mM 的 TEAA 水溶液；缓冲溶液 B 是浓度为 0.1M TEAA 水溶液与乙腈按体积比 3:1 的混合溶液；

流速：0.9 mL/min；

检测器：荧光检测器，光源：150W Xenon 灯；激发谱带宽：15 nm；发射谱带宽：15.3 nm；检测灵敏度：在波长 350 nm 积分 2 s；

上样量：PCR 产物 5μL。

检测结束后，根据 DHPLC 检测图谱中特征色谱峰的峰形、保留时间以及与阳性对照谱图的对照，来确定样品的染菌情况。

本发明采用 mPCR-DHPLC 技术，针对乳制品中 8 种致病菌建立灵敏便捷的检测方法，并建立应用于该方法的快速检测试剂盒。使用本发明的检测试剂盒及检测方法，可以高灵敏度地检测乳制品中 8 种致病菌，并且检测耗时短，操作简捷，可以节省大量的劳力和财力，适合快速检测的要求。

附图说明

本发明附图 7 幅：

图 1 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌单一菌 PCR-电泳检测结果；

图 2 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌四重 PCR-电泳检测电泳图；

图 3 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的四重 PCR-DHPLC 检测谱图；

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1864

图 4 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌单一菌 PCR-电泳检测结果；

图 5 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌四重 PCR-电泳检测电泳图；

图 6 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌四重 PCR-DHPLC 检测谱图；

图 7 是阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌 mPCR-DHPLC 灵敏度试验结果；

图 8 是奇异变形杆菌、肺炎克雷伯氏菌、气味沙雷氏菌和致病性嗜水气单胞菌 mPCR-DHPLC 灵敏度试验结果；

上述附图中，横坐标均为保留时间（单位：分钟 min），纵坐标表示吸收峰信号强度（单位：毫伏 mV）。

具体实施方式

下面为本发明的具体实施例，其对本方法的建立及其应用作进一步的说明，但并不以任何形式限制本发明的内容。

本部分试验所用的营养肉汤培养液，普通琼脂培养平板，以及所使用的各菌株的增菌、分离和生化鉴定培养基等均按照相关的国家标准（GB）、检验检疫行业标准（SN）或国际权威标准方法中的配方，购于美国 BD 公司。

细菌基因组 DNA 提取试剂（TakaRa MiniBEST Bacterial Genomic DNA Extraction kit）；Taq DNA 聚合酶，10×PCR buffer，dNTPs 等试剂购自宝生物工程（大连）有限公司；三乙胺乙酰盐（TEAA，色谱纯）购自 Transgenomic 公司；乙腈（色谱纯）购自 Fisher 公司。

本部分试验所用的主要仪器和设备包括基因扩增仪（美国 ABI 公司）和变性高效液相色谱（简称 DHPLC，美国 Transgenomic 公司）。

本研究所用标准菌株均购自美国典型菌种保藏中心（ATCC）和中国医学微生物菌种保藏管理中心（CMCC）。

实施例 1、乳制品中 8 种致病菌检测试剂盒及其检测方法的建立

（1）引物的设计、合成与试剂盒的组装：

菌株	上、下游引物序列（5′-3′）	扩增片段

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1865

阪崎肠杆菌	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG	228bp
粘质沙雷氏菌	GGAAGCGACTGCATTATTACCAT TGGTGGTGGCGATCACTT	332bp
绿脓杆菌（ETA 基因）	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396 bp
普通变形杆菌	GATGGCAAGTACAAGTAAG GACGCTGAGATTGACCTA	413 bp
奇异变形杆菌	CAACGTGAGATTAGTGGTGA CTGCTTTAAGTTCACAAATTAAGTG	241 bp
肺炎克雷伯氏菌	TGGCCCGCGCCCAGGGTTCGAAA GATGTCGTCATCGTTGATGCCGAG	368bp
气味沙雷氏菌	TCGAGCGGTAGCACAGGA CAACGTATTAAGCTATTG	412bp
致病性嗜水气单胞菌（aerA 基因）	CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG	209 bp

在此基础上，设计用于乳制品中 8 种致病菌检测的试剂盒，该试剂盒包括两组检测溶剂：检测溶液 A 和检测溶液 B；

其中，检测溶液 A 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌的引物对各 10 μM；

检测溶液 B 中含 10 mM Tris·Cl、50 mM KCl、25mM $MgCl_2$、dNTP 混合物（包括 dATP、dGTP、dCTP 和 dTTP 各 2.5 mM）、Taq DNA 聚合酶 5U/μL 及致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌的引物对各 10 μM。

（2）mPCR-DHPLC 检测方法

本检测方法使用本实施例建立的检测试剂盒，包括如下检测步骤：

① 待检样品的制备：

a. 奶制品样品：无菌方法称取 100 克样品，加到已预热的（44℃）装有 900mL 改良月桂基硫酸盐胰蛋白胨肉汤（mLST）的 2L 三角瓶中，振摇使样品充分混匀后，44℃恒温培养 22-24h。在液面 1cm 以下取 mLST 增菌液 0.2mL 加到装有 5mL 心脑浸液肉汤（BHI）的小试管中，混匀，36℃恒温水浴 4h，水面要高于

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1866

试管中培养基高度。取 BHI 增菌液 1.5mL，使用细菌基因组提取试剂盒提取其基因组 DNA，制取 PCR 模板。标记，直接作为 PCR 模板或-20℃保存。

b. 标准菌株：挑取单个菌落于营养肉汤中培养 18～24h，使用细菌基因组提取试剂盒提取其基因组 DNA，制取 PCR 模板。标记，直接作为 PCR 模板或-20℃保存。；

② PCR 扩增：

取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 A 和 14ul 灭菌超纯水，总体积 25ul；另取 1ul 待测样品 DNA 溶液，加入 10ul 检测溶液 B 和 14ul 灭菌超纯水，总体积 25ul；两个反应体系分别在 5000 r/min 离心 10s，然后按下列参数分别进行 PCR 扩增：

预变性：94℃，3 min；

进入循环：94℃变性 60s，56℃退火 60s，72℃延伸 60s，35 次循环；

终止延伸：72℃，7 min；

③ DHPLC 分析：

色谱柱：PS-DVB & C18 DNASep 色谱柱，4.6 mm×50 mm，粒度 3 μm；

柱温：50℃；

流动相（体积比）：0.0 min，51.0% 缓冲溶液 A，49.0% 缓冲溶液 B；
0.5 min，45.7% 缓冲溶液 A，54.3% 缓冲溶液 B；
2.7 min，41.3% 缓冲溶液 A，58.7% 缓冲溶液 B；
4.9 min，38.7% 缓冲溶液 A，61.3% 缓冲溶液 B；
7.1 min，37.1% 缓冲溶液 A，62.9% 缓冲溶液 B；
9.3 min，35.9% 缓冲溶液 A，64.1% 缓冲溶液 B；

其中，缓冲溶液 A 是浓度 0.1mM 的 TEAA 水溶液；缓冲溶液 B 是浓度为 0.1M TEAA 水溶液与乙腈按体积比 3:1 的混合溶液；

流速：0.9 mL/min；

检测器：荧光检测器，光源：150W Xenon 灯；激发谱带宽：15 nm；发射谱带宽：15.3 nm；检测灵敏度：在波长 350 nm 积分 2 s；

上样量：PCR 产物 5μL。

实施例 2、与常规 PCR-电泳检测方法的比较

实施例 1 所建立的检测试剂盒及检测方法涉及两组 PCR 体系，第一组复合

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1867

PCR 体系一次检测阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌；第二组复合 PCR 体系一次检测致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌。

按照实施例 1 所建立的方法提取标准菌株的 DNA 作为模板，按照实施例 1 所建立的方法的 PCR 条件进行扩增：第一组阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌四种菌的预期扩增片段分别为：228bp、332bp、396 bp、413 bp；第二组致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌四种菌的预期扩增片段分别为：209bp、241bp、368bp、412bp；对扩增后的扩增产物分别采用电泳及实施例 1 所建立的 DHPLC 检测条件检测。

第一组阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌各自单一 PCR 扩增的电泳检测结果见附图 1 所示；四重 PCR 扩增的电泳结果见附图 2 所示；四重 PCR 扩增的 DHPLC 检测结果见附图 3 所示；第二组致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌各自单一 PCR 扩增的电泳检测结果见附图 4 所示；四重 PCR 扩增的电泳结果见附图 5 所示；四重 PCR 扩增的 DHPLC 检测结果见附图 6 所示。

由图 1～3 可见，当第一组四种细菌作单一 PCR 扩增和电泳检测时，均能明显地检测出各自单一的条带；而当该四种细菌作复合 PCR 扩增和电泳检测时，396 bp、413 bp 的扩增条带却无法分开；但 DHPLC 检测中它们能清晰地分离并被准确检测；由图 4～6 可见，当第二组四种细菌作单一 PCR 扩增和电泳检测时，均能明显地检测出各自单一的条带；而当该四种细菌作复合 PCR 扩增和电泳检测时，209 bp 和 241 bp 的扩增条带很难分开，368bp、412bp 的扩增条带很难分开，但 DHPLC 检测中它们能清晰地分离并被准确检测。由此可见，本发明实施例 1 所建立的 mPCR-DHPLC 有更好的检测效果。

实施例 3、PCR-DHPLC 检测方法特异性试验

取表 1 中所列试验菌种，经培养后分别提取基因组 DNA，建立模板库。然后以这些基因组 DNA 为模板，采用实施例 1 所建立的方法进行 PCR-DHPLC 分析检测。

表 1

菌株名称	拉丁名

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1868

普通变形杆菌	*Proteus vulgaris*
奇异变形杆菌	*Proteus mirabilis*
致病性嗜水气单胞菌	*Aeromonas hydrophila*
非致病性嗜水气单胞菌	*Aeromonas hydrophila*
类志贺邻单胞菌	*Plesiomonas shigelloides*
副溶血性弧菌	*Vibrio parahaemolyticus*
副溶血性弧菌	*Vibrio parahaemolyticus*
副溶血性弧菌	*Vibrio parahaemolyticus*
溶藻性弧菌	*Vibrio alginolyticus*
创伤弧菌	*Vibrio vulnificus*
拟态弧菌	*Vibrio mimicus*
拟态弧菌	*Vibrio mimicus*
梅氏弧菌	*Vibrio metschnikouii*
河流弧菌	*Vibrio fluvialis*
单核细胞增生李斯特氏菌	*Listeria monocytogenes*
单核细胞增生李斯特氏菌	*Listeria monocytogenes*
单核细胞增生李斯特氏菌	*Listeria monocytogenes*
绵羊李斯特氏菌	*Listeria ivanovii*
英诺克李斯特氏菌	*Listeria innocua*
威尔斯李斯特氏菌	*Listeria welshimeri*
西尔李斯特氏菌	*Listeria seeligeri*
格氏李斯特氏菌	*Listeria grayi*
脱氮李斯特氏菌	*listeria denitrificans*
沙门氏菌	*Salmonella*
鼠伤寒沙门氏菌	*Salmonella. typhimurium*
甲型副伤寒沙门氏菌	*Salmonella. paratyphi A*
乙型副伤寒沙门氏菌	*Salmonella. paratyphi B*
丙型副伤寒沙门氏菌	*Salmonella. paratyphi C*
猪霍乱沙门氏菌	*Salmonella enterica subsp.*
猪霍乱沙门氏菌	*Salmonella enterica subsp.*
猪霍乱沙门氏菌	*Salmonella. cholerae*
肠炎沙门氏菌	*Salmonella. enteritidis*
亚利桑那沙门氏菌	*Salmonella enterica subsp. arizonae*
阿巴特图巴沙门氏菌	*Salmonella enterica subsp. enterica serovar Abaetetuba*
汤卜逊沙门氏菌	*Salmonella.thompson*
圣保罗沙门氏菌	*Salmonella.taint-paul*

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1869

海德堡沙门氏菌	*Salmonella.heidelberg*
都柏林沙门氏菌	*Salmonella dublin*
绿脓杆菌（铜绿假单胞菌）	*Pseudomonas aeruginosa*
绿脓杆菌（铜绿假单胞菌）	*Pseudomonas aeruginosa*
荧光假单胞菌	*Pseudomonas fluorescens*
恶臭假单胞菌	*Pseudomonas putida*
产碱假单胞菌	*Pseudomonas alcaligenes*
类产碱假单胞菌	*Pseudomonas pseodoalcaligenes*
洋葱假单胞菌	*Pseudomonas cepacia*
绿针假单胞菌	*Pseudomonas choloraphis*
稻草假单胞菌	*Pseudomonas straminea*
皮氏假单胞菌	*Pseudomonas pickettii*
门多萨假单胞菌	*Pseudomonas mendocina*
施氏假单胞菌	*Pseudomonas stutzeri*
阪崎肠杆菌	*Enterobacter.sakazakii*
大肠埃希氏菌	*Escherichia coli*
大肠埃希氏菌	*Escherichia coli*
产肠毒素大肠埃希氏菌	*Enterotoxigenic E. coli*
肠致病性大肠埃希氏菌	*Enteropathogenic E.coli*
肠侵袭性大肠埃希氏菌	*Enteroinvasive E.coli*
摩根氏摩根氏菌	*Morganella morganii*
产酸克雷伯氏菌	*Klebsiella oxytoca*
肺炎克雷伯氏菌	*Klebsiella peneumoniae*
弗劳地柠檬酸杆菌	*Citrobacter freundii*
弗地劳枸橼酸杆菌	*Citrobacter freundii*
弗劳地柠檬酸杆菌	*Citrobacter freundii*
阴沟肠杆菌	*Enterobacter cloacae*
阪崎肠杆菌	*Enterobacter.sakazakii*
产气肠杆菌	*Enterobacter aerogenes*
福氏志贺氏菌	*Shigella flexneri*
金黄色葡萄球菌	*Staphylococcus aureus*
金黄色葡萄球菌	*Staphylococcus aureus subsp. aureus*
小肠结肠炎耶尔森氏菌	*Yersinia enterocolitica*
溶血性链球菌	*Streptococcus hemolyticus*

特异性试验结果表明，采用实施例 1 所建立的检测试剂盒及检测方法，两

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1870

次检测即可特异地检测 8 种目标致病菌；采用检测试剂 A 进行的检测，只有阪崎肠杆菌、粘质沙雷氏菌、绿脓杆菌和普通变形杆菌检测出特异样品峰；采用检测试剂 B 进行的检测，只有致病性嗜水气单胞菌、奇异变形杆菌、肺炎克雷伯氏菌和气味沙雷氏菌检测出特异样品峰，其它对照细菌的检测结果都为阴性，没有假阳性和假阴性结果，具有很高的特异性。

实施例 4、PCR-DHPLC 模拟污染样品灵敏度试验

以奶粉为例，制备乳制品中微生物模拟污染样品，用比浊法定量所模拟污染的菌液浓度，单位为 CFU/mL。将致病菌的模拟污染肉汤进行系列稀释得到 10^{-3}、10^{-4}、10^{-5}、10^{-6}、10^{-7}、10^{-8}、10^{-9}、10^{-10} 的菌液，然后采用实施例 1 所建立的试剂盒及方法提取 DNA，各取 2 μL 做为模板，同样按照实施例 1 的方法进行 mPCR-DHPLC 检测，将检测结果与稀释梯度结果比较，确定检测灵敏度。检测结果如附图 7（第一组）和附图 8（第二组）所示。

表 1 和表 2 为致病菌在不同稀释度下的细菌数量。

表 1 第一组模拟污染液梯度稀释细菌数量表

序号	
L01	阪崎肠杆菌 29000\粘质沙雷氏菌 51000\绿脓杆菌 35000\普通变形杆菌 17000
L02	阪崎肠杆菌 2900\粘质沙雷氏菌 5100\绿脓杆菌 3500\普通变形杆菌 1700
L03	阪崎肠杆菌 290\粘质沙雷氏菌 510\绿脓杆菌 350\普通变形杆菌 170
L04	阪崎肠杆菌 29\粘质沙雷氏菌 51\绿脓杆菌 35\普通变形杆菌 17

表 2 第二组模拟污染液梯度稀释细菌数量表

序号	
L05	奇异变形杆菌 16000\肺炎克雷伯氏菌 40000\ 气味沙雷氏菌 29000\致病性嗜水气单胞菌 120000
L06	奇异变形杆菌 1600\肺炎克雷伯氏菌 4000\ 气味沙雷氏菌 2900\致病性嗜水气单胞菌 12000
L07	奇异变形杆菌 160\肺炎克雷伯氏菌 400\ 气味沙雷氏菌 290\致病性嗜水气单胞菌 1200
L08	奇异变形杆菌 16\肺炎克雷伯氏菌 40\ 气味沙雷氏菌 29\致病性嗜水气单胞菌 120

如附图 7 所示，第一组可以检测到表 2 中 L03 梯度的阪崎肠杆菌 290 CFU/mL，粘质沙雷氏菌 510 CFU/mL，绿脓杆菌 350 CFU/mL，普通变形杆菌 170 CFU/mL，即可以检测到 10^2 的数量级的菌浓度；而 PCR-凝胶电泳却只能检

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1871

测到 L02 梯度（10^3 的数量级）的菌浓度。

如附图 8 所示，第二组可以检测到表 2 中 L07 梯度的奇异变形杆菌 160 CFU/mL，肺炎克雷伯氏菌 400 CFU/mL，气味沙雷氏菌 290 CFU/mL，致病性嗜水气单胞菌 1200 CFU/mL，即可以检测到 10^2 的数量级的菌浓度；而 PCR-凝胶电泳却只能检测到 L06 梯度（10^3 的数量级）的菌浓度。

结果表明，本研究所建立的乳粉中致病菌 PCR-DHPLC 高通量检测方法的灵敏度非常高，可以达到 10^2 CFU/mL 的数量级。

实施例 5、应用于实际样品的检测试验

自 2007 年 12 月至 2008 年 6 月的 7 个月期间，采用实施例 1 建立的多重 PCR-DHPLC 方法快速筛选，同时采用检验检疫行业标准（SN/T 1632.1-3-2005、SN/T 0751-1999、SN/T 0738-1997）的培养鉴定方法进行验证比较，同时检测实际样品，共检测 2675 份乳制品样品。结果表明，用多重 PCR-DHPLC 方法筛选检出的 21 份阳性样本，其中检出普通变形杆菌 13 份，奇异变形杆菌 3 份，4 份样品检出阪崎肠杆菌，1 份样品检出粘质沙雷氏菌，采用经典的生化方法进行验证，均证实多重 PCR-DHPLC 方法检测准确率 100%。

本实施例中，采用检验检疫行业标准 SN/T 1632.1-3-2005、SN/T 0751-1999、SN/T 0738-1997）的方法，对 8 种菌均进行检测，平均总耗时（包括样品制备）576 h，检测耗时（不包括样品制备）575 h；使用 mPCR-DHPLC 方法，检测每个样品平均总耗时（包括样品制备）29 h，检测耗时（不包括样品制备）0.5 h。

实施例 6、应用于实际阳性样品的检测试验

对既往检测工作中，已经由行标检测确认为阳性的样品应用实施例 1 的 PCR-DHPLC 方法进行检测。

（1）在普通变形杆菌和奇异变形杆菌阳性样品检测中的应用

① 乳制品中普通变形杆菌和奇异变形杆菌检测结果

留存样品中，有 3 份样品已证实同时检出普通变形杆菌和奇异变形杆菌。用实施例 1 建立的方法检测这 3 份样品，经多重 PCR-DHPLC 检出 3 份样品普通变形杆菌和奇异变形杆菌阳性。

本试验采用普通变形杆菌和奇异变形杆菌阳性标准菌株、大肠杆菌阴性对照菌株（ATCC 17803）及水空白对照进行检测的结果表明，阳性对照检测出

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1872

DHPLC 的阳性吸收峰，且出峰时间和峰型重现性较好，阴性及水对照结果未见 DHPLC 吸收峰。多重 PCR-DHPLC 同时检测 3 株乳制品中的普通变形杆菌和奇异变形杆菌，均具有很好的阳性吸收峰重现性。

② 乳制品中普通变形杆菌和奇异变形杆菌分离株常规鉴定结果

5 株乳制品中的普通变形杆菌和奇异变形杆菌，常规分离培养鉴定结果与 PCR-DHPLC 检测结果比较，符合率为 100 %。

结果表明：本实施例 1 建立的多重 PCR-DHPLC 检测乳制品致病菌的方法，与经典的培养生化鉴定方法具有很好的符合性和结果重现性。

（2）在普通变形杆菌和阪崎肠杆菌阳性样品检测中的应用

留存样品中，其中有 2 份样品已证实同时检出普通变形杆菌和阪崎肠杆菌。用实施例 1 建立的方法检测这 2 份样品，经多重 PCR-DHPLC 检出 2 份样品普通变形杆菌和阪崎肠杆菌阳性。取从样品中分离出的所留存的普通变形杆菌和阪崎肠杆菌分离株，提取 DNA 进行 PCR-DHPLC 的验证试验，普通变形杆菌和阪崎肠杆菌均经 DHPLC 检测到了扩增吸收峰，且出峰时间和峰型重现性较好，以大肠杆菌为阴性对照的检测结果则为阴性。

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1873

SEQUENCE LISTING

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1874

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1875

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1876

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1877

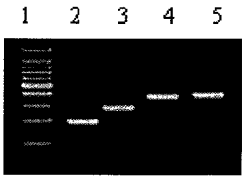


图 1



图 2

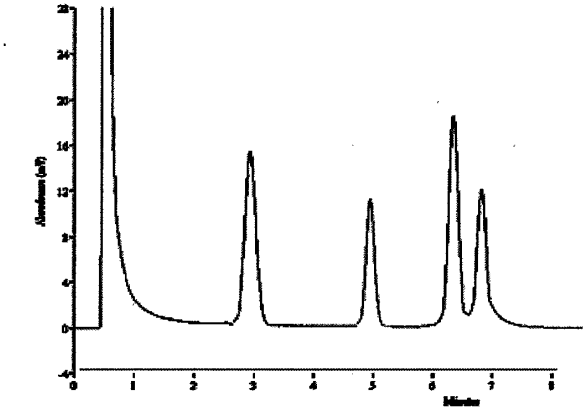


图 3

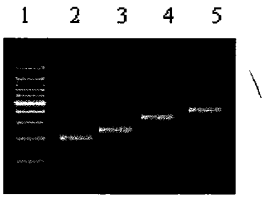


图 4



图 5

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1878

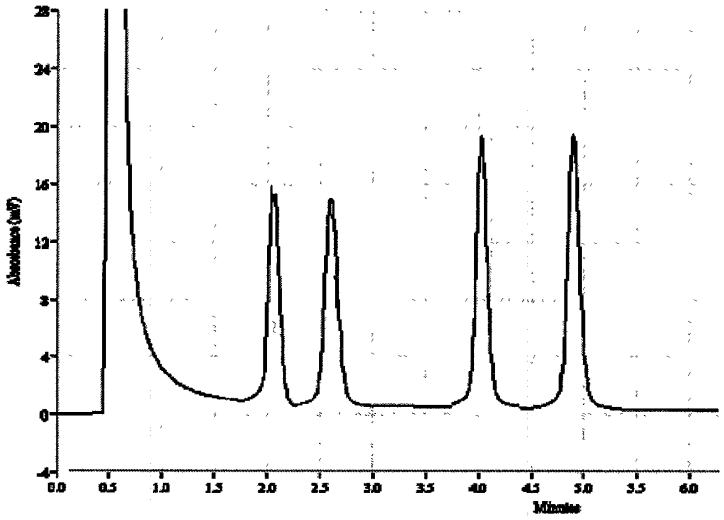


图 6

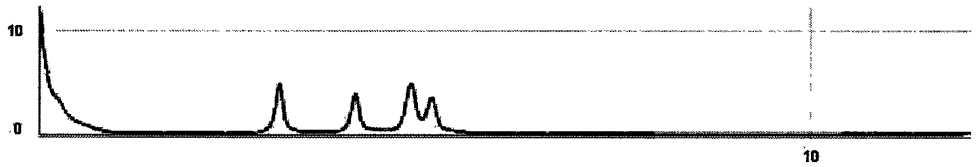


图 7

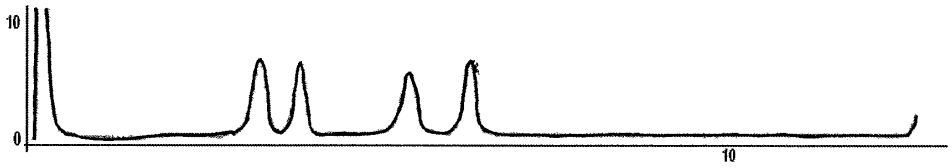


图 8

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1879

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV; Emil et al.	Group Art Unit:	1631
Serial Number:	14/451,876	Examiner:	RUSSELL SCOTT NEGIN
Filing or 371 (c) Date:	2014-08-05	**CONFIRMATION NO:**	1065
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: January 25, 2016

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1880

A. ☒ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☒ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☐ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution on the merits, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☐ a fee of $180.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113 or (2) a notice of allowance under § 1.311, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $180.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37Â CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37Â CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☐ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 is NOT enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☒ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☒ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1882

I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 23-2415 (Docket No.38075-716.301).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: January 25, 2016

By: /Reena Bajpai/
Reena Bajpai Ph.D., Patent Agent
Reg. No. 69,996

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1883

UNITED STATES PATENT AND TRADEMARK OFFICE

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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/451,876	08/05/2014	Emil Kartalov	38075-716.301	1065

21971 7590 05/12/2016

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1631	

NOTIFICATION DATE	DELIVERY MODE
05/12/2016	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):

patentdocket@wsgr.com

Office Action Summary	Application No. 14/451,876	Applicant(s) KARTALOV ET AL.	
	Examiner Russell S. Negin	Art Unit 1631	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 2 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 _____.

☐ 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) *See Continuation Sheet* 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) _____ is/are rejected.

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

9)☒ Claim(s) *See Continuation Sheet* 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 _____ 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: _____.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1885

Continuation of Disposition of Claims: Claims pending in the application are 1,2,4,5,9,13,17-19,22,27-29,34,35,37-39,42,43,45,47,49,51,53,55 and 56.

Continuation of Disposition of Claims: Claims subject to restriction and/or election requirement are 1,2,4,5,9,13,17-19,22,27-29,34,35,37-39,42,43,45,47,49,51,53,55 and 56.

DETAILED ACTION

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-2, 4-5, 9, 13, 17-19, 22, 27-29, 34-35, 37-39, 42-43, 45, and 47, drawn to an assay that does not use immobilization of analytes, separation of analytes, melting curve analysis, or mass spectrometry, classified in G06F 19/20.

II. Claims 49, 51, 53, and 55-56 drawn to using cumulative measurements to determine the presence or absence of each analyte in a plurality of analytes, classified in G06F 19/20.

The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are directed to related processes/assays. The related inventions are distinct if: (1) the inventions as claimed are either not capable of use together or can have a materially different design, mode of operation, function, or effect; (2) the inventions do not overlap in scope, i.e., are mutually exclusive; and (3) the inventions as claimed are not obvious variants. See MPEP § 806.05(j). In the instant case, the inventions as claimed serve different functions. In this instance, Invention I has the limitations of not using immobilization of analytes, separation of analytes, melting curve analysis, or mass spectrometry, which are not in Invention II. Likewise, Invention II has the limitation of using cumulative measurements to determine the presence or absence of each analyte in a plurality of analytes, which is not in Invention

I. Furthermore, the inventions as claimed do not encompass overlapping subject matter and there is nothing of record to show them to be obvious variants.

Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and/or examination burden if restriction were not required because one or more of the following reasons apply:

Each invention requires a different field of search (e.g. searching different classes/subclasses or electronic resources, or employing different search strategies or search queries).

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention.**

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other invention.

Species Election Requirement

This application contains claims directed to the following patentably distinct species:

Species A: the plurality of fluorescent probes comprise an identical fluorophore (claim 18)

Species B: the plurality of fluorescent probes comprise different fluorophores (claim 19)

The species are independent or distinct because using different versus identical fluorophores are mutually exclusive. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species, or a single grouping of patentably indistinct species, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 1-2, 4-5, 9, 13, 17, 22, 27-29, 34-35, 37-39, 42-43, 45, and 47 are generic to Invention I.

There is a search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply:

The species or groupings of patentably indistinct species require a difference filed of search (e.g., searching different classes/subclasses or electronic resources, or employing different search strategies or search queries).

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species or grouping of patentably indistinct species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

The election may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species or grouping of patentably indistinct species.

Should applicant traverse on the ground that the species, or groupings of patentably indistinct species from which election is required, are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing them

to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other species.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 10:30 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Peter Paras, Supervisory Patent Examiner, can be reached at (571) 272-4517.

Information regarding the status of the 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.

/Russell S. Negin/
Primary Examiner, Art Unit 1631
9 May 2016

Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	NEGIN, RUSSELL SCOTT
Attorney Docket Number	38075-716.301

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.	Document Number 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
	001	US-20020146734	10-10-2002	ORTYN; William E. et al.	
	002	US-20080124705	05-29-2008	KRAMER; Fred R.	
	003	US-20100151443	06-17-2010	XIANG; Qing et al.	
	004	US-20110151550	06-23-2011	SAGNER; Gregor et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Patent Document Country Code[3]-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]
	001	WO-03002979-A2	01-09-2003	ILLUMINA INC [US], et al.		☐
	002	WO-2013096851-A1	06-27-2013	HARVARD COLLEGE [US]		☐

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[2]
	001	CHAPIN et al., Rapid microRNA profiling on encoded gel microparticles. Angewandte Chemie International Edition, 50(10):2289-2293, 2011.	☐

Examiner Signature		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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 2 hours 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, 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 (1-800-786-9199) and select option 2.

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 2 of 2

Complete if Known	
Application Number	14451876
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First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	NEGIN, RUSSELL SCOTT
Attorney Docket Number	38075-716.301

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[2]
	002	European Patent Application No. 13744261.2 Extended European Search Report dated May 3, 2016.	☐
	003	HAN et al., Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nature Biotechnology, 19(7):631-635, 2001.	☐
	004	KLOSTRANEC et al., Convergence of quantum dot barcodes with microfluidics and signal processing for multiplexed high-throughput infectious disease diagnostics. Nano Letters, 7(9):2812-2818, 2007. (Abstract only)	☐
	005	LI et al., Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. Nature Biotechnology, 23(7):885-889, 2005.	☐
	006	LIN et al., Self-assembled combinatorial encoding nanoarrays for multiplexed biosensing. Nano Letters, 7(2):507-512, 2007.	☐
	007	XU et al., Multiplexed SNP genotyping using the Qbead™ system: a quantum dot-encoded microspere-based assay. Nucleic Acids Research, 31(8):e43, 2003.	☐

Examiner Signature		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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 2 hours 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, 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 (1-800-786-9199) and select option 2.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 January 2003 (09.01.2003)

PCT

(10) International Publication Number
WO 03/002979 A2

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(25) Filing Language: English

(26) Publication Language: English

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60/302,213 28 June 2001 (28.06.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/302,213 (CIP)
Filed on 28 June 2001 (28.06.2001)

(71) Applicant *(for all designated States except US)*: **ILLUMINA, INC.** [US/US]; 9885 Towne Centre Drive, San Diego, CA 92121 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CHEE, Mark, S.** [AU/US]; 155 15th Street, No. 22, Del Mar, CA 92014 (US). **STUELPNAGEL, John, R.** [US/US]; 38 Briggs Avenue, Encinitas, CA 92024 (US). **CZARNIK, Anthony, W.** [US/US]; 11017 Camino Abrojo, San Diego, CA 92127 (US).

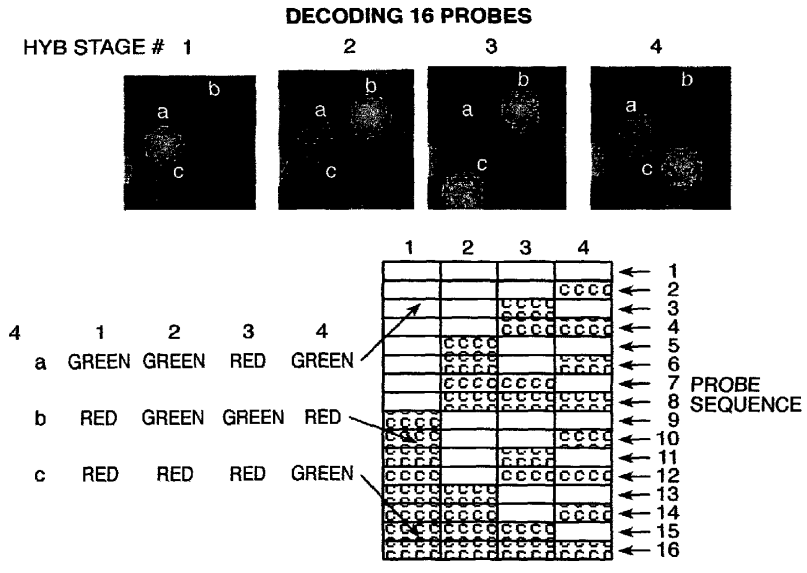
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(54) Title: MULTIPLEX DECODING OF ARRAY SENSORS WITH MICROSPHERES



(57) Abstract: The invention relates to compositions and methods for multiplex decoding of microsphere array sensors.

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1895

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MULTIPLEX DECODING OF ARRAY SENSORS WITH MICROSPHERES

This application claims the benefit of U.S.S.N. 60/302, 213, filed June 28, 2001.

FIELD OF THE INVENTION

The invention relates to compositions and methods for decoding microsphere array sensors.

BACKGROUND OF THE INVENTION

There are a number of assays and sensors for the detection of the presence and/or concentration of specific substances in fluids and gases. Many of these rely on specific ligand/antiligand reactions as the mechanism of detection. That is, pairs of substances (i.e. the binding pairs or ligand/antiligands) are known to bind to each other, while binding little or not at all to other substances. This has been the focus of a number of techniques that utilize these binding pairs for the detection of the complexes. These generally are done by labeling one component of the complex in some way, so as to make the entire complex detectable, using, for example, radioisotopes, fluorescent and other optically active molecules, enzymes, etc.

Of particular use in these sensors are detection mechanisms utilizing luminescence. Recently, the use of optical fibers and optical fiber strands in combination with light absorbing dyes for chemical analytical determinations has undergone rapid development, particularly within the last decade. The use of optical fibers for such purposes and techniques is described by Milanovich et al., "Novel Optical Fiber Techniques For Medical Application", Proceedings of the SPIE 28th Annual International Technical Symposium On Optics and Electro-Optics, Volume 494, 1980; Seitz, W.R., "Chemical

Sensors Based On Immobilized Indicators and Fiber Optics" in *C.R.C. Critical Reviews In Analytical Chemistry*, Vol. 19, 1988, pp. 135-173; Wolfbeis, O.S., "Fiber Optical Fluorosensors In Analytical Chemistry" in *Molecular Luminescence Spectroscopy, Methods and Applications* (S. G. Schulman, editor), Wiley & Sons, New York (1988); Angel, S.M., *Spectroscopy 2* (4):38 (1987); Walt, et al., "Chemical Sensors and Microinstrumentation", *ACS Symposium Series*, Vol. 403, 1989, p. 252, and Wolfbeis, O.S., *Fiber Optic Chemical Sensors*, Ed. CRC Press, Boca Raton, FL, 1991, 2nd Volume.

When using an optical fiber in an *in vitro/in vivo* sensor, one or more light absorbing dyes are located near its distal end. Typically, light from an appropriate source is used to illuminate the dyes through the fiber's proximal end. The light propagates along the length of the optical fiber; and a portion of this propagated light exits the distal end and is absorbed by the dyes. The light absorbing dye may or may not be immobilized; may or may not be directly attached to the optical fiber itself; may or may not be suspended in a fluid sample containing one or more analytes of interest; and may or may not be retainable for subsequent use in a second optical determination.

Once the light has been absorbed by the dye, some light of varying wavelength and intensity returns, conveyed through either the same fiber or collection fiber(s) to a detection system where it is observed and measured. The interactions between the light conveyed by the optical fiber and the properties of the light absorbing dye provide an optical basis for both qualitative and quantitative determinations.

Of the many different classes of light absorbing dyes which conventionally are employed with bundles of fiber strands and optical fibers for different analytical purposes are those more common compositions that emit light after absorption termed "fluorophores" and those which absorb light and internally convert the absorbed light to heat, rather than emit it as light, termed "chromophores."

Fluorescence is a physical phenomenon based upon the ability of some molecules to absorb light (photons) at specified wavelengths and then emit light of a longer wavelength and at a lower energy. Substances able to fluoresce share a number of common characteristics: the ability to absorb light energy at one wavelength $_{ab}$; reach an excited energy state; and subsequently emit light at another light wavelength, $_{em}$. The absorption and fluorescence emission spectra are individual for each fluorophore and are often graphically represented as two separate curves that are slightly overlapping. The same fluorescence emission spectrum is generally observed irrespective of the wavelength of the exciting light and, accordingly, the wavelength and energy of the exciting light may be varied within limits; but the light emitted by the fluorophore will always provide the same emission spectrum. Finally, the strength of the fluorescence signal may be measured as the quantum yield of light emitted. The fluorescence quantum yield is the ratio of the number of photons emitted in comparison to the number of photons initially absorbed by the fluorophore. For more detailed information regarding each of these characteristics, the following references are recommended: Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983; Freifelder, D., Physical Biochemistry, second edition, W. H. Freeman and Company, New York, 1982; "Molecular Luminescence

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1898

Spectroscopy Methods and Applications: Part I" (S.G. Schulman, editor) in *Chemical Analysis*, vol. 77, Wiley & Sons, Inc., 1985; *The Theory of Luminescence*, Stepanov and Gribkovskii, Iliffe Books, Ltd., London, 1968.

In comparison, substances which absorb light and do not fluoresce usually convert the light into heat or kinetic energy. The ability to internally convert the absorbed light identifies the dye as a "chromophore." Dyes which absorb light energy as chromophores do so at individual wavelengths of energy and are characterized by a distinctive molar absorption coefficient at that wavelength. Chemical analysis employing fiber optic strands and absorption spectroscopy using visible and ultraviolet light wavelengths in combination with the absorption coefficient allow for the determination of concentration for specific analyses of interest by spectral measurement. The most common use of absorbance measurement via optical fibers is to determine concentration which is calculated in accordance with Beers' law; accordingly, at a single absorbance wavelength, the greater the quantity of the composition which absorbs light energy at a given wavelength, the greater the optical density for the sample. In this way, the total quantity of light absorbed directly correlates with the quantity of the composition in the sample.

Many of the recent improvements employing optical fiber sensors in both qualitative and quantitative analytical determinations concern the desirability of depositing and/or immobilizing various light absorbing dyes at the distal end of the optical fiber. In this manner, a variety of different optical fiber chemical sensors and methods have been reported for specific analytical determinations and applications such as pH measurement, oxygen detection, and carbon dioxide analyses. These developments are exemplified by the following publications: Freeman, et al., *Anal Chem.* 53:98 (1983); Lippitsch et al., *Anal. Chem. Acta.* 205:1, (1988); Wolfbeis et al., *Anal. Chem.* 60:2028 (1988); Jordan, et al., *Anal. Chem.* 59:437 (1987); Lubbers et al., *Sens. Actuators* 1983; Munkholm et al., *Talanta* 35:109 (1988); Munkholm et al., *Anal. Chem.* 58:1427 (1986); Seitz, W. R., *Anal. Chem.* 56:16A-34A (1984); Peterson, et al., *Anal. Chem.* 52:864 (1980): Saari, et al., *Anal. Chem.* 54:821 (1982); Saari, et al., *Anal. Chem.* 55:667 (1983); Zhujun et al., *Anal. Chem. Acta.* 160:47 (1984); Schwab, et al., *Anal. Chem.* 56:2199 (1984); Wolfbeis, O.S., "Fiber Optic Chemical Sensors", *Ed. CRC Press, Boca Raton, FL*, 1991, 2nd Volume; and Pantano, P., Walt, D.R., *Anal. Chem.*, 481A-487A, Vol. 67, (1995).

More recently, fiber optic sensors have been constructed that permit the use of multiple dyes with a single, discrete fiber optic bundle. U.S. Pat. Nos. 5,244,636 and 5,250,264 to Walt, *et al.* disclose systems for affixing multiple, different dyes on the distal end of the bundle, the teachings of each of these patents being incorporated herein by this reference. The disclosed configurations enable separate optical fibers of the bundle to optically access individual dyes. This avoids the problem of deconvolving the separate signals in the returning light from each dye, which arises when the signals from two or more dyes are combined, each dye being sensitive to a different analyte, and there is significant overlap in the dyes' emission spectra.

U.S.S.N.s 08/818,199 and 09/151,877 describe array compositions that utilize microspheres or beads on a surface of a substrate, for example on a terminal end of a fiber optic bundle, with each individual fiber comprising a bead containing an optical signature. Since the beads go down randomly, a unique optical signature is needed to "decode" the array; i.e. after the array is made, a correlation of the location of an individual site on the array with the bead or bioactive agent at that particular site can be made. This means that the beads may be randomly distributed on the array, a fast and inexpensive process as compared to either the *in situ* synthesis or spotting techniques of the prior art. Once the array is loaded with the beads, the array can be decoded, or can be used, with full or partial decoding occurring after testing, as is more fully outlined below.

One drawback with the previous system is that it requires a set of unique optical signatures. While large sets of such signatures are available, for example by using different ratios of different dyes, it would be preferable to use decoding systems that do not rely on the use of sets of optical signatures. Accordingly, it is an object of the invention to provide methods to allow decoding of bead arrays without relying solely on unique optical signatures.

SUMMARY OF THE INVENTION

In accordance with the above objects, the present invention provides array compositions comprising a substrate with a surface comprising discrete sites. The composition further comprises a population of microspheres comprising at least a first and a second subpopulation; each subpopulation comprises a bioactive agent; and an identifier binding ligand that will bind a decoder binding ligand such that the identity of the bioactive agent can be elucidated. The microspheres are distributed on the surface.

In a further aspect, the present invention provides array compositions comprising a substrate with a surface comprising discrete sites, and a population of microspheres comprising at least a first and a second subpopulation. Each subpopulation comprises a bioactive agent and does not comprise an optical signature.

In an additional aspect, the present invention provides methods of making an array composition as outlined above. The methods comprise forming a surface comprising individual sites on a substrate and distributing microspheres on said surface such that said individual sites contain microspheres. The microspheres comprise at least a first and a second subpopulation each comprising a bioactive agent and do not comprise an optical signature.

In a further aspect, the invention provides methods of making a composition comprising forming a surface comprising individual sites on a substrate and distributing microspheres on the surface such that the individual sites contain microspheres. The microspheres comprise at least a first and a second subpopulation each comprising a bioactive agent and an identifier binding ligand that will bind a decoder binding ligand such that the identification of the bioactive agent can be elucidated.

In an additional aspect, the invention provides methods of decoding an array composition comprising providing an array composition as outlined above, and adding a plurality of decoding binding ligands to the array composition to identify the location of at least a plurality of the bioactive agents.

In a further aspect, the invention provides methods of determining the presence of a target analyte in a sample. The methods comprise contacting the sample with an array composition as outlined herein, and determining the presence or absence of the target analyte.

In a further aspect, the invention provides a method comprising providing an array composition comprising a population of microspheres comprising at least a first and a second subpopulation, wherein each subpopulation comprises a bioactive agent and at least a first and a second decoding attribute, and detecting each of said first and second decoding attributes to identify each of said bioactive agents.

In a further aspect the invention provides a method of increasing the information obtained in a decoding step. The method includes the use of degenerate probes as DBL-IBL combinations. In addition the invention provides the use of multiple decoding attributes on a bead.

In further aspect, the invention provides a method of increasing the confidence of decoding. The method includes using the decoding as a quality control measure. In addition, the invention provides quality control fibers. In addition, the invention provides for parity analysis of decoding data.

In a further aspect, the invention provides a method of decoding an array composition comprising providing an array composition comprising a population of microspheres comprising at least 50 subpopulations, wherein each subpopulation comprises a bioactive agent adding a plurality of decoding binding ligands to said population of microspheres to identify at least 50 of the bioactive agents.

In a further aspect, the invention provides a method of determining the presence of a target analyte in a sample comprising contacting said sample with a composition comprising a population of microspheres comprising at least 50 subpopulations, wherein each subpopulation comprises a bioactive agent adding a plurality of decoding binding ligands to said population of microspheres to identify at least 50 of the bioactive agents and determining the presence or absence of said target analyte.

FIGURES

Fig. 1 Illustrates a two color decoding process wherein either FAM-labeled or Cy3-labeled oligo complements are use to "paint" (label) the different bead types on the array.

Fig. 2 Depicts the decoding 128 different bead types with four colors and four decode stages. (inset shows a single decode stage using four different dyes to decode 16 bead types.)

Fig. 3. Grey scale decoding of 16 different bead types. (A) Combinatorial pooling scheme for complementary decoding oligos. A (B) Two independent normalizing images were acquired, and the resulting bead intensities compared. (C) The alpha values (ratio of bead intensity in indicated decode stage to intensity in normalization image) are plotted for three decodes stage described in (A).

Fig. 4 Depicts the use of fluorescence resonance energy transfer as a code for a probe on a random array. A. Depicts FRET oligos with linkers of different lengths. B. Depicts a plot of the ration of emission of the fluors from the oligos in Fig. 4A.

Figure 5 Use of fluorescence resonance energy transfer to discriminate between linkers of varying length.

Figure 6 depicts clustering in hyperspectral alpha space ($\Delta_1 = I_1/6I_I$, $\Delta_2 = I_2/6I_i$, $\Delta_3 = I_3/6I_i$, etc.). A set of 128 different bead types present on a fiber bundle were decoded with by hybridizing set of complementary oligonucleotides labeled with four dyes: Bodipy-493, Bodipy-R6G, Bodipy-TXR, and Bod-564 (only one dye per oligonucleotide). Shown is the second stage of a four stage decode in which 4013 beads were decoded. Ovals are drawn around zones of hue clusters.

Figure 7 depicts an error checking scheme.

Figure 8 depicts an example of primer extension labeling of decoder oligonucleotides.

Figure 9 depicts two color decoding of 16 beads with 16 distinct IBLs.

Figure 10 depicts primer extension labeling of degenerate IBL-DBL probes.

Figure 11 depicts a scatter plot of signals obtained from three distinct labels recorded in two channels.

Figure 12 depicts decoding by sequencing. A polymerase (e.g. Sequenase, Thermosequenase) and dye-labeled chain-terminating nucleotides are used to sequence the encoding region of a DNA strand present on the bead. The four nucleotides are each labeled with a discrete label. Because only 4 colors are detected at once, the colors are removed after each extension step. In subsequent extension steps, the primers are indexed or extended by one base so that the primer can query the next base in the encoding region.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1902

Figure 13 Depicts construction of probes on bead containing encoding sequences, zipcodes, and a gene-specific sequence. Two different encoding cassettes are employed to facilitate the primer extension reaction using primers with universal or degenerate bases. Using four color sequencing and a single base code, 6 bases generates 4^6 = 4096 codes, likewise four color hybridization using single hybridization colors also generates 4096 codes. The grand total number of codes is 4096*4096 = >16 million. If only a single color/two state scheme is employed for hybridization, than 2^6 = 64 codes are generated. The grand total is 4096*64 = 262,144 codes. The zipcode sequences can also be constructed so as to be overlapping to reduce the length of the overall sequence (i.e. cZip#1 vs. cZip#2).

Figure 14 Depicts incorporation of a cleavable linker between encoding sequences and gene-specific sequence. A disulfide cleavable linker is shown for illustration purposes. If the linker is not present, the terminal portion of the gene-specific sequence can be included as part of the encoding sequence to reduce overall length of the probe.

Figure 15 depicts decoding with exonuclease.

Figure 16 depicts a graph showing decoding of about 1500 bead subpopulation on each of several different fiber bundles.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is generally based on previous work comprising a bead-based analytic chemistry system in which beads, also termed microspheres, carrying different chemical functionalities are distributed on a substrate comprising a patterned surface of discrete sites that can bind the individual microspheres. Since the beads are generally put onto the substrate randomly, the previous work relied on the incorporation of unique optical signatures, generally fluorescent dyes, that could be used to identify the chemical functionality on any particular bead. This allows the synthesis of the candidate agents (i.e. compounds such as nucleic acids and antibodies) to be divorced from their placement on an array, i.e. the candidate agents may be synthesized on the beads, and then the beads are randomly distributed on a patterned surface. Since the beads are first coded with an optical signature, this means that the array can later be "decoded", i.e. after the array is made, a correlation of the location of an individual site on the array with the bead or candidate agent at that particular site can be made. This means that the beads may be randomly distributed on the array, a fast and inexpensive process as compared to either the in situ synthesis or spotting techniques of the prior art.

However, the drawback to these methods is that for a large array, the system requires a large number of different optical signatures, which may be difficult or time-consuming to utilize. Accordingly, the present invention provides several improvements over these methods, generally directed to methods of coding and decoding the arrays. That is, as will be appreciated by those in the art, the placement of

the bioactive agents is generally random, and thus a coding/decoding system is required to identify the bioactive agent at each location in the array. This may be done in a variety of ways, as is more fully outlined below, and generally includes: a) the use of decoding binding ligands (DBLs), generally directly labeled, that binds to either the bioactive agent or to identifier binding ligands (IBLs) attached to the beads; b) positional decoding, for example by either targeting the placement of beads (for example by using photoactivatible or photocleavable moieties to allow the selective addition of beads to particular locations), or by using either sub-bundles or selective loading of the sites, as are more fully outlined below; c) selective decoding, wherein only those beads that bind to a target are decoded; or d) combinations of any of these. In some cases, as is more fully outlined below, this decoding may occur for all the beads, or only for those that bind a particular target analyte. Similarly, this may occur either prior to or after addition of a target analyte.

Once the identity of the bioactive agent and its location in the array has been fixed, the array is exposed to samples containing the target analytes, although as outlined below, this can be done prior to or during the analysis as well. The target analytes will bind to the bioactive agents as is more fully outlined below, and result in a change in the optical signal of a particular bead.

In the present invention, "decoding" does not rely on the use of optical signatures, but rather on the use of decoding binding ligands that are added during a decoding step. The decoding binding ligands will bind either to a distinct identifier binding ligand partner that is placed on the beads, or to the bioactive agent itself, for example when the beads comprise single-stranded nucleic acids as the bioactive agents. The decoding binding ligands are either directly or indirectly labeled, and thus decoding occurs by detecting the presence of the label. By using pools of decoding binding ligands in a sequential fashion, it is possible to greatly minimize the number of required decoding steps.

In one embodiment, the invention provides methods for multiplex decoding of bead-arrays. That is, microspheres with bioactive agents are decoded to elucidate the identity of the bioactive agent on the bead. In a preferred embodiment at least 50 different bead types or subpopulations are simultaneously decoded, or decoded in a single decoding experiment. More preferably at least 100; more preferably at least 500; more preferably at least 1000; or preferably at least 1500 bead types are simultaneously decoded or decoded in a single decoding experiment by the methods of the invention. Preferably at least 5000 bead types are simultaneously decoded or decoded in a single decoding experiment.

Accordingly, the present invention provides array compositions comprising at least a first substrate with a surface comprising individual sites. By "array" herein is meant a plurality of candidate agents in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different bioactive agents (i.e. different beads) to many millions can be made, with very large fiber optic arrays being possible. Generally, the array will comprise from two to as many as a billion or more, depending on the size of the beads and the substrate, as well as the end use of the array, thus very high density, high density, moderate density, low density and very low

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1904

density arrays may be made. Preferred ranges for very high density arrays are from about 10,000,000 to about 2,000,000,000 (all numbers are per square cm), with from about 100,000,000 to about 1,000,000,000 being preferred. High density arrays range about 100,000 to about 10,000,000, with from about 1,000,000 to about 5,000,000 being particularly preferred. Moderate density arrays range from about 10,000 to about 100,000 being particularly preferred, and from about 20,000 to about 50,000 being especially preferred. Low density arrays are generally less than 10,000, with from about 1,000 to about 5,000 being preferred. Very low density arrays are less than 1,000, with from about 10 to about 1000 being preferred, and from about 100 to about 500 being particularly preferred. In some embodiments, the compositions of the invention may not be in array format; that is, for some embodiments, compositions comprising a single bioactive agent may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

In addition, one advantage of the present compositions is that particularly through the use of fiber optic technology, extremely high density arrays can be made. Thus for example, because beads of 200 m or less (with beads of 200 nm possible) can be used, and very small fibers are known, it is possible to have as many as 40,000 or more (in some instances, 1 million) different fibers and beads in a 1 mm^2 fiber optic bundle, with densities of greater than 15,000,000 individual beads and fibers (again, in some instances as many as 25-50 million) per 0.5 cm^2 obtainable.

By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of beads and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not themselves appreciably fluoresce.

Generally the substrate is flat (planar), although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the beads in a porous block of plastic that allows sample access to the beads and using a confocal microscope for detection. Similarly, the beads may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred substrates include optical fiber bundles as discussed below, and flat planar substrates such as glass, polystyrene and other plastics and acrylics.

In a preferred embodiment, the substrate is an optical fiber bundle or array, as is generally described in U.S.S.N.s 08/944,850 and 08/519,062, PCT US98/05025, and PCT US98/09163, all of which are expressly incorporated herein by reference. Preferred embodiments utilize preformed unitary fiber optic arrays. By "preformed unitary fiber optic array" herein is meant an array of discrete individual fiber optic strands that are co-axially disposed and joined along their lengths. The fiber strands are generally individually clad. However, one thing that distinguished a preformed unitary array from other fiber optic formats is that the fibers are not individually physically manipulatable; that is, one strand generally cannot be physically separated at any point along its length from another fiber strand.

Generally, the array of array compositions of the invention can be configured in several ways; see for example U.S.S.N. 09/473,904, and WO 00/39587, both of which are hereby expressly incorporated by reference. In a preferred embodiment, as is more fully outlined below, a "one component" system is used. That is, a first substrate comprising a plurality of assay locations (sometimes also referred to herein as "assay wells"), such as a microtiter plate, is configured such that each assay location contains an individual array. That is, the assay location and the array location are the same. For example, the plastic material of the microtiter plate can be formed to contain a plurality of "bead wells" in the bottom of each of the assay wells. Beads containing the capture probes of the invention can then be loaded into the bead wells in each assay location as is more fully described below.

Alternatively, a "two component" system can be used. In this embodiment, the individual arrays are formed on a second substrate, which then can be fitted or "dipped" into the first microtiter plate substrate. A preferred embodiment utilizes fiber optic bundles as the individual arrays, generally with "bead wells" etched into one surface of each individual fiber, such that the beads containing the capture probes are loaded onto the end of the fiber optic bundle. The composite array thus comprises a number of individual arrays that are configured to fit within the wells of a microtiter plate.

By "composite array" or "combination array" or grammatical equivalents herein is meant a plurality of individual arrays, as outlined above. Generally the number of individual arrays is set by the size of the microtiter plate used; thus, 96 well, 384 well and 1536 well microtiter plates utilize composite arrays comprising 96, 384 and 1536 individual arrays, although as will be appreciated by those in the art, not each microtiter well need contain an individual array. It should be noted that the composite arrays can comprise individual arrays that are identical, similar or different. That is, in some embodiments, it may be desirable to do the same 2,000 assays on 96 different samples; alternatively, doing 192,000 experiments on the same sample (i.e. the same sample in each of the 96 wells) may be desirable. Alternatively, each row or column of the composite array could be the same, for redundancy/quality control. As will be appreciated by those in the art, there are a variety of ways to configure the system. In addition, the random nature of the arrays may mean that the same population of beads may be added to two different surfaces, resulting in substantially similar but perhaps not identical arrays.

At least one surface of the substrate is modified to contain discrete, individual sites for later association of microspheres. These sites may comprise physically altered sites, i.e. physical configurations such as wells or small depressions in the substrate that can retain the beads, such that a microsphere can rest in the well, or the use of other forces (magnetic or compressive), or chemically altered or active sites, such as chemically functionalized sites, electrostatically altered sites, hydrophobically/ hydrophilically functionalized sites, spots of adhesive, etc.

The sites may be a pattern, i.e. a regular design or configuration, or randomly distributed. A preferred embodiment utilizes a regular pattern of sites such that the sites may be addressed in the X-Y coordinate plane. "Pattern" in this sense includes a repeating unit cell, preferably one that allows a high density of beads on the substrate. However, it should be noted that these sites may not be discrete sites. That is, it is possible to use a uniform surface of adhesive or chemical functionalities, for example, that allows the association of beads at any position. That is, the surface of the substrate is modified to allow association of the microspheres at individual sites, whether or not those sites are contiguous or non-contiguous with other sites. Thus, the surface of the substrate may be modified such that discrete sites are formed that can only have a single associated bead, or alternatively, the surface of the substrate is modified and beads may go down anywhere, but they end up at discrete sites.

In a preferred embodiment, the surface of the substrate is modified to contain wells, i.e. depressions in the surface of the substrate. This may be done as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, molding techniques and microetching techniques. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the substrate.

In a preferred embodiment, physical alterations are made in a surface of the substrate to produce the sites. In a preferred embodiment, the substrate is a fiber optic bundle and the surface of the substrate is a terminal end of the fiber bundle, as is generally described in 08/818,199 and 09/151,877, both of which are hereby expressly incorporated by reference. In this embodiment, wells are made in a terminal or distal end of a fiber optic bundle comprising individual fibers. In this embodiment, the cores of the individual fibers are etched, with respect to the cladding, such that small wells or depressions are formed at one end of the fibers. The required depth of the wells will depend on the size of the beads to be added to the wells.

Generally in this embodiment, the microspheres are non-covalently associated in the wells, although the wells may additionally be chemically functionalized as is generally described below, cross-linking agents may be used, or a physical barrier may be used, i.e. a film or membrane over the beads.

In a preferred embodiment, the surface of the substrate is modified to contain chemically modified sites, that can be used to associate, either covalently or non-covalently, the microspheres of the invention to the discrete sites or locations on the substrate. "Chemically modified sites" in this context includes, but is not limited to, the addition of a pattern of chemical functional groups including amino groups, carboxy groups, oxo groups and thiol groups, that can be used to covalently attach microspheres, which generally also contain corresponding reactive functional groups; the addition of a pattern of adhesive that can be used to bind the microspheres (either by prior chemical functionalization for the addition of the adhesive or direct addition of the adhesive); the addition of a pattern of charged groups (similar to the chemical functionalities) for the electrostatic association of the microspheres, i.e. when the microspheres comprise charged groups opposite to the sites; the addition of a pattern of chemical functional groups that renders the sites differentially hydrophobic or hydrophilic, such that the addition of similarly hydrophobic or hydrophilic microspheres under suitable experimental conditions will result in association of the microspheres to the sites on the basis of hydroaffinity. For example, the use of hydrophobic sites with hydrophobic beads, in an aqueous system, drives the association of the beads preferentially onto the sites. As outlined above, "pattern" in this sense includes the use of a uniform treatment of the surface to allow association of the beads at discrete sites, as well as treatment of the surface resulting in discrete sites. As will be appreciated by those in the art, this may be accomplished in a variety of ways.

The compositions of the invention further comprise a population of microspheres. By "population" herein is meant a plurality of beads as outlined above for arrays. Within the population are separate subpopulations, which can be a single microsphere or multiple identical microspheres. That is, in some embodiments, as is more fully outlined below, the array may contain only a single bead for each bioactive agent; preferred embodiments utilize a plurality of beads of each type.

By "microspheres" or "beads" or "particles" or grammatical equivalents herein is meant small discrete particles. The composition of the beads will vary, depending on the class of bioactive agent and the method of synthesis. Suitable bead compositions include those used in peptide, nucleic acid and organic moiety synthesis, including, but not limited to, plastics, ceramics, glass, polystyrene, methylstyrene, acrylic polymers, paramagnetic materials, thoria sol, carbon graphite, titanium dioxide, latex or cross-linked dextrans such as Sepharose, cellulose, nylon, cross-linked micelles and Teflon may all be used. *"Microsphere Detection Guide"* from Bangs Laboratories, Fishers IN is a helpful guide.

The beads need not be spherical; irregular particles may be used. In addition, the beads may be porous, thus increasing the surface area of the bead available for either bioactive agent attachment or IBL attachment. The bead sizes range from nanometers, i.e. 100 nm, to millimeters, i.e. 1 mm, with

beads from about 0.2 micron to about 200 microns being preferred, and from about 0.5 to about 5 micron being particularly preferred, although in some embodiments smaller beads may be used.

It should be noted that a key component of the invention is the use of a substrate/bead pairing that allows the association or attachment of the beads at discrete sites on the surface of the substrate, such that the beads do not move during the course of the assay.

Each microsphere comprises a bioactive agent, although as will be appreciated by those in the art, there may be some microspheres which do not contain a bioactive agent, depending on the synthetic methods. By "candidate bioactive agent" or "bioactive agent" or "chemical functionality" or "binding ligand" herein is meant as used herein describes any molecule, *e.g.*, protein, oligopeptide, small organic molecule, coordination complex, polysaccharide, polynucleotide, etc. which can be attached to the microspheres of the invention. It should be understood that the compositions of the invention have two primary uses. In a preferred embodiment, as is more fully outlined below, the compositions are used to detect the presence of a particular target analyte; for example, the presence or absence of a particular nucleotide sequence or a particular protein, such as an enzyme, an antibody or an antigen. In an alternate preferred embodiment, the compositions are used to screen bioactive agents, i.e. drug candidates, for binding to a particular target analyte.

Bioactive agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 Daltons. Bioactive agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The bioactive agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Bioactive agents are also found among biomolecules including peptides, nucleic acids, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are nucleic acids and proteins.

Bioactive agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical

modifications, such as acylation, alkylation, esterification and/or amidification to produce structural analogs.

In a preferred embodiment, the bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and norleucine are considered amino acids for the purposes of the invention. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradations.

In one preferred embodiment, the bioactive agents are naturally occurring proteins or fragments of naturally occuring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening in the systems described herein. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

In a preferred embodiment, the bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized bioactive proteinaceous agents.

In a preferred embodiment, a library of bioactive agents are used. The library should provide a sufficiently structurally diverse population of bioactive agents to effect a probabilistically sufficient range of binding to target analytes. Accordingly, an interaction library must be large enough so that at least one of its members will have a structure that gives it affinity for the target analyte. Although it is difficult to gauge the required absolute size of an interaction library, nature provides a hint with the immune response: a diversity of 10^7-10^8 different antibodies provides at least one combination with sufficient affinity to interact with most potential antigens faced by an organism. Published in vitro

selection techniques have also shown that a library size of 10^7 to 10^8 is sufficient to find structures with affinity for the target. Thus, in a preferred embodiment, at least 10^6, preferably at least 10^7, more preferably at least 10^8 and most preferably at least 10^9 different bioactive agents are simultaneously analyzed in the subject methods. Preferred methods maximize library size and diversity.

In a preferred embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment, the bioactive agents are nucleic acids (generally called "probe nucleic acids" or "candidate probes" herein). By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage, *et al.*, Tetrahedron, **49**(10):1925 (1993) and references therein; Letsinger, J. Org. Chem., **35**:3800 (1970); Sprinzl, *et al.*, Eur. J. Biochem., **81**:579 (1977); Letsinger, *et al.*, Nucl. Acids Res., **14**:3487 (1986); Sawai, *et al.*, Chem. Lett., 805 (1984), Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); and Pauwels, *et al.*, Chemica Scripta, **26**:141 (1986)), phosphorothioate (Mag, *et al.*, Nucleic Acids Res., **19**:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu, *et al.*, J. Am. Chem. Soc., **111**:2321 (1989)), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc., **114**:1895 (1992); Meier, *et al.*, Chem. Int. Ed. Engl., **31**:1008 (1992); Nielsen, Nature, **365**:566 (1993); Carlsson, *et al.*, Nature, **380**:207 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpcy, *et al.*, Proc. Natl. Acad. Sci. USA, **92**:6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowshi, *et al.*, Angew. Chem. Intl. Ed. English, **30**:423 (1991); Letsinger, *et al.*, J. Am. Chem. Soc., **110**:4470 (1988); Letsinger, *et al.*, Nucleosides & Nucleotides, **13**:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, Bioorganic & Medicinal Chem. Lett., **4**:395 (1994); Jeffs, *et al.*, J. Biomolecular NMR, **34**:17 (1994); Tetrahedron Lett., **37**:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of

nucleic acids (see Jenkins, *et al.*, Chem. Soc. Rev., (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, C & E News, June 2, 1997, page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments; for example, PNA is particularly preferred. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthanine, hypoxanthanine, isocytosine, isoguanine, and base analogs such as nitropyrrole and nitroindole, etc.

In a preferred embodiment, the bioactive agents are libraries of clonal nucleic acids, including DNA and RNA. In this embodiment, individual nucleic acids are prepared, generally using conventional methods (including, but not limited to, propagation in plasmid or phage vectors, amplification techniques including PCR, etc.). The nucleic acids are preferably arrayed in some format, such as a microtiter plate format, and beads added for attachment of the libraries.

Attachment of the clonal libraries (or any of the nucleic acids outlined herein) may be done in a variety of ways, as will be appreciated by those in the art, including, but not limited to, chemical or affinity capture (for example, including the incorporation of derivatized nucleotides such as AminoLink or biotinylated nucleotides that can then be used to attach the nucleic acid to a surface, as well as affinity capture by hybridization), cross-linking, and electrostatic attachment, etc.

In a preferred embodiment, affinity capture is used to attach the clonal nucleic acids to the beads. For example, cloned nucleic acids can be derivatized, for example with one member of a binding pair, and the beads derivatized with the other member of a binding pair. Suitable binding pairs are as described herein for IBL/DBL pairs. For example, the cloned nucleic acids may be biotinylated (for example using enzymatic incorporate of biotinylated nucleotides, for by photoactivated cross-linking of biotin). Biotinylated nucleic acids can then be captured on streptavidin-coated beads, as is known in the art. Similarly, other hapten-receptor combinations can be used, such as digoxigenin and anti-digoxigenin antibodies. Alternatively, chemical groups can be added in the form of derivatized nucleotides, that can them be used to add the nucleic acid to the surface.

Preferred attachments are covalent, although even relatively weak interactions (i.e. non-covalent) can be sufficient to attach a nucleic acid to a surface, if there are multiple sites of attachment per each

nucleic acid. Thus, for example, electrostatic interactions can be used for attachment, for example by having beads carrying the opposite charge to the bioactive agent.

Similarly, affinity capture utilizing hybridization can be used to attach cloned nucleic acids to beads. For example, as is known in the art, polyA+RNA is routinely captured by hybridization to oligo-dT beads; this may include oligo-dT capture followed by a cross-linking step, such as psoralen crosslinking). If the nucleic acids of interest do not contain a polyA tract, one can be attached by polymerization with terminal transferase, or via ligation of an oligoA linker, as is known in the art.

Alternatively, chemical crosslinking may be done, for example by photoactivated crosslinking of thymidine to reactive groups, as is known in the art.

In general, special methods are required to decode clonal arrays, as is more fully outlined below.

As described above generally for proteins, nucleic acid bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eukaryotic genomes may be used as is outlined above for proteins.

In general, probes of the present invention are designed to be complementary to a target sequence (either the target analyte sequence of the sample or to other probe sequences, as is described herein), such that hybridization of the target and the probes of the present invention occurs. This complementarily need not be perfect; there may be any number of base pair mismatches that will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under the selected reaction conditions. High stringency conditions are known in the art; see for example Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2d Edition, 1989, and *Short Protocols in Molecular Biology*, ed. Ausubel, et al., both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10 C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are

occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 C for short probes (e.g. 10 to 50 nucleotides) and at least about 60 C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Maniatis and Ausubel, supra, and Tijssen, supra.

The term 'target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

In a preferred embodiment, the bioactive agents are organic chemical moieties, a wide variety of which are available in the literature.

In a preferred embodiment, each bead comprises a single type of bioactive agent, although a plurality of individual bioactive agents are preferably attached to each bead. Similarly, preferred embodiments utilize more than one microsphere containing a unique bioactive agent; that is, there is redundancy built into the system by the use of subpopulations of microspheres, each microsphere in the subpopulation containing the same bioactive agent.

As will be appreciated by those in the art, the bioactive agents may either be synthesized directly on the beads, or they may be made and then attached after synthesis. In a preferred embodiment, linkers are used to attach the bioactive agents to the beads, to allow both good attachment, sufficient flexibility to allow good interaction with the target molecule, and to avoid undesirable binding reactions.

In a preferred embodiment, the bioactive agents are synthesized directly on the beads. As is known in the art, many classes of chemical compounds are currently synthesized on solid supports, including beads, such as peptides, organic moieties, and nucleic acids.

In a preferred embodiment, the bioactive agents are synthesized first, and then covalently attached to the beads. As will be appreciated by those in the art, this will be done depending on the composition

of the bioactive agents and the beads. The functionalization of solid support surfaces such as certain polymers with chemically reactive groups such as thiols, amines, carboxyls, etc. is generally known in the art. Accordingly, "blank" microspheres may be used that have surface chemistries that facilitate the attachment of the desired functionality by the user. Some examples of these surface chemistries for blank microspheres include, but are not limited to, amino groups including aliphatic and aromatic amines, carboxylic acids, aldehydes, amides, chloromethyl groups, hydrazide, hydroxyl groups, sulfonates and sulfates.

These functional groups can be used to add any number of different candidate agents to the beads, generally using known chemistries. For example, candidate agents containing carbohydrates may be attached to an amino-functionalized support; the aldehyde of the carbohydrate is made using standard techniques, and then the aldehyde is reacted with an amino group on the surface. In an alternative embodiment, a sulfhydryl linker may be used. There are a number of sulfhydryl reactive linkers known in the art such as SPDP, maleimides, -haloacetyls, and pyridyl disulfides (see for example the 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference) which can be used to attach cysteine containing proteinaceous agents to the support. Alternatively, an amino group on the candidate agent may be used for attachment to an amino group on the surface. For example, a large number of stable bifunctional groups are well known in the art, including homobifunctional and heterobifunctional linkers (see Pierce Catalog and Handbook, pages 155-200). In an additional embodiment, carboxyl groups (either from the surface or from the candidate agent) may be derivatized using well known linkers (see the Pierce catalog). For example, carbodiimides activate carboxyl groups for attack by good nucleophiles such as amines (see Torchilin et al., Critical Rev. Therapeutic Drug Carrier Systems, 7(4):275-308 (1991), expressly incorporated herein). Proteinaceous candidate agents may also be attached using other techniques known in the art, for example for the attachment of antibodies to polymers; see Slinkin et al., Bioconj. Chem. 2:342-348 (1991); Torchilin et al., *supra*; Trubetskoy et al., Bioconj. Chem. 3:323-327 (1992); King et al., Cancer Res. 54:6176-6185 (1994); and Wilbur et al., Bioconjugate Chem. 5:220-235 (1994), all of which are hereby expressly incorporated by reference). It should be understood that the candidate agents may be attached in a variety of ways, including those listed above. Preferably, the manner of attachment does not significantly alter the functionality of the candidate agent; that is, the candidate agent should be attached in such a flexible manner as to allow its interaction with a target.

Specific techniques for immobilizing enzymes on microspheres are known in the prior art. In one case, NH_2 surface chemistry microspheres are used. Surface activation is achieved with a 2.5% glutaraldehyde in phosphate buffered saline (10 mM) providing a pH of 6.9. (138 mM NaCl, 2.7 mM, KCl). This is stirred on a stir bed for approximately 2 hours at room temperature. The microspheres are then rinsed with ultrapure water plus 0.01% tween 20 (surfactant) -0.02%, and rinsed again with a

pH 7.7 PBS plus 0.01% tween 20. Finally, the enzyme is added to the solution, preferably after being prefiltered using a 0.45 m amicon micropure filter.

In some embodiments, the microspheres may additionally comprise identifier binding ligands for use in certain decoding systems. By "identifier binding ligands" or "IBLs" herein is meant a compound that will specifically bind a corresponding decoder binding ligand (DBL) to facilitate the elucidation of the identity of the bioactive agent attached to the bead. That is, the IBL and the corresponding DBL form a binding partner pair. By "specifically bind" herein is meant that the IBL binds its DBL with specificity sufficient to differentiate between the corresponding DBL and other DBLs (that is, DBLs for other IBLs), or other components or contaminants of the system. The binding should be sufficient to remain bound under the conditions of the decoding step, including wash steps to remove non-specific binding. In some embodiments, for example when the IBLs and corresponding DBLs are proteins or nucleic acids, the dissociation constants of the IBL to its DBL will be less than about 10^{-4}-10^{-6} M^{-1}, with less than about 10^{-5} to 10^{-9} M^{-1} being preferred and less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred.

IBL-DBL binding pairs are known or can be readily found using known techniques. For example, when the IBL is a protein, the DBLs include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)) or small molecules, or vice versa (the IBL is an antibody and the DBL is a protein). Metal ion- metal ion ligands or chelators pairs are also useful. Antigen-antibody pairs, enzymes and substrates or inhibitors, other protein-protein interacting pairs, receptor-ligands, complementary nucleic acids (including nucleic acid molecules that form triple helices), and carbohydrates and their binding partners are also suitable binding pairs. Nucleic acid - nucleic acid binding proteins pairs are also useful, including single-stranded or double-stranded nucleic acid binding proteins, and small molecule nucleic acid binding agents. Similarly, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptamers" can be developed for binding to virtually any target; such an aptamer-target pair can be used as the IBL-DBL pair. Similarly, there is a wide body of literature relating to the development of binding pairs based on combinatorial chemistry methods.

In a preferred embodiment, the IBL is a protein, particularly an enzyme, that is conjugated to a bead. One advantage of using proteins/enzymes as IBLs is that many proteins and enzymes are commercially available in relatively pure preparations. Proteins contain many suitable functional groups for attachment to beads. Moreover, the identity of many small molecules including substrates that bind the proteins/enzymes are known. Such small molecules serve as the DBL. In addition, the small molecule can serve as the IBL and the enzyme as the DBL. As will be appreciated by those in the art, combinations of enzymes and small molecules can be used as well, or multiple enzymes and small molecules. Another potential advantage relates to the purification of the labeled proteins. If one

conjugates a fluorophore to a recrystallizable protein, then the unlabeled protein can be removed from labeled protein by use of crystallization. By filtering off the crystalline solid, the labeled protein will be concentrated in the filtrate.

In an alternative embodiment, antigen-antibody pairs are used as DBL-IBL combinations. In one embodiment antibodies are conjugated to different labels, defined below, that are then used to decode antigen-labeled beads.

In a preferred embodiment, the IBL is a molecule whose color or luminescence properties change in the presence of a selectively-binding DBL.

In one embodiment, the DBL may be attached to a bead, i.e. a "decoder bead", that may carry a label such as a fluorophore.

In a preferred embodiment, the IBL-DBL pair comprise substantially complementary single-stranded nucleic acids. In this embodiment, the binding ligands can be referred to as "identifier probes" and "decoder probes". Generally, the identifier and decoder probes range from about 4 basepairs in length to about 1000, with from about 6 to about 100 being preferred, and from about 8 to about 40 being particularly preferred. What is important is that the probes are long enough to be specific, i.e. to distinguish between different IBL-DBL pairs, yet short enough to allow both a) dissociation, if necessary, under suitable experimental conditions, and b) efficient hybridization.

In a preferred embodiment, as is more fully outlined below, the IBLs do not bind to DBLs. Rather, the IBLs are used as identifier moieties ("IMs") that are identified directly, for example through the use of mass spectroscopy.

Alternatively, in a preferred embodiment, the IBL and the bioactive agent are the same moiety; thus, for example, as outlined herein, particularly when no optical signatures are used, the bioactive agent can serve as both the identifier and the agent. For example, in the case of nucleic acids, the bead-bound probe (which serves as the bioactive agent) can also bind decoder probes, to identify the sequence of the probe on the bead. Thus, in this embodiment, the DBLs bind to the bioactive agents. This is particularly useful as this embodiment can give information about the array or the assay in addition to decoding. For example, as is more fully described below, the use of the DBLs allows array calibration and assay development. This may be done even if the DBLs are not used as such; for example in non-random arrays, the use of these probe sets can allow array calibration and assay development even if decoding is not required.

In a preferred embodiment, the microspheres do not contain an optical signature. That is, as outlined in U.S.S.N.s 08/818,199 and 09/151,877, previous work had each subpopulation of microspheres comprising a unique optical signature or optical tag that is used to identify the unique bioactive agent of that subpopulation of microspheres; that is, decoding utilizes optical properties of the beads such that a bead comprising the unique optical signature may be distinguished from beads at other locations with different optical signatures. Thus the previous work assigned each bioactive agent a unique optical signature such that any microspheres comprising that bioactive agent are identifiable on the basis of the signature. These optical signatures comprised dyes, usually chromophores or fluorophores, that were entrapped or attached to the beads themselves. Diversity of optical signatures utilized different fluorochromes, different ratios of mixtures of fluorochromes, and different concentrations (intensities) of fluorochromes.

Thus, the present invention does not rely solely on the use of optical properties to decode the arrays. However, as will be appreciated by those in the art, it is possible in some embodiments to utilize optical signatures as an additional coding method, in conjunction with the present system. Thus, for example, as is more fully outlined below, the size of the array may be effectively increased while using a single set of decoding moieties in several ways, one of which is the use in combination with optical signatures one beads. Thus, for example, using one "set" of decoding molecules, the use of two populations of beads, one with an optical signature and one without, allows the effective doubling of the array size. The use of multiple optical signatures similarly increases the possible size of the array.

In a preferred embodiment, each subpopulation of beads comprises a plurality of different IBLs. By using a plurality of different IBLs to encode each bioactive agent, the number of possible unique codes is substantially increased. That is, by using one unique IBL per bioactive agent, the size of the array will be the number of unique IBLs (assuming no "reuse" occurs, as outlined below). However, by using a plurality of different IBLs per bead, n, the size of the array can be increased to 2^n, when the presence or absence of each IBL is used as the indicator. For example, the assignment of 10 IBLs per bead generates a 10 bit binary code, where each bit can be designated as "1" (IBL is present) or "0" (IBL is absent). A 10 bit binary code has 2^{10} possible variants However, as is more fully discussed below, the size of the array may be further increased if another parameter is included such as concentration or intensity; thus for example, if two different concentrations of the IBL are used, then the array size increases as 3^n. Thus, in this embodiment, each individual bioactive agent in the array is assigned a combination of IBLs, which can be added to the beads prior to the addition of the bioactive agent, after, or during the synthesis of the bioactive agent, i.e. simultaneous addition of IBLs and bioactive agent components.

Alternatively, when the bioactive agent is a polymer of different residues, i.e. when the bioactive agent is a protein or nucleic acid, the combination of different IBLs can be used to elucidate the sequence of the protein or nucleic acid.

Thus, for example, using two different IBLs (IBL1 and IBL2), the first position of a nucleic acid can be elucidated: for example, adenosine can be represented by the presence of both IBL1 and IBL2; thymidine can be represented by the presence of IBL1 but not IBL2, cytosine can be represented by the presence of IBL2 but not IBL1, and guanosine can be represented by the absence of both. The second position of the nucleic acid can be done in a similar manner using IBL3 and IBL4; thus, the presence of IBL1, IBL2, IBL3 and IBL4 gives a sequence of AA; IBL1, IBL2, and IBL3 shows the sequence AT; IBL1, IBL3 and IBL4 gives the sequence TA, etc. The third position utilizes IBL5 and IBL6, etc. In this way, the use of 20 different identifiers can yield a unique code for every possible 10-mer.

The system is similar for proteins but requires a larger number of different IBLs to identify each position, depending on the allowed diversity at each position. Thus for example, if every amino acid is allowed at every position, five different IBLs are required for each position. However, as outlined above, for example when using random peptides as the bioactive agents, there may be bias built into the system; not all amino acids may be present at all positions, and some positions may be preset; accordingly, it may be possible to utilize four different IBLs for each amino acid.

In this way, a sort of "bar code" for each sequence can be constructed; the presence or absence of each distinct IBL will allow the identification of each bioactive agent.

In addition, the use of different concentrations or densities of IBLs allows a "reuse" of sorts. If, for example, the bead comprising a first agent has a 1X concentration of IBL, and a second bead comprising a second agent has a 10X concentration of IBL, using saturating concentrations of the corresponding labelled DBL allows the user to distinguish between the two beads.

In addition, the use of different ratios of labeled to unlabeled IBLs allows for a differentiation of the intensity rather than the concentration of IBLs. For example, if a bead comprising a first agent has a ratio of 1:1 (labeled: unlabeled IBL), while a second bead comprising a second agent has a ration of 1:10 (labeled: unlabeled IBL), the user will distinguish the different intensities between the two beads. This procedure offers the advantage that a broad spectrum of intensities of labels can be created using only a single label.

Once the microspheres comprising the candidate agents and the unique IBLs are generated, they are added to the substrate to form an array. It should be noted that while most of the methods described

herein add the beads to the substrate prior to the assay, the order of making, using and decoding the array can vary. For example, the array can be made, decoded, and then the assay done. Alternatively, the array can be made, used in an assay, and then decoded; this may find particular use when only a few beads need be decoded. Alternatively, the beads can be added to the assay mixture, i.e. the sample containing the target analytes, prior to the addition of the beads to the substrate; after addition and assay, the array may be decoded. This is particularly preferred when the sample comprising the beads is agitated or mixed; this can increase the amount of target analyte bound to the beads per unit time, and thus (in the case of nucleic acid assays) increase the hybridization kinetics. This may find particular use in cases where the concentration of target analyte in the sample is low; generally, for low concentrations, long binding times must be used.

In addition, adding the beads to the assay mixture can allow sorting or selection. For example, a large library of beads may be added to a sample, and only those beads that bind the sample may be added to the substrate. For example, if the target analyte is fluorescently labeled (either directly (for example by the incorporation of labels into nucleic acid amplification reactions) or indirectly (for example via the use of sandwich assays)), beads that exhibit fluorescence as a result of target analyte binding can be sorted via Fluorescence Activated Cell Sorting (FACS) and only these beads added to an array and subsequently decoded. Similarly, the sorting may be accomplished through affinity techniques; affinity columns comprising the target analytes can be made, and only those beads which bind are used on the array. Similarly, two bead systems can be used; for example, magnetic beads comprising the target analytes can be used to "pull out" those beads that will bind to the targets, followed by subsequent release of the magnetic beads (for example via temperature elevation) and addition to an array.

In general, the methods of making the arrays and of decoding the arrays is done to maximize the number of different candidate agents that can be uniquely encoded. The compositions of the invention may be made in a variety of ways. In general, the arrays are made by adding a solution or slurry comprising the beads to a surface containing the sites for association of the beads. This may be done in a variety of buffers, including aqueous and organic solvents, and mixtures. The solvent can evaporate, and excess beads removed.

In a preferred embodiment, when non-covalent methods are used to associate the beads to the array, a novel method of loading the beads onto the array is used. This method comprises exposing the array to a solution of particles (including microspheres and cells) and then applying energy, e.g. agitating or vibrating the mixture. This results in an array comprising more tightly associated particles, as the agitation is done with sufficient energy to cause weakly-associated beads to fall off (or out, in the case of wells). These sites are then available to bind a different bead. In this way, beads that exhibit a high affinity for the sites are selected. Arrays made in this way have two main advantages as

compared to a more static loading: first of all, a higher percentage of the sites can be filled easily, and secondly, the arrays thus loaded show a substantial decrease in bead loss during assays. Thus, in a preferred embodiment, these methods are used to generate arrays that have at least about 50% of the sites filled, with at least about 75% being preferred, and at least about 90% being particularly preferred. Similarly, arrays generated in this manner preferably lose less than about 20% of the beads during an assay, with less than about 10% being preferred and less than about 5% being particularly preferred.

In this embodiment, the substrate comprising the surface with the discrete sites is immersed into a solution comprising the particles (beads, cells, etc.). The surface may comprise wells, as is described herein, or other types of sites on a patterned surface such that there is a differential affinity for the sites. This differnetial affinity results in a competitive process, such that particles that will associate more tightly are selected. Preferably, the entire surface to be "loaded" with beads is in fluid contact with the solution. This solution is generally a slurry ranging from about 10,000:1 beads:solution (vol:vol) to 1:1. Generally, the solution can comprise any number of reagents, including aqueous buffers, organic solvents, salts, other reagent components, etc. In addition, the solution preferably comprises an excess of beads; that is, there are more beads than sites on the array. Preferred embodiments utilize two-fold to billion-fold excess of beads.

The immersion can mimic the assay conditions; for example, if the array is to be "dipped" from above into a microtiter plate comprising samples, this configuration can be repeated for the loading, thus minimizing the beads that are likely to fall out due to gravity.

Once the surface has been immersed, the substrate, the solution, or both are subjected to a competitive process, whereby the particles with lower affinity can be disassociated from the substrate and replaced by particles exhibiting a higher affinity to the site. This competitive process is done by the introduction of energy, in the form of heat, sonication, stirring or mixing, vibrating or agitating the solution or substrate, or both.

A preferred embodiment utilizes agitation or vibration. In general, the amount of manipulation of the substrate is minimized to prevent damage to the array; thus, preferred embodiments utilize the agitation of the solution rather than the array, although either will work. As will be appreciated by those in the art, this agitation can take on any number of forms, with a preferred embodiment utilizing microtiter plates comprising bead solutions being agitated using microtiter plate shakers.

The agitation proceeds for a period of time sufficient to load the array to a desired fill. Depending on the size and concentration of the beads and the size of the array, this time may range from about 1 second to days, with from about 1 minute to about 24 hours being preferred.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1921

In a preferred embodiment, the substrate is pressed into a mixture of dry beads (although slurries may be used as well), and then tapped to remove excess beads.

It should be noted that not all sites of an array may comprise a bead; that is, there may be some sites on the substrate surface which are empty. In addition, there may be some sites that contain more than one bead, although this is not preferred.

In some embodiments, for example when chemical attachment is done, it is possible to associate the beads in a non-random or ordered way. For example, using photoactivatible attachment linkers or photoactivatible adhesives or masks, selected sites on the array may be sequentially rendered suitable for attachment, such that defined populations of beads are laid down.

The arrays of the present invention are constructed such that information about the identity of the candidate agent is built into the array, such that the random deposition of the beads in the fiber wells can be "decoded" to allow identification of the candidate agent at all positions. This may be done in a variety of ways, and either before, during or after the use of the array to detect target molecules.

Thus, after the array is made, it is "decoded" in order to identify the location of one or more of the bioactive agents, i.e. each subpopulation of beads, on the substrate surface.

In a preferred embodiment, a selective decoding system is used. In this case, only those microspheres exhibiting a change in the optical signal as a result of the binding of a target analyte are decoded. This is commonly done when the number of "hits", i.e. the number of sites to decode, is generally low. That is, the array is first scanned under experimental conditions in the absence of the target analytes. The sample containing the target analytes is added, and only those locations exhibiting a change in the optical signal are decoded. For example, the beads at either the positive or negative signal locations may be either selectively tagged or released from the array (for example through the use of photocleavable linkers), and subsequently sorted or enriched in a fluorescence-activated cell sorter (FACS). That is, either all the negative beads are released, and then the positive beads are either released or analyzed in situ, or alternatively all the positives are released and analyzed. Alternatively, the labels may comprise halogenated aromatic compounds, and detection of the label is done using for example gas chromatography, chemical tags, isotopic tags, or mass spectral tags.

As will be appreciated by those in the art, this may also be done in systems where the array is not decoded; i.e. there need not ever be a correlation of bead composition with location. In this embodiment, the beads are loaded on the array, and the assay is run. The "positives", i.e. those beads displaying a change in the optical signal as is more fully outlined below, are then "marked" to

distinguish or separate them from the "negative" beads. This can be done in several ways, preferably using fiber optic arrays. In a preferred embodiment, each bead contains a fluorescent dye. After the assay and the identification of the "positives" or "active beads", light is shown down either only the positive fibers or only the negative fibers, generally in the presence of a light-activated reagent (typically dissolved oxygen). In the former case, all the active beads are photobleached. Thus, upon non-selective release of all the beads with subsequent sorting, for example using a fluorescence activated cell sorter (FACS) machine, the non-fluorescent active beads can be sorted from the fluorescent negative beads. Alternatively, when light is shown down the negative fibers, all the negatives are non-fluorescent and the the postives are fluorescent, and sorting can proceed. The characterization of the attached bioactive agent may be done directly, for example using mass spectroscopy.

Alternatively, the identification may occur through the use of identifier moieties ("IMs"), which are similar to IBLs but need not necessarily bind to DBLs. That is, rather than elucidate the structure of the bioactive agent directly, the composition of the IMs may serve as the identifier. Thus, for example, a specific combination of IMs can serve to code the bead, and be used to identify the agent on the bead upon release from the bead followed by subsequent analysis, for example using a gas chromatograph or mass spectroscope.

Alternatively, rather than having each bead contain a fluorescent dye, each bead comprises a non-fluorescent precursor to a fluorescent dye. For example, using photocleavable protecting groups, such as certain ortho-nitrobenzyl groups, on a fluorescent molecule, photoactivation of the fluorochrome can be done. After the assay, light is shown down again either the "positive" or the "negative" fibers, to distinguish these populations. The illuminated precursors are then chemically converted to a fluorescent dye. All the beads are then released from the array, with sorting, to form populations of fluorescent and non-fluorescent beads (either the positives and the negatives or vice versa).

In an alternate preferred embodiment, the sites of association of the beads (for example the wells) include a photopolymerizable reagent, or the photopolymerizable agent is added to the assembled array. After the test assay is run, light is shown down again either the "positive" or the "negative" fibers, to distinquish these populations. As a result of the irradiation, either all the positives or all the negatives are polymerized and trapped or bound to the sites, while the other population of beads can be released from the array.

In a preferred embodiment, the location of every bioactive agent is determined using decoder binding ligands (DBLs). As outlined above, DBLs are binding ligands that will either bind to identifier binding ligands, if present, or to the bioactive agents themselves, preferably when the bioactive agent is a nucleic acid or protein.

In a preferred embodiment, as outlined above, the DBL binds to the IBL.

In a preferred embodiment, the bioactive agents are single-stranded nucleic acids and the DBL is a substantially complementary single-stranded nucleic acid that binds (hybridizes) to the bioactive agent, termed a decoder probe herein. A decoder probe that is substantially complementary to each candidate probe is made and used to decode the array. In this embodiment, the candidate probes and the decoder probes should be of sufficient length (and the decoding step run under suitable conditions) to allow specificity; i.e. each candidate probe binds to its corresponding decoder probe with sufficient specificity to allow the distinction of each candidate probe.

In a preferred embodiment, the DBLs are either directly or indirectly labeled. By "labeled" herein is meant that a compound has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) magnetic, electrical, thermal; and c) colored or luminescent dyes; although labels include enzymes and particles such as magnetic particles as well. Examples of such lables include: peroxidase; alkaline phosphatase; biotin; urease; E alactosidase; BSA/KLH; gold particles; quantum dots; redox indicators; pH indicators; E-lactamase; luciferase; TSA; SPA; chemiluminescence; sonoluminescence; fluorophores; phosphors; and, fluorescent metal ion sensors. Preferred labels include luminescent labels. In a preferred embodiment, the DBL is directly labeled, that is, the DBL comprises a label. In an alternate embodiment, the DBL is indirectly labeled; that is, a labeling binding ligand (LBL) that will bind to the DBL is used. In this embodiment, the labeling binding ligand-DBL pair can be as described above for IBL-DBL pairs. Suitable labels include, but are not limited to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue™, Texas Red, FITC, PE, cy3, cy5 and others described in the 6th Edition of the Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference. In addition, ion-sensitive dyes such as bis-BITC, Newport green, fluo 3, fura red find use in the invention.

In one embodiment, the label is a molecule whose color or luminescence properties change in the presence of the IBL, due to a change in the local environment. For example, the label may be: (1) a fluorescent pH indicator whose emission intensity changes with pH; (2) a fluorescent ion indicator, whose emission properties change with ion concentration; or (3) a fluorescent molecule such as an ethidium salt whose fluorescence intensity increases in hydrophobic environments.

In one embodiment ion sensitive dyes are used for decoding. That is, dyes that fluoresce in the presence of certain ions are used. In a preferred embodiment the ion sensitive dyes are attached to the beads. An advantage of attaching dyes directly to microspheres is that the cost associated with oligonucleotide synthesis is avoided.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1924

Accordingly, when such dyes are attached to the beads they can be used to encode a specific nucleotide sequence, or the identity of the bioactive agent. Once the beads are distributed on the array, the array is contacted with buffers that include certain ions. In the presence of appropriate ion, certain dyes will fluoresce and fluorescence is detected. In a preferred embodiment, the array is contacted with different buffers sequentially. Each buffer contains a different ion. Preferably the array is washed between buffer solutions so that any signal produced as a result of the contact with the ion solution is diminished.

In this embodiment multiple different ion sensitive dyes can be used. That is, preferably, more than 2 with more than 3 or 4 dyes are attached to the microspheres. In some embodiments, more than 10 dyes are attached to the microsphere.

One advantage to using ion sensitive dyes is that fluorescence bleed-through is diminished. That is, because the response of the dyes is either positive or negative, i.e. they interact or they don't, bleed-through is avoided.

In a preferred embodiment, decoding of self-assembled random arrays is done on the basis of pH titration. In this embodiment, in addition to bioactive agents, the beads comprise optical signatures, wherein the optical signatures are generated by the use of pH-responsive dyes (sometimes referred to herein as "pH dyes") such as fluorophores. This embodiment is similar to that outlined in PCT US98/05025 and U.S.S.N. 09/151,877, both of which are expressly incorporated by reference, except that the dyes used in the present invention exhibits changes in fluorescence intensity (or other properties) when the solution pH is adjusted from below the pKa to above the pKa (or vice versa). In a preferred embodiment, a set of pH dyes is used, each with a different pKa, preferably separated by at least 0.5 pH units. Preferred embodiments utilize a pH dye set of pKa's of 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11, and 11.5. Each bead can contain any subset of the pH dyes, and in this way a unique code for the bioactive agent is generated. Thus, the decoding of an array is achieved by titrating the array from pH 1 to pH 13, and measuring the fluorescence signal from each bead as a function of solution pH.

In one embodiment, the decoder probes are labeled in situ; that is, they need not be labeled prior to the decoding reaction. In this embodiment, the incoming decoder probe is shorter than the candidate probe, creating a 5' "overhang" on the decoding probe. The addition of labeled ddNTPs (each labeled with a unique tag) and a polymerase will allow the addition of the tags in a sequence specific manner, thus creating a sequence-specific pattern of signals. Similarly, other modifications can be done, including ligation, etc.

In one embodiment, a series of sequential extension and denaturing stages allows for positional decoding of the beads. That is, following one round of extension, the DBL is removed or stripped from

the IBL and a second round of extension is performed. The contents of any given primer extension reaction is shown in Figure 8.

In the primer extension method, up to four fluorescently-labeled dideoxynucleotide terminators each with a different colored label (e.g. using ABI's dRhodamine® terminators such as dR110,dR6G, dTAMRA, dROX or Big Dye® Terminators) could be used for 'painting'. The number of unique beads that can be decoded is given by $N= y^x$, where n= the number of unique codes, y= the number of colors and x= the number of stages or primer extension reactions. This formula yields 4096 unique codes using 6 primer extension stages and four colors (i.e. $4096 = 4^6$).

In a preferred embodiment the bioactive agent is synthesized with encoding sequences that allows decoding to be accomplished independent of the useable portion of the bioactive agent, i.e. nucleic acid sequence. By "useable" portion is meant the adapter sequence or target sequence. In a preferred embodiment the bioactive agent also is synthesized with a primer region to facilitate sequencing or primer extension analysis of the encoding sequence (see Figure 12). As shown in Figure 12, a primer complementary to a portion of the ssDNA on the bead is extended by single base polymerase extension using dye-labeled chain terminating nucleotides. After incorporation of a labeled, i.e. fluorescent, chain terminator and reading of the array, i.e. detecting the label, the fluorescence is removed. Removal of the fluorescence is accomplished directly when reversible chain terminators are used (see Canard and Sarfati, Gene, 148(1): 1-6, 1994, which is expressly incorporated herein by reference). However, in a preferred embodiment the primer is dissociated from the bead and a subsequent primer is annealed. Preferably the subsequent primer is 1 nucleotide longer than the former primer so that an additional nucleotide in the encoding sequence can be interrogated. In a preferred embodiment the subsequent primer is lengthened by the addition of a degenerate base. This can be accomplished either by incorporating all 4 nucleotides into the additional position of the primer, or by the incorporation of a universal base into the primer. The new primer is then subject to extension as described above. The primer extension can be repeated as many times as are necessary to decode the microsphere.

Decoding by sequencing has the advantage that encoding a vast number of distinct codes by using only a small number of nucleotides. That is, the information content of the encoding region depends on the number of combinations of the bases used, i.e. 4 when A, C, G and T are used, that are present at each position and the length of the encoding region. When 4 nucleotides are used, there are up to fifteen possible combinations of nucleotides at each position (A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T, A/C/G, A/C/T, A/G/T, C/G/G and A/C/G/T). As such, the information content is 15^n, where n is equal to the length of the encoding sequence. As such, for example, with just 3 base positions, 3375 different codes can be generated.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1926

In addition an additional preferred embodiment includes the combination of decoding by sequencing with decoding by hybridization. In one application of decoding by sequencing, universal primers, each one base longer than the previous, are sequentially hybridized to the array. The base adjacent to the 3' end of the universal primer is determined by a single base extension reaction employing labeled-chain terminators, preferably using four different color labels one for each base. Since a hybridization event must be performed for each base read, once can improve the decoding power by using the hybridization event to also decode. However, rather than using universal primers, zipcode sequences would be used instead (see Figure 13)

The placement of the encoding sequences can be either on the "bead-side" of the gene-specific sequence or on the terminal side. Placement of the encoding sequences on the terminal side offers that advantage of incorporating a cleavable linker between the encoding sequences and the gene-specific sequence (Fig. 14). After completion of decoding, the encoding sequences can be removed to prevent possible interference in the analytical assay.

In one embodiment following decoding, the label is extinguished. By "extinguished" is meant that the signal from the label is eliminated. This is accomplished by a variety of techniques including cleaving the label or destroying the signal of the label. That is, in one embodiment, the label is attached to the DBL via a cleavable linker. Following a decoding step, the linker is cleaved thereby releasing the label. In one embodiment cleavage of the label results in deprotection of the bioactive agent, for example when the bioactive agent contains a protecting group. Alternatively, cleavage of the label preserves the nature of the bioactive agent. That is, what is important is that cleavage of the label does not interfere with the bioactive agent.

In an alternative embodiment, the label is not cleaved, but rather is eliminated by destroying the signal of the label. That is, following decoding, the label is contacted with an agent that degrades or destroys the signal. For example, organic fluorescent dyes are subject to chemical degradation under appropriate conditions. Thus, following a decoding step where the fluorescent signal of the dyes are detected, the fluorophore is destroyed by an appropriate chemical solution that degrades the fluorescent molecule sufficient leaving no trace of fluorescence. In this way, beads are tagged with fluorescent labels, i.e. barcodes of sorts, their location or identity deciphered and recorded. The beads are then exposed to the "fluorescent destroying solution" and the fluorescence is eliminated, allowing an analytical assay to be conducted. Suitable fluorescent dyes include but are not limited to various bodipy, or Alexa dyes from Molecular Probes. The "fluorescent destroying solution" is a reducing or oxidizing solution, such as 0.01 SM sodium periodate.

In a preferred embodiment not only is the label cleaved, but the encoding nucleic acid is cleaved. This method finds particular use in removing residual signal following a decoding step. Accordingly, in a preferred embodiment a cleavable linker can be used to bridge the adapter and beads. After

decoding, the adapter oligonucleotide(s) can be easily removed through cleavable linkers. There are many commercially available cleavable linkers. They are routinely used in the combinatorial solid-phase synthesis field. The linkers can be cleaved under acid, base or photo radiation conditions.

In addition, restriction enzymes can be used to cleave a linker. Alternatively, because of the sequence specificity of restriction enzymes, they also are used to cleave encoding nucleic acids. A change in signal following incubation with a sequence specific enzyme decodes the bead. An example is illustrative.

Consider the decoding of 6 bead types with three zip codes or adapters. In general, the strategy will have B=(2^n)-2. Where B is the number of types encoded and n is the number of zip codes. The bead types are z1, z2, z3, z1z2, z1z3, and z2z3. Here zizj indicates that both codes are on a bead. Every code is already dye labeled.

Generally,each code is designed with a site that can be specifically cut with some agent. This includes, but is not limited to, restriction enzymes. For ease of description, the process using restriction enzymes and zip codes that contain specific restriction sites is described. When restriction enzymes are used, the zip codes are double stranded. Each stage would consist of sequentially adding one restriction enzyme. An image is taken prior to stage 1, and the intensity of every bead is measured. Restriction enzyme 1 (Re1) is added. New intensity measurements are taken. The information of which beads decrease in intensity, and which remain the same. Detecting the pattern of beads for which the signal "decreased" and "stayed the same" uniquely determines the bead type. If we denote "decreased" by 1 and "stayed the same" by 0, then the decoding would give the following codes:

z1 : 1,0,0
z2 : 0,1,0
z3 : 0,0,1
z1z2 : 1,1,0
z1z3 : 1,0,1
z2z3 : 0,1,1.

The general approach uses n codes, which would give (2^n)-2 types. Subtracting 2 leaves off the all off state, and the all zip codes used state.

In another embodiment the array is decoded by using exonuclease enzyme. In this embodiment the microspheres are encoded with dye-labeled nucleotides. Preferably each nucleotide is labeled with a discrete label. In this particular embodiment it is desirable to protect the bioactive agent from cleavage. This can be accomplished by a variety of known methods including hybridizing the bioactive agent to a complementary sequence or blocking the terminus of the bioactive agent. Alternatively, if

the exonuclease enzyme operates from 5' to 3', one can design zipcodes from 5' to 3' and SNP-gene specific oligos from 3' to 5'. This way zipcode sequences will be operated on by the exonuclease, but the SNP/gene-specific sequences will remain intact.

The microsphere is then contacted with an exonuclease that successively removes the labeled nucleotides of the encoding sequence. Monitoring of the signal of the microspheres should begin upon addition of the exonuclease and monitored in all channels that correspond to the signal emitted by the labels in use. For example, assume the microsphere has all the zipcodes available (with the above specifications), and is in a solution. At time T=0, the exonuclease enzyme is applied to the solution. At the same time, the acquisition system (CCD) starts taking images every few seconds (Figure 15). The assumption is that the reaction of the exonuclease is slow enough, so one would have ample time to take several images, as the reaction is proceeding. If this is not the case, the reaction can be slowed down by increasing the stringency conditions.

The exonuclease removes the nucleotides, one at a time. Therefore, ideally, one would notice a step reduction in the signal of the appropriate channel, once the nucleotide is removed (and moved away from the focal zone). The complete set of waveforms (i.e., the four color channels, and from T0 to Tend) will provide a unique signature for every zipcode.

In practice, the removal of the nucleotides will not be completely synchronized on different oligos (as shown in Figure 15). For instance, for two oligos on the same bead (same zipcode), the exonuclease may have removed one nucleotide from one, and two nucleotides from the other. However, since one monitors many oligos from each bead, the overall trend should be preserved, i.e., in general the nucleotides closer to the end should be removed first. This means that in general, although the transitions on the waveforms may not be sharp, the signatures could still be unique.

Accordingly, the identification of the location of the individual beads (or subpopulations of beads) is done using one or more decoding steps comprising a binding between the labeled DBL and either the IBL or the bioactive agent (i.e. a hybridization between the candidate probe (sometimes referred to herein as a "decoding sequence) and the decoder probe when the bioactive agent is a nucleic acid). After decoding, the DBLs can be removed and the array can be used; however, in some circumstances, for example when the DBL binds to an IBL and not to the bioactive agent, the removal of the DBL is not required (although it may be desirable in some circumstances). In addition, as outlined herein, decoding may be done either before the array is used in an assay, during the assay, or after the assay.

In one embodiment, a single decoding step is done. In this embodiment, each DBL is labeled with a unique label, such that the the number of unique labels is equal to or greater than the number of bioactive agents (although in some cases, "reuse" of the unique labels can be done, as described herein; similarly, minor variants of candidate probes can share the same decoder, if the variants are

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1929

encoded in another dimension, i.e. in the bead size or label). For each bioactive agent or IBL, a DBL is made that will specifically bind to it and contains a unique label, for example one or more fluorochromes. Thus, the identity of each DBL, both its composition (i.e. its sequence when it is a nucleic acid) and its label, is known. Then, by adding the DBLs to the array containing the bioactive agents under conditions which allow the formation of complexes (termed hybridization complexes when the components are nucleic acids) between the DBLs and either the bioactive agents or the IBLs, the location of each DBL can be elucidated. This allows the identification of the location of each bioactive agent; the random array has been decoded. The DBLs can then be removed, if necessary, and the target sample applied.

In a preferred embodiment, the number of unique labels is less than the number of unique bioactive agents, and thus a sequential series of decoding steps are used. To facilitate the discussion, this embodiment is explained for nucleic acids, although other types of bioactive agents and DBLs are useful as well. In this embodiment, decoder probes are divided into **n** sets for decoding. The number of sets corresponds to the number of unique tags. Each decoder probe is labeled in **n** separate reactions with **n** distinct tags. All the decoder probes share the same **n** tags. Each pool of decoders contains only one of the **n** tag versions of each decoder, and no two decoder probes have the same sequence of tags across all the pools. The number of pools required for this to be true is determined by the number of decoder probes and the **n**. Hybridization of each pool to the array generates a signal at every address comprising an IBL. The sequential hybridization of each pool in turn will generate a unique, sequence-specific code for each candidate probe. This identifies the candidate probe at each address in the array. For example, if four tags are used, then 4 X **n** sequential hybridizations can ideally distinguish 4^n sequences, although in some cases more steps may be required. After the hybridization of each pool, the hybrids are denatured and the decoder probes removed, so that the probes are rendered single-stranded for the next hybridization (although it is also possible to hybridize limiting amounts of target so that the available probe is not saturated. Sequential hybridizations can be carried out and analyzed by subtracting pre-existing signal from the previous hybridization).

An example is illustrative. Assuming an array of 16 probe nucleic acids (numbers 1-16), and four unique tags (four different fluors, for example; labels A-D). Decoder probes 1-16 are made that correspond to the probes on the beads. The first step is to label decoder probes 1-4 with tag A, decoder probes 5-8 with tag B, decoder probes 9-12 with tag C, and decoder probes 13-16 with tag D. The probes are mixed and the pool is contacted with the array containing the beads with the attached candidate probes. The location of each tag (and thus each decoder and candidate probe pair) is then determined. The first set of decoder probes are then removed. A second set is added, but this time, decoder probes 1, 5, 9 and 13 are labeled with tag A, decoder probes 2, 6, 10 and 14 are labeled with tag B, decoder probes 3, 7, 11 and 15 are labeled with tag C, and decoder probes 4, 8, 12 and 16 are labeled with tag D. Thus, those beads that contained tag A in both decoding steps contain candidate probe 1; tag A in the first decoding step and tag B in the second decoding step contain candidate probe 2; tag A in the first decoding step and tag C in the second step contain candidate probe 3; etc.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1930

As will be appreciated by those in the art, the decoder probes can be made in any order and added in any order.

In addition, since the size of the array will be set by the number of unique decoding binding ligands, there are a number of techniques that allow new combinations and the "reuse" of DBLs to allow for a greater number of test sites. This may be done in a wide variety of ways, as outlined below.

In a preferred embodiment, a spatial or positional coding system is done. In this embodiment, there are sub-bundles or subarrays (i.e. portions of the total array) that are utilized. By analogy with the telephone system, each subarray is an "area code", that can have the same labels (i.e. telephone numbers) of other subarrays, that are separated by virtue of the location of the subarray. Thus, for example, the same unique labels can be reused from bundle to bundle. Thus, the use of 50 unique labels in combination with 100 different subarrays can form an array of 5000 different bioactive agents. In this embodiment, it becomes important to be able to identify one bundle from another; in general, this is done either manually or through the use of marker beads; these can be beads containing unique tags for each subarray, or the use of the same marker bead in differing amounts, or the use of two or more marker beads in different ratios.

In a preferred embodiment, the coding and decoding is accomplished by sequential loading of the microspheres into the array. As outlined above for spatial coding, in this embodiment, the optical signatures can be "reused". In this embodiment, the library of microspheres each comprising a different bioactive agent (or the subpopulations each comprise a different bioactive agent), is divided into a plurality of sublibraries; for example, depending on the size of the desired array and the number of unique tags, 10 sublibraries each comprising roughly 10% of the total library may be made, with each sublibrary comprising roughly the same unique tags. Then, the first sublibrary is added to the fiber optic bundle comprising the wells, and the location of each bioactive agent is determined, generally through the use of DBLs. The second sublibrary is then added, and the location of each bioactive agent is again determined. The signal in this case will comprise the signal from the "first" DBL and the "second" DBL; by comparing the two matrices the location of each bead in each sublibrary can be determined. Similarly, adding the third, fourth, etc. sublibraries sequentially will allow the array to be filled.

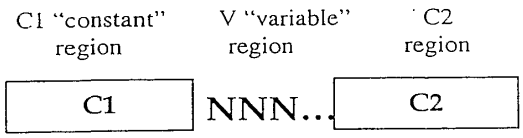
In a preferred embodiment, codes can be "shared" in several ways. In a first embodiment, a single code (i.e. IBL/DBL pair) can be assigned to two or more agents if the target analytes different sufficiently in their binding strengths. For example, two nucleic acid probes used in an mRNA quantitation assay can share the same code if the ranges of their hybridization signal intensities do not overlap. This can occur, for example, when one of the target sequences is always present at a much higher concentration than the other. Alternatively, the two target sequences might always be present at a similar concentration, but differ in hybridization efficiency.

Alternatively, a single code can be assigned to multiple agents if the agents are functionally equivalent. For example, if a set of oligonucleotide probes are designed with the common purpose of detecting the presence of a particular gene, then the probes are functionally equivalent, even though they may differ in sequence. Similarly, if classes or "families" of analytes are desired, all probes for different members of a class such as kinases or G-protein coupled receptors could share a code. Similarly, an array of this type could be used to detect homologs of known genes. In this embodiment, each gene is represented by a heterologous set of probes, hybridizing to different regions of the gene (and therefore differing in sequence). The set of probes share a common code. If a homolog is present, it might hybridize to some but not all of the probes. The level of homology might be indicated by the fraction of probes hybridizing, as well as the average hybridization intensity. Similarly, multiple antibodies to the same protein could all share the same code.

In a preferred embodiment, the set of codes can be increased using a kind of degeneracy on the IBLs on the beads. For most of the systems described herein, particularly when the IBL is a nucleic acid (including the case where the nucleic acid IBL is also the candidate agent), any single bead contains a single IBL sequence (decoding sequence). However, in this embodiment, it is possible to have a single bead comprise two or more decoding sequences, that differ at a particular nucleotide position. For a degeneracy of two at position X, there are 10 different possible combinations: the decoding sequence has an A at position X, a T, a C, a G, or a mixture of decoding sequences with either A/T, A/G, A/C, C/G, C/T or G/T. By using decoder probes that have different labels for each different nucleotide at the position, the number of codes can be increased, since beads containing the two different sequences will exhibit a mixture of signals. In addition, as will be appreciated by those in the art, rather than a "degeneracy" of two, degeneracies of three or four can be done as well. Thus, a bead containing three different decoding sequences will exhibit a signal comprising three different colors, etc.

Decoding the degenerate probes is done by a series of hybridizations to labeled oligonucleotides. Each hybridization decodes one position in the probe so the total number of hybridizations needed to decode a library is equal to the length of the variable region. Each hybridization is done with four oligonucleotides, each designed to hybridize specifically to a sequence containing a specific base at the interrogated position and each tagged with a distinctly colored fluorescent dye.

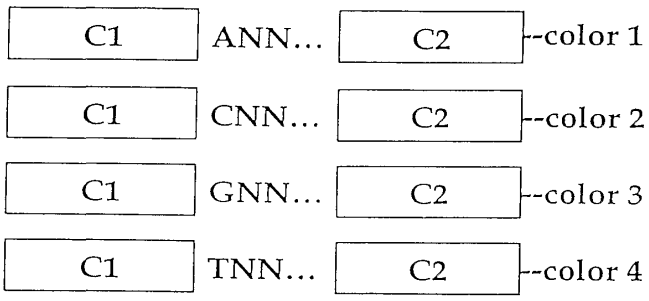
In a preferred embodiment, a degenerate probe sequence is attached to one or more constant probe regions. By "constant" probe region is meant a portion of a probe that is invariant among a population of decoder probes. In a particularly preferred embodiment, the degenerate or variable region is flanked on either side by constant regions. As such, each oligonucletoide has the following structure:



Although the C1 and C2 regions may be any length, preferably each is from 5 to 50 nucleotides in length. More preferably each is from 7 to 30 nucleotides in length and most preferably, each is about 10 bp in length. In one embodiment, the constant regions do not vary in sequence among the probes designed to decode a particular set of beads. As such, by using constant and variable regions (i.e. "CV" or "CVC" when two constant regions are used) in a set of probes, an array specific set or library of probes is generated.

The variable region contains a sequence that is unique to each address. Each "N" or variable position within the variable region can contain one of ten possibilities as described above. As such, the number of unique addresses in a library is determined by the length of the variable region: if L is length of the variable region, the number of unique addresses, A, is $A=10^{L}$.

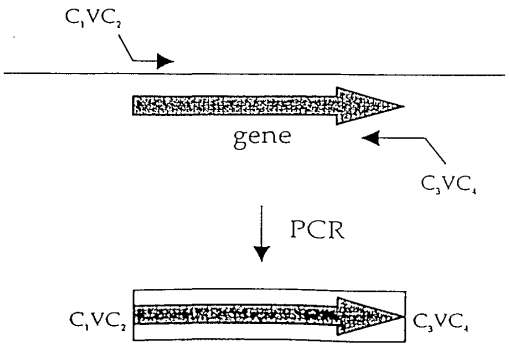
Accordingly, decoding the first base of the variable region of a decoding sequence is illustrated below:



Any address with an A at the first position would hybridize with the fourth oligonucleotide and give the corresponding color. An address with an A/C at the first position would hybridize with both the third and fourth decoders and give the corresponding combination of colors. By serial hybridizations, each using four decoders and each interrogating a different position of the V region, the entire sequence of each address can be determined. The "N' positions in the decoders are synthesized with degeneracies of the four standard bases.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1933

In one embodiment, CVC probes are incorporated into the probes of an oligonucleotide array during synthesis as is described for the addition of adapters; see U.S.S.Ns 09/553,993, filed April 20, 2000, 09/556,463, filed April 21, 2000, 60/160,917, filed October 22, 1999, and 60/135,123, filed May 20, 1999 and WO 00/63437, all of which are expressly incorporated herein by reference. In a similar fashion, the CVC probes could be synthesized in the 5-prime ends of PCR primers used to generate a PCR-product-based array. By incorporating different probes into the two primers of each PCR reaction, a combinatorial expansion of addresses is possible:



The constant regions in the C_1VC_2 addresses would be different in sequence from the C_3VC_4 addresses. Each would therefore be decoded with its own set of CVC complement decoders. If C_1VC_2 has a 3-base V region (1000 addresses) and C_3VC_4 has a 3-base V region (1,000 addresses), then the combination results in 1,000 x 1,000, or one million addresses.

In a preferred embodiment, there are additional ways to increase the number of unique or distinct tags. That is, the use of distinct attributes on each bead can be used to increase the number of codes. In addition, sequential decoding allows a reuse of codes in new ways. These attributes are independent of each other, thus allowing the number of codes to grow exponentially as a function of the number of decoding steps and the number of attributes (e.g. distinct codes). However, by increasing the amount of decoding information obtained in a single decoding step, the number of decoding steps is markedly reduced. By increasing the number of attributes per decoding step, fewer decoding steps are required for a given number of codes. Thus, in a preferred embodiment, a variety of methods are used to generate a number of codes for use in the process of decoding the arrays, while minimizing the necessary decoding steps. For example, a variety of different coding strategies can be combined: thus, different "colors", combinations of colors ("hues"), different intensities of colors or hues or both, different colors and different bead sizes, etc. can all be combined. Alternatively, the number of distinct codes is markedly increased.

In a preferred embodiment DBLs rely on attaching or embedding a quantitative or discrete set of physical attributes to the bead, i.e. labeling the bead. Preferred physical attributes of a bead include but are not limited to: surface "smoothness" or "roughness", color (fluorescent and otherwise), color

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1934

intensity, size, detectable chemical moieties, chemical reactivity, magnetization, pH sensitivity, energy transfer efficiency between dyes present, hydrophobicity, hydrophilicity, absorptivity, charge, pH sensitivity, etc. In general, physical attributes that can be measured optically are preferred, including color, size, surface smoothness etc.

In a preferred embodiment, the DBLs may be reused by having some subpopulations of beads comprise optical signatures. In a preferred embodiment, the optical signature is generally a mixture of reporter dyes, preferably fluoroscent. By varying both the composition of the mixture (i.e. the ratio of one dye to another) and the concentration of the dye (leading to differences in signal intensity), matrices of unique optical signatures may be generated. This may be done by covalently attaching the dyes to the surface of the beads, or alternatively, by entrapping the dye within the bead. The dyes may be chromophores or phosphors but are preferably fluorescent dyes, which due to their strong signals provide a good signal-to-noise ratio for decoding. Suitable dyes for use in the invention include those listed for labeling DBLs, above.

In a preferred embodiment, the encoding can be accomplished in a ratio of at least two dyes, although more encoding dimensions may be added in the size of the beads, for example. In addition, the labels are distinguishable from one another; thus two different labels may comprise different molecules (i.e. two different fluors) or, alternatively, one label at two different concentrations or intensity.

In a preferred embodiment, the dyes are covalently attached to the surface of the beads. This may be done as is generally outlined for the attachment of the bioactive agents, using functional groups on the surface of the beads. As will be appreciated by those in the art, these attachments are done to minimize the effect on the dye.

In a preferred embodiment, the dyes are non-covalently associated with the beads, generally by entrapping the dyes in the pores of the beads.

Additionally, encoding in the ratios of the two or more dyes, rather than single dye concentrations, is preferred since it provides insensitivity to the intensity of light used to interrogate the reporter dye's signature and detector sensitivity.

In alternative embodiments, additional encoding parameters can be added, such as microsphere size. For example, the use of different size beads may also allow the reuse of sets of DBLs; that is, it is possible to use microspheres of different sizes to expand the encoding dimensions of the microspheres. Optical fiber arrays can be fabricated containing pixels with different fiber diameters or cross-sections; alternatively, two or more fiber optic bundles, each with different cross-sections of the individual fibers, can be added together to form a larger bundle; or, fiber optic bundles with fiber of the same size cross-sections can be used, but just with different sized beads. With different diameters, the largest wells can be filled with the largest microspheres and then moving onto progressively

smaller microspheres in the smaller wells until all size wells are then filled. In this manner, the same dye ratio could be used to encode microspheres of different sizes thereby expanding the number of different oligonucleotide sequences or chemical functionalities present in the array. Although outlined for fiber optic substrates, this as well as the other methods outlined herein can be used with other substrates and with other attachment modalities as well.

A bead decoding scheme includes assigning/imbuing a single quantifiable attribute to each bead type wherein each bead type differs in the quantifiable value of that attribute. For instance, one can attach a given number of fluorophores to a bead and quantitate the number of attached fluorophores in the decoding process; however, in practice, attaching a "given amount" of an attribute to a bead and accurately measuring the attribute may be problematic. Typically, the coefficient of variation (CV) seen in associating an attribute and the resultant measurement of this attribute is quite large (> 20%). By coefficient of variation is meant the variability in labeling a bead in successive labelings. This CV can be determined by labeling beads with a defined given number of label (fluorophore, for example) in multiple tests and measuring the resulting signal emitted by the bead. This large CV limits the number of useable and resolvable "levels" for any given attribute.

A more robust decoding scheme employs ratiometric rather than absolute measurements for segmenting a quantitative attribute into codes. By ratiometric decoding is meant labeling a bead with a ratio of labels or attributes (i.e. 1:10, 1:1, and 10:1). In theory any number of ratios can be used so long as the difference in signals between the ratios is detectable. This process produced smaller CVs and allowing more attribute segmentation within a given dynamic range. Thus, in a preferred embodiment, the use of ratiometric decoding reduces the coefficient of variability.

In addition, as will be appreciated by those in the art, ratiometric decoding can be accomplished in a different way. In this embodiment, rather than add a given number of beads with a first dye (or dye combination) intensity in the first decoding reaction and a second number with a second dye intensity in the sequential second decoding reaction, this ratiometric analysis may be done by using a ratio of labelled:unlabelled beads. That is, given a set saturating concentration of decoding beads, for example 100,000 beads/reaction, the first intensity decoding step may be done by adding 100,000 labelled beads and the second step can be done by adding 10,000 labelled beads and 90,000 unlabeled beads. Equilibrium dictates that the second step will give one tenth the signal intensity.

Because of the spread in values of a quantitatively measured attribute value, the number of distinct codes is practically limited to less than a dozen or so codes. However, by serially "painting" (i.e. temporarily attaching an attribute level to a bead) and "stripping" (removing the attribute level) a bead with different attribute values, the number of possible codes grows exponentially with the number of serial stages in the decoding process.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1936

An example is illustrative. For instance, 9 different bead types and three distinguishable attribute distributions (Table 1). "Painting" (labeling) the beads with different attribute values in a combinatorially distinct pattern in the two different stages, generates a unique code for each bead type, i.e. nine distinct codes are generated. Thus, in a preferred embodiment beads are labeled with different attributes in a combinatorially distinct pattern in a plurality of stages. This generates unique codes for each bead type. Examples of different attributes are described above. Labeling of beads with different attributes is performed by methods known in the art.

	stage 1	stage 2	
Bead Type	attribute value	attribute value	Code
1	L	L	(L, L)
2	L	M	(L, M)
3	L	H	(L, H)
4	M	L	(M, L)
5	M	M	(M, M)
6	M	H	(M, H)
7	H	L	(H, L)
8	H	M	(H, M)
9	H	H	(H, H)

Number of unique codes =

Number of attributes^Number of stages

Table 1 Serial decode generates unique codes using a small number of attribute levels.

As outlined herein, fluorescent colors are a particularly convenient and preferable attribute to use in a decoding scheme. Fluorescent colors can be attached to any agent that recognizes an IBL to form a labeled DBL. A fluorescently labeled oligonucleotide is a particularly useful DBL since it can specifically and reversibly "paint" (label) any desired subset of beads with a particular color simply by the process of hybridization and dehybridization (i.e. to the DBL with a complementary sequence). Moreover, fluorescence is easily imaged and quantitated using standard optical hardware and software. In order to "paint" a given bead type with a particular color, the bead type must be labeled

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1937

with a unique hybridizable DNA sequence (IBL) and the decoding solution must contain the color-labeled complement of that sequence.

One consideration in implementing a decoding scheme is to minimize the number of images collected. In a color-based scheme, the number of images collected is the product of the number of colors and the number of stages. The number of images can be reduced by "painting" a bead with multiple colors for each given stage. By assigning multiple colors to a bead, the number of effective codes is increased. As an example, in a 24 bit three color scheme (e.g. red, green, blue) coloring process used by computers, a total of 256*256*256 = 16.7 million different "hues" can be generated from just three colors (red, green, blue).

Thus, in a preferred embodiment DBLs are labeled with a combination of colored fluorophores. As such, this method finds use in increasing the number of available codes for labeling DBLs using only a handful of different dyes (colors). Increasing the number of codes available at each decoding step will greatly decrease the number of decoding steps required in a given decoding process.

In one embodiment a population of oligonucleotides encoding a single DBL is labeled with a defined ratio of colors such that each bead to which the DBL binds is identified based on a characteristic "hue" formulated from the combination of the colored fluorophores. In a preferred embodiment two distinct colors are used. In a preferred embodiment, three or more distinct dyes (colors) are available for use. In this instance the number of differentiable codes generated by labeling a population of oligonucleotides encoding a single DBL with any given color is three. However by allowing combinations of colors and color levels in the labeling, many more codes are generated.

For decoding by hybridization, a preferred number of distinguishable color shades is from 2 to 2000; a more preferred number of distinguishable color shades is from 2 to 200 and a most preferred number of distinguishable color shades is from 2 to 20. Utilizing three different color shades (intensities) and three colors, the number of different hues will be $3^4 = 81$. Combining a hue with sequential decoding allows a virtually limitless number of codes to be generated.

As previously described, the DBL can be any agent that binds to the IBL. In a preferred embodiment, a single DBL is labeled with a pre-determined ratio of colors. This ratio is varied for each DBL thus allowing for a unique "hue" for each DBL labeled as such. Following treatment of the beads with the DBL, the bead is analyzed to determine the "hue" associated with each bead, thereby identifying the bead with its associated bioactive agent.

For instance, with four primary colors and two intensity levels (color is present or absent), fifteen different hues/stage are possible. If four dyes and three different intensity levels are used (absent, half-present, fully present), then 73 different hues/stage are possible. In this case, acquisition of only 4 color images is sufficient to obtain information on 73 different coding hues.

In a preferred embodiment the DBL relies on a Fluorescence Resonance Energy Transfer (FRET) identifier. Fluorescence resonance energy transfer (FRET) is well known in the art and has been employed for various biotechnological applications. FRET requires that the energy of the donor excited state overlap with the ground state of the acceptor, implying that the donor emission spectrum overlaps with the acceptor absorbance spectrum. In a FRET experiment, the donor is excited with light; some of the energy of the excited state is released as light emission while some of the energy is transferred to the acceptor, which becomes excited emitting light at a characteristic wavelength. The distance over which energy transfer may occur can be varied by the choice of energy donor and acceptor, wherein the use of various different donor and acceptor molecules is contemplated. The formula for the efficiency of energy transfer is:

$$T = R_o^6/(R_o^6+R^6)$$

Where R is the distance between the donor and acceptor, and R_0 is a constant related to the characteristic overlap of the absorption and emission spectra of the donor-acceptor pair.

A FRET identifier comprises a linker region that is labeled at each terminus with a fluorophore. The first fluorophore serves as a donor fluorophore, whose signal is detectable; the other acts as an acceptor fluorophore. The emission of the acceptor fluorophore also is a detectable signal, the intensity of which varies proportionally with the distance between the two fluorophores. That is, by varying the distance between the two fluorophores different codes can be generated.

Identification of the decoding oligonucleotide, therefore, is accomplished by identifying the "efficiency" or ratio of intensities of the acceptor fluorophore and the donor fluorophore. This ratio should be invariant with regard to the number of decoding oligonucleotides bound to a particle (bead); rather, without being bound by any theory, the distance between the fluorophores determines the ratio. Thus, by varying the distance between the fluorophores (i.e. increasing or decreasing the length of the linker), unique "codes" can be assigned to the DBLs. Each code is characterized by the ratio of emission intensities between the two fluorophores.

An even greater advantage is realized when different bead attributes can be collected in a single image rather than from multiple images. For instance, if the colors of red, green and blue are used as attributes, three separate images need to be collected. However, if different shades or intensities of each color are used in the coding process, a much larger number of attributes can be realized in a single image. The difficulty with using mixtures of colors, is that the color ratios can vary somewhat between bead types since individual components are mixed together to determine the color ratio. If on the other hand, the color ratio is built into the decoding label , as with the above described FRET decoding oligonucleotides, the decoding process should be more reliable and more efficient.

In preferred embodiments of the present invention, the first component may be a lanthanide such as a chelate of europium(III) (Eu(III)) or terbium(III) (Tb(III)) which serves as a fluorescent donor and the second component may be an activated ester of cyanine 5 (Cy5) or tetramethylrhodamine (TMR) which serve as energy acceptors of Eu(III) and Tb(III). Other first and second component pairs that will find use for FRET include, for example, fluorescein and rhodamine; FITC and rhodamine; and fluorescein and trinitrophenyl, and other components as are well known in the art.

The linker is comprised of any spacer element including alkyl linkers, polymer linkers, etc. For ease of synthesis, preferably the linker comprises nucleic acids (including nucleic acid analogs).

The FRET decoding oligonucleotide comprises a spacer or linker region that is labeled with fluorophores (dyes). In general, this may be used in two general ways. In a preferred embodiment, as generally depicted in Figure 4, the linker is attached to the terminus of a decoding nucleotide that hybridizes with (is complementary to) the IBL. In this embodiment, the linker length can be varied to adjust signal intensity. Thus, in a preferred embodiment a FRET oligonucleotide is adjoined to an oligonucleotide DBL. In one embodiment the FRET oligonucleotide is synthesized independent of the DBL oligo. Subsequently the FRET oligonucleotide is ligated to the DBL oligo according to methods well known in the art. Alternatively, the DBL oligo and FRET oligo are synthesized as a single oligonucleotide comprising the FRET fluorophores at the desired positions.

Alternatively, in a second embodiment the linker is itself the oligonucleotide that hybridizes with (is complementary to) the IBL. That is, fluorophores are attached to the decoding oligonucleotide at different basepair lengths; some may be 10 nucleotides apart, some 20, etc. In a preferred embodiment the oligonucleotide DBL is itself labeled with fluors; as such the oligonucleotide serves as both DBL and FRET oligonucleotide.

The linker region of a FRET oligonucleotide comprises any set of nucleotides; in a preferred embodiment, for example when the linker does not hybridize to anything, the linker comprises a single repeated nucleotide. A single repeated nucleotide is less likely to hybridize with the IBL or bioactive agent on the bead because of the reduced likelihood of the presence of a complementary nucleotide sequence in either the IBL or bioagent on the bead. In a preferred embodiment, the linker comprises a polynucleotide linker such as poly T, poly A, poly G or poly C, or combinations.

The linker can be of any length so long as the emission of the first fluorophore is absorbed by the second fluorophore. In a preferred embodiment the linker is formed of from 1 to at least 100 nucleotides. More preferably the linker is formed from 1 to 70 nucleotides. Most preferably the linker is formed from 1 to 40 nucleotides, with from 5 to 20 being especially preferred.

In a preferred embodiment, for a given DBL sequence, several codes can be generated by varying the distance of separation of the two fluorophores and hence generating several energy transfer "efficiency" ratios.

In another preferred embodiment, for a given DBL sequence, a single code can be generated by varying the distance of separation of the two flourophores and hence generating a single energy transfer ratio for a single DBL. However, numerous DBLs can be labeled with distinct FRET oligos thus allowing for the simultaneous determination or identification of multiple DBLs in a single imaging session as a result of the plurality of energy transfer ratios.

For example, given a 40-mer decoding oligo, the donor and acceptor fluorophores can be located as close as one or as far apart as 40 bases. In principle this should allow 39 codes to be generated for a single decoding sequence. Empirically, the number of codes generated depends on dispersion in the distributions for the ratiometric measurements.

Also, given a 40-mer decoding oligo, 39 unique codes can be generated which will identify 39 different oligos.

The present invention provides array compositions comprising a substrate with a surface comprising discrete sites. A population of microspheres is distributed on the sites, and the population comprises at least a first and a second subpopulation. Each subpopulation comprises a bioactive agent, and, in addition, at least one optical dye with a given pKa. The pKas of the different optical dyes are different.

In a preferred embodiment, when for example the array comprises cloned nucleic acids, there are several methods that can be used to decode the arrays. In a preferred embodiment, when some sequence information about the cloned nucleic acids is known, specific decoding probes can be made as is generally outlined herein.

In a preferred embodiment, "random" decoding probes can be made. By sequential hybridizations or the use of multiple labels, as is outlined above, a unique hybridization pattern can be generated for each sensor element. This allows all the beads representing a given clone to be identified as belonging to the same group. In general, this is done by using random or partially degenerate decoding probes, that bind in a sequence-dependent but not highly sequence-specific manner. The process can be repeated a number of times, each time using a different labeling entity, to generate a different pattern of signals based on quasi-specific interactions. In this way, a unique optical signature is eventually built up for each sensor element. By applying pattern recognition or clustering algorithms to the optical signatures, the beads can be grouped into sets that share the same signature (i.e. carry the same probes).

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1941

In order to identify the actual sequence of the clone itself, additional procedures are required; for example, direct sequencing can be done. By using an ordered array containing the clones, such as a spotted cDNA array, a "key" can be generated that links a hybridization pattern to a specific clone whose position in the set is known. In this way the clone can be recovered and further characterized.

Alternatively, clonal arrays can be decoded using binary decoding with vector tags. For example, partially randomized oligos are cloned into a nucleic acid vector (e.g. plasmid, phage, etc.). Each oligonucleotide sequence consists of a subset of a limited set of sequences. For example, if the limites set comprises 10 sequences, each oligonucleotide may have some subset (or all of the 10) sequences. Thus each of the 10 sequences can be present or absent in the oligonucleotide. Therefore, there are 2^{10} or 1,024 possible combinations. The sequences may overlap, and minor variants can also be represented (e.g. A, C, T and G substitutions) to increase the number of possible combinations. A nucleic acid library is cloned into a vector containing the random code sequences. Alternatively, other methods such as PCR can be used to add the tags. In this way it is possible to use a small number of oligo decoding probes to decode an array of clones.

There are a variety of methods used to evaluate decoding. In a preferred embodiment, discriminant analysis and cluster algorithms and computer apparatus are used to analyze the decoding data from the arrays of the invention. The potentially large number of codes utilized in the invention, coupled with the use of different intensities and "hues" of fluorophores in multi-step decoding processes, requires good classification of the data. The data, particularly intensity data, is acquired in a multi-step process during which beads are "painted" (for example by hybridizing dye-labeled complementary decoding oligonucleotides to the IBL probes on the beads, or the formation of binding ligand pairs for non-nucleic acid IBL-DBL pairs) with different colors or mixtures of colors ("hues") at each stage. The challenge is to accurately classify a bead as to which color with which it was painted at each step. The more closely related the "paints" (colors) are to one another (as determined by the optical imaging system), the more difficult the classification.

The proximity of the dyes as seen by the imaging system is determined by the spectral properties of the decoding dyes and the spectral channel separation of the imaging system. Better color separation is achieved by employing fluorescent dyes with narrow emission spectra, and by employing an optical system with narrow band pass excitation and emission filters which are designed to excite the dye "on peak" and measure its emission "on peak". The process of optically imaging the dyes on the beads is similar to the human vision process in which our brain sees color by measuring the ratio of excitation in the three different cone types within our eye. However, with an optical imaging system, the number of practical color channels is much greater than the three present in the human eye. CCD based imaging systems can "see" color from 350 nm up to 850 nm whereas the cones in the eye are tuned to the visible spectrum from 500 - 600 nm.

The problem of decoding bead arrays is essentially a discriminant analysis classification problem. Thus, in a preferred embodiment, an analysis of variance in hyperspectral alpha space is performed on a known set of bead colors or hues. The center of the bead clusters in alpha space are termed the centroids of the clusters, and the scatter of the points within a cluster determines the spread of the cluster. A robust classification scheme requires that the distance between the centroids of the different bead classes (hues) is much greater than the spread of any cluster class. Moreover, the location of the centroids should remain invariant from substrate to substrate and from experiment to experiment.

Thus, in a preferred embodiment, a hue "zone" is defined as a region in alpha space surrounding the hue centroid and extending out to the spread radius of the cluster. Given a reference set of hue centroids and spread radii, as determined empirically, the classification of a new set of data can be accomplished by asking whether a given bead point falls closest to or within the "zone" of a hue cluster. This is accomplished by calculating the Mahalanobis distance (in this case, it is simply a Euclidean distance metric) of the bead point from the centroids of the different hue classes. For the data shown in Fig. 6, the location of the centroids and their distances from one another are indicated in Table 2.

Table 2	Centroid position				Distance between centroids			
dye/channel	Blue	Green	Yellow	Red	Bod-493	Bod-R6G	Bod-564	Bod-TXR
Bod-493	0.63	0.22	0.11	0.03	0.00			
Bod-R6G	0.03	0.51	0.37	0.09	0.72	0.00		
Bod-564	0.06	0.04	0.57	0.32	0.81	0.55	0.00	
Bod-TXR	0.09	0.05	0.04	0.82	0.99	0.93	0.73	0.00

For classifying the different beads into a particular hue class, a Euclidean distance cutoff of 0.3 was chosen. The closest two centroids, the Bod-R6G and Bod-564 (dist = 0.55), have a slight overlap in their decoding zones when using a Euclidean or Mahalanobis distance of 0.3. An improvement in classification can be achieved by decreasing this distance, and by weighting the different coordinate axes appropriately.

Accordingly, the present invention provides computer methods for analyzing and classifying the color of a bead. The classification of the color of the bead is done by viewing the bead in hyperspectral "alpha" space ($\Delta_1 = I_1/6I_i$, $\Delta_2 = I_2/6I_i$, $\Delta_3 = I_3/6I_i$,, etc.) in which each coordinate axis represents the fraction of the bead intensity within a given imaging channel. For instance, if four imaging channels are used to image the beads, the color or hue of a bead can be represented by a point in 3-D alpha space (the fourth dimension is not necessary since $Sa_i = 1$). Given a set of different primary dyes by which to "paint" the beads, the number of hues that can be generated from these dyes is unlimited since the dyes can be combined in varying ratios and in varying combinatorial patterns. The

number of practical hues is experimentally determined by the separation of the different hue clusters in hyperspectral alpha space.

Fig. 6 shows a hyperspectral alpha plot of beads "painted" with four different hues imaged in four separate imaging channels. Note that the beads form four distinct clusters. The fact that these four clusters are well separated allows a robust decode classification scheme to be implemented.

In a preferred embodiment, a quality control analysis of the decoding process is done. This is achieved by performing a cluster analysis of alpha space for each decoding stage. The number of clusters determined will be fixed by the expected number of hues. The positions of the cluster centroids will be monitored and any deviations from the expected position will be noted.

Thus the invention provides an apparatus for decoding the arrays of the invention. In addition to the compositions outlined herein, the apparatus includes a central processing unit which communicates with a memory and a set of input/output devices (e.g., keyboard, mouse, monitor, printer, etc.) through a bus. The general interaction between a central processing unit, a memory, input/output devices, and a bus is known in the art. One aspect of the present invention is directed toward the hyperspectral "alpha" space classification system stored in the memory.

The classification system program includes a data acquisition module that receives data from the optical reader or confocal microscope (or other imaging system). In general, the classification program also includes an analysis module, that can analyze the variance in hyperspectral alpha space, calculate the centroids of the clusters, calculate the scatter of the cluster (the spread) and define the hue zone and distance cutoff. In general, the analysis module will further determine whether a data point falls within the hue zone by calculating the Mahalanobis distance.

Finally, the analysis module will analyze the different sequential decoding information to finally assign a bioactive agent to a bead location.

In this way, sequential decoding steps are run, with each step utilizing the discriminant analysis calculations to assign each bead in the array to a hue cluster at each step. The buildup of the sequential decoding information allows the correlation of the location of a bead and the chemistry contained on it.

In one embodiment, the invention includes modifying the number of detection channels available for analysis relative to the number of labels to be detected. That is, in one embodiment, the number of detection channels is decreased relative to the number of labels used. In an alternative embodiment the number of detection channels is increased relative to the number of labels used.

When fewer detection channels are used, fewer images are required during decoding. As such the invention provides a method of reducing the number of images obtained during decoding. Fluorescent dyes have broad band absorption and emission spectra. Accordingly, the emission of certain dyes is detected in multiple channels, generally by choosing channels to maximize the signal. However by choosing the dyes and the channels carefully, fewer channels that dyes may be used. As such, decoding with fewer channels than dyes, allows for detection of signals that are maximally detected in a particular channel, and overlapping in several channels. Thus, for example, beads with dye-1 are read in channel-1, beads with dye-2 are read in channel-2 and beads with dye-3 show signals in both channel-1 and channel-2. Example 7 is illustrative and the results of the experiment are found in Figure 11. It is noted, however, that three dyes are clearly detected and distinguished following detection in only two channels. Accordingly, using fewer detection channels than the number of dyes to be analyzed, allows for a reduction in the number of images and steps required for decoding.

In an alternative embodiment, the invention provides for using additional channels to increase the number of detectable codes. That is, additional readout channels are used to increase the coding/decoding capacity of arrays by analyzing attributes of the bead in addition to an optical signal such as fluorescence. For example, bead size, shape, density and surface characteristics can alter the light-scattering properties of the beads and can be detected optically. Additional channels that provide information independent of fluorescence are forward-scatter and side-scatter. Low-angle forward light scatter provides some information on the relative size of beads, whereas side light scatter provides some information on the relative granularity or texture of individual beads. By analogy these channels are often used in combination to distinguish the different major categories of white cells in unseparated mammalian blood, but are useful in a wide variety of other assays as well.

With respect to particle size, particles may simply be varied in diameter. Alternatively, particles could be assembled as doublets, triplets, or other higher-order multimers. Doublets and triplets often occur spontaneously in a population, and can be enriched for by fractionation.

With respect to surface properties, in one embodiment, the invention provides the use of metallization. This can dramatically alter the scattering properties of a surface. One way to accomplish this is to attach gold particles (e.g. colloidal gold) to an affinity molecule, such as streptavidin or DNA, and then to bind the molecule at defined loadings to specific sites on the bead. For example, ten different loadings could give ten new codes. Together with 100 fluorescence codes, this would allow the recognition of ~ 1000 bead types in an array. Alternatively, metallization could be used for the assay readout, and fluorescence for the encoding.

With respect to magnetism, superparamagnetic beads could be deflected according to the amount of magnetism, and directed to different readout channels -i.e. this mechanism could be used as a

"pre-sorter" prior to fluorescence readout. In one version, bead subpopulations could be localized to different regions of a reaction vessel (e.g. microtiter well) by the use of small magnets. Each sub-population could be released sequentially for analysis.

Although the previously described method involving additional detection channels finds use in planar bead arrays, it also finds particular use in liquid arrays. By "liquid arrays" is meant a collection of beads that are not spatially organized, but are typically encoded by fluorescence labeling and "decoded" by readout on a flow- cytometer or equivalent instrument. Assays are carried out in bulk in solution. Generally, liquid arrays are designed such that the assay result and decoding result are obtained essentially simultaneously. However, because only optical images are detected simultaneously, the number of codes that can be analyzed is limited. That is, there is a limit to the number of codes that can be used since the number of assay signals and encoding signals must be determined at different wavelengths at essentially the same time. However, by using additional detection channels as described above, the number of codes that can be analyzed is markedly increased.

This method finds particular use in analyzing the results of genomics based assays such as gene expression profiling, genotyping and SNP analysis. In such genomics based assays, it is frequreintly desirable to make hundreds to millions of measurements simultaneously. Thus, by increasing the number of codes that can be simultaneously detected, analysis of such assays is enhanced.

This invention provides methods of maximizing the information that is obtained from a single bead. That is, by providing ways of multiplexing assays on a single bead, multiple parameters can be simultaneously detected. That is, after a cycle of performing a particular assay, decoding and reading the assay results, the bead population is recovered. The detection molecules are removed from the bead and the cycle is repeated.

In a preferred embodiment, parity analysis is used during decoding to increase the robustness and accuracy of the system. By "parity analysis"is meant a decoding step wherein the signal of a particular element is analyzed across a plurality of decoding stages. That is, following at least one decoding step, but most preferably following multiple decoding steps, the signal of an array element across the decoding stages is analyzed. That is, the signal from a particular bead is evaluated across multiple stages. Although the analysis includes any parameter that can be obtained from the signals such as evaluating the total signal obtained across the stages, in a preferred embodiment, the parity of the signals across the stages is analyzed.

By "parity" is meant the digital or modular readout of signals, i.e. odd or even, when binary signals are used. As depicted in Figure 7, the digit sum of the signals across a plurality of stages is translated into a parity determination. The parity determination is then useful in evaluating the decoding process. An

example is illustrative. Codes are designed to have an odd number of a particular signal, for example a red signal, when viewed across all stages or decoding steps, or a pre-determined plurality of stages or decoding steps. The detection of an even number of red stages, provides an indication that an error has occurred at some point in decoding. When this result is obtained, the faulty code can either be discarded, or the analysis repeated.

In a preferred embodiment the invention includes introducing a "redundant stage" into the decoding system. By "redundant stage" is meant a stage that serves as a parity check. That is, following the decoding stages, an additional stage is included to analyze the parity. This analysis provides an indication of the competence or validity of the decoding. That is, when codes are designed with a pre-determined parity, the redundant stage is used to detect the parity of the signals obtained from the decoding step. That is, the redundant stage detects errors in parity because if there has been an error in decoding, the parity detected following the redundant stage will be different from the parity designed into the codes.

An example is illustrative. In the following illustration, array elements are decoded in two stages with two colors. This results in the detection of 4 (2^2) codes. The codes are designed to result in odd parity following the analysis with the redundant stage. That is, the code sequences can be made such that each code will contain an odd number of "red" colors (when viewed across all stages, or when viewed across the pre-determined number of stages). In the illustration below, only the stage where the "red" signal is detected is indicated.

Code	Stage 1	Stage 2	Redundant Stage	Parity (number of "red" stages)
1	0	0	red	1 (odd)
2	0	red	0	1 (odd)
3	red	0	0	1 (odd)
4	red	red	red	3 (odd)

However, if there is an error during decoding, this will be reflected as detecting a parity that is different from that designed into the codes. In the following illustration the same codes as above are used however, an error is detected. In this case if one detects an even number of "red" stages it can be inferred that there has been a decoding error.

Code	Stage 1	Stage 2	Redundant Stage	Parity (number of "red" stages)
1	0	0	red	1 (odd)
2	0	red	0	1 (odd)

3	red	0	0	1 (odd)
4	red	0 (error)	red	2 (even)

Although in some embodiments, the parity is binary, i.e. odd or even, in other embodiments the parity is modular or based on numbers other than 2. That is, the parity can be based on detection of 3 or more signals. In a preferred embodiment, the signals are multiple colors. In a particularly preferred embodiment the colors are red, green and blue.

An example is illustrative. In the following illustration, codes are decoded in two steps with three colors. This results in the detection of 3^2 (9) signals. In the illustration, red is indicated by 1, green is indicated by 2 and blue is indicated by 3. The parity is based on a modulus 3. By including a third stage as a redundant stage the parity can be checked as described above.

Code	Stage 1	Stage 2	Redundant stage	Parity
1	1	1	1	3
2	1	2	3	6
3	1	3	2	6
4	2	1	3	6
5	2	2	2	6
6	2	3	1	6
7	3	1	2	6
8	3	2	1	6
9	3	3	3	9

However, when there is a decoding error, the parity will be incorrect; in the illustration the parity readout is not divisible by 3.

Code	Stage 1	Stage 2	Redundant stage	Parity
1	1	1	1	3
2	1	2	3	6
3	1	3	2	6
4	2	1	3	6
5	2	2	2	6

6	2	3	1	6
7	3	1	2	6
8	3	2	1	6
9	3	2 (error)	3	8

Thus, a redundant stage allows for the detection of errors on the array or in the signal obtained from an array.

Although described above for detecting errors on arrays, the method of using redundant stages is not limited to arrays. That is, the method finds use in detecting errors in a variety of systems that send, receive and/or interpret or analyze data such as communications systems including modems etc. In particular, the method of using the modular redundant stages allows for processing of signals of vastly increased complexity as compared to sending bits of data. That is, when using modular redundant stages, characters as opposed to bits, can be sent across modem lines in the communications field. The modular redundant stage is used to detect errors in the communication or processing of the signals.

In an additional embodiment a redundant channel is used during the decoding. By "redundant channel" is meant either the use of multiple filters to analyze a particular signal or repeated use of the same filter to analyze the signal, i.e. multiple signals from the same channel. Redundant channels find use as a source of a "back-up" signal of sorts for a particular array element. That is, when multiple images are obtained as a result of redundant channels, the likelihood of obtaining the correct signal increases. That is, when it is realized that one of the results for an element is compromised for some reason, the image obtained from the redundant channel is used instead. When the initial image is not compromised, the result from the redundant channel need not be used, although it may be used, for example as a way to confirm the initial signal.

An example is illustrative. During decoding, when it is apparent that one stage fails, for example as a result of mis-registration of a channel, the use of a redundant channel serves to rescue the decoding at that particular stage. Mis-registration can also be addressed as outlined in USSN 09/636,387, filed August 9, 2000, which is expressly incorporated herein by reference. As noted above, the redundant channel can either be an image separate filter to analyze the signal, or alternatively, it can be multiple images taken from the same filter.

In one embodiments, the number of redundant channels increases as the number of decoding stages increases. Because the investment in decoding increases as each decoding stage or step proceeds, it is important in this embodiment to ensure the competence of decoding at later decoding stages.

In a preferred embodiment, redundant systems are used to avoid the loss of information. It is appreciated that when "S" decoding steps (sometimes referred to herein as "stages") and "C" colors are used, the maximum number of distinct signals is C^S (C raised to the "S"th power). In some instances, the corruption of a stage results in the loss of C^S- (C-1)x$C^{(S-1)}$ codes. Depending on the values of C and S, the loss can be substantial. Accordingly, the present invention provides for methods of improving the robustness of the decoding system. That is, the invention provides for a method of confirming the results of a decoding analysis or detecting errors in a decoding analysis during or after the decoding procedure. The method also decreases the number of false positive or false negative signals; similarly, the number of true positives and/or true negatives increases.

In a preferred embodiment, several levels of redundancy are built into the arrays of the invention. Building redundancy into an array gives several significant advantages, including the ability to make quantitative estimates of confidence about the data and signficant increases in sensitivity. Thus, preferred embodiments utilize array redundancy. As will be appreciated by those in the art, there are at least two types of redundancy that can be built into an array: the use of multiple identical sensor elements (termed herein "sensor redundancy"), and the use of multiple sensor elements directed to the same target analyte, but comprising different chemical functionalities (termed herein "target redundancy"). For example, for the detection of nucleic acids, sensor redundancy utilizes of a plurality of sensor elements such as beads comprising identical binding ligands such as probes. Target redundancy utilizes sensor elements with different probes to the same target: one probe may span the first 25 bases of the target, a second probe may span the second 25 bases of the target, etc. By building in either or both of these types of redundancy into an array, significant benefits are obtained. For example, a variety of statistical mathematical analyses may be done.

In addition, while this is generally described herein for bead arrays, as will be appreciated by those in the art, this techniques can be used for any type of arrays designed to detect target analytes.

In a preferred embodiment, sensor redundancy is used. In this embodiment, a plurality of sensor elements, e.g. beads, comprising identical bioactive agents are used. That is, each subpopulation comprises a plurality of beads comprising identical bioactive agents (e.g. binding ligands). By using a number of identical sensor elements for a given array, the optical signal from each sensor element can be combined and any number of statistical analyses run, as outlined below. This can be done for a variety of reasons. For example, in time varying measurements, redundancy can significantly reduce the noise in the system. For non-time based measurements, redundancy can significantly increase the confidence of the data.

In a preferred embodiment, a plurality of identical sensor elements are used. As will be appreciated by those in the art, the number of identical sensor elements will vary with the application and use of the sensor array. In general, anywhere from 2 to thousands may be used, with from 2 to 100 being preferred, 2 to 50 being particularly preferred and from 5 to 20 being especially preferred. In general,

preliminary results indicate that roughly 10 beads gives a sufficient advantage, although for some applications, more identical sensor elements can be used.

Once obtained, the optical response signals from a plurality of sensor beads within each bead subpopulation can be manipulated and analyzed in a wide variety of ways, including baseline adjustment, averaging, standard deviation analysis, distribution and cluster analysis, confidence interval analysis, mean testing, etc.

In a preferred embodiment, the first manipulation of the optical response signals is an optional baseline adjustment. In a typical procedure, the standardized optical responses are adjusted to start at a value of 0.0 by subtracting the integer 1.0 from all data points. Doing this allows the baseline-loop data to remain at zero even when summed together and the random response signal noise is canceled out. When the sample is a fluid, the fluid pulse-loop temporal region, however, frequently exhibits a characteristic change in response, either positive, negative or neutral, prior to the sample pulse and often requires a baseline adjustment to overcome noise associated with drift in the first few data points due to charge buildup in the CCD camera. If no drift is present, typically the baseline from the first data point for each bead sensor is subtracted from all the response data for the same bead. If drift is observed, the average baseline from the first ten data points for each bead sensor is substracted from the all the response data for the same bead. By applying this baseline adjustment, when multiple bead responses are added together they can be amplified while the baseline remains at zero. Since all beads respond at the same time to the sample (e.g. the sample pulse), they all see the pulse at the exact same time and there is no registering or adjusting needed for overlaying their responses. In addition, other types of baseline adjustment may be done, depending on the requirements and output of the system used.

Once the baseline has been adjusted, a number of possible statistical analyses may be run to generate known statistical parameters. Analyses based on redundancy are known and generally described in texts such as Freund and Walpole, Mathematical Statistics, Prentice Hall, Inc. New Jersey, 1980, hereby incorporated by reference in its entirety.

In a preferred embodiment, signal summing is done by simply adding the intensity values of all responses at each time point, generating a new temporal response comprised of the sum of all bead responses. These values can be baseline-adjusted or raw. As for all the analyses described herein, signal summing can be performed in real time or during post-data acquisition data reduction and analysis. In one embodiment, signal summing is performed with a commercial spreadsheet program (Excel, Microsoft, Redmond, WA) after optical response data is collected.

In a preferred embodiment, cummulative response data is generated by simply adding all data points in successive time intervals. This final column, comprised of the sum of all data points at a particular

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1951

time interval, may then be compared or plotted with the individual bead responses to determine the extent of signal enhancement or improved signal-to-noise ratios.

In a preferred embodiment, the mean of the subpopulation (i.e. the plurality of identical beads) is determined, using the well known Equation 1:

Equation 1

$$\mu = \sum \frac{x_i}{n}$$

In some embodiments, the subpopulation may be redefined to exclude some beads if necessary (for example for obvious outliers, as discussed below).

In a preferred embodiment, the standard deviation of the subpopulation can be determined, generally using Equation 2 (for the entire subpopulation) and Equation 3 (for less than the entire subpopulation):

Equation 2

$$\sigma = \sqrt{\frac{\sum (x_i - \mu)^2}{n}}$$

Equation 3

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

As for the mean, the subpopulation may be redefined to exclude some beads if necessary (for example for obvious outliers, as discussed below).

In a preferred embodiment, statistical analyses are done to evaluate whether a particular data point has statistical validity within a subpopulation by using techniques including, but not limited to, t distribution and cluster analysis. This may be done to statistically discard outliers that may otherwise skew the result and increase the signal-to-noise ratio of any particular experiment. This may be done using Equation 4:

Equation 4

$$t = \frac{\bar{x} - \mu}{s/\sqrt{n}}$$

In a preferred embodiment, the quality of the data is evaluated using confidence intervals, as is known in the art. Confidence intervals can be used to facilitate more comprehensive data processing to measure the statistical validity of a result.

In a preferred embodiment, statistical parameters of a subpopulation of beads are used to do hypothesis testing. One application is tests concerning means, also called mean testing. In this application, statistical evaluation is done to determine whether two subpopulations are different. For example, one sample could be compared with another sample for each subpopulation within an array to determine if the variation is statistically significant.

In addition, mean testing can also be used to differentiate two different assays that share the same code. If the two assays give results that are statistically distinct from each other, then the subpopulations that share a common code can be distinguished from each other on the basis of the assay and the mean test, shown below in Equation 5:

Equation 5

$$z = \frac{\overline{x_1} - \overline{x_2}}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

Furthermore, analyzing the distribution of individual members of a subpopulation of sensor elements may be done. For example, a subpopulation distribution can be evaluated to determine whether the distribution is binomial, Poisson, hypergeometric, etc.

In addition to the sensor redundancy, a preferred embodiment utilizes a plurality of sensor elements that are directed to a single target analyte but yet are not identical. For example, a single target nucleic acid analyte may have two or more sensor elements each comprising a different probe. This adds a level of confidence as non-specific binding interactions can be statistically minimized. When nucleic acid target analytes are to be evaluated, the redundant nucleic acid probes may be overlapping, adjacent, or spatially separated. However, it is preferred that two probes do not compete for a single binding site, so adjacent or separated probes are preferred. Similarly, when proteinaceous target analytes are to be evaluated, preferred embodiments utilize bioactive agent binding agents that bind to different parts of the target. For example, when antibodies (or antibody fragments) are used as bioactive agents for the binding of target proteins, preferred embodiments utilize antibodies to different epitopes.

In this embodiment, a plurality of different sensor elements may be used, with from about 2 to about 20 being preferred, and from about 2 to about 10 being especially preferred, and from 2 to about 5

being particularly preferred, including 2, 3, 4 or 5. However, as above, more may also be used, depending on the application.

As above, any number of statistical analyses may be run on the data from target redundant sensors.

One benefit of the sensor element summing (referred to herein as "bead summing" when beads are used), is the increase in sensitivity that can occur.

Once made, the compositions of the invention find use in a number of applications. In a preferred embodiment, the compositions are used to probe a sample solution for the presence or absence of a target analyte, including the quantification of the amount of target analyte present. By "target analyte" or "analyte" or grammatical equivalents herein is meant any atom, molecule, ion, molecular ion, compound or particle to be either detected or evaluated for binding partners. As will be appreciated by those in the art, a large number of analytes may be used in the present invention; basically, any target analyte can be used which binds a bioactive agent or for which a binding partner (i.e. drug candidate) is sought.

Suitable analytes include organic and inorganic molecules, including biomolecules. When detection of a target analyte is done, suitable target analytes include, but are not limited to, an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc. Particularly preferred analytes are nucleic acids and proteins.

In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected or evaluated for binding partners using the present invention. Suitable protein target analytes include, but are not limited to, (1) immunoglobulins; (2) enzymes (and other proteins); (3) hormones and cytokines (many of which serve as ligands for cellular receptors); and (4) other proteins.

In a preferred embodiment, the target analyte is a nucleic acid. These assays find use in a wide variety of applications. Such applications are described in more detail in USSNs 60/130,089, filed April 20, 1999; 60/160,927, filed October 22, 1999, 09/513,362, filed February 25, 2000, 60/135,051 filed May 20, 1999, 60/161,148, filed October 22, 1999, 09/517, 945, filed March 3, 2000, 09/425,633, filed October 22, 1999, 09/535,854, filed March 27, 2000, 09/535,993, filed April 20, 2000, 09/556,463, filed April 21, 2000, 60/224,119, filed October 26, 2000, all of which are expressly incorporated herein by reference.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1954

In a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, cytochrome p450s or any of the others well known in the art.

In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly highly conserved HIV sequences. In addition, this allows direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, chlamydia and other sexually transmitted diseases, may also be detected.

In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid, and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*, enterotoxic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the probes in an array are used for sequencing by hybridization.

The present invention also finds use as a methodology for the detection of mutations or mismatches in target nucleic acid sequences, as is described in more detail in USSNs 09/425,633, filed October 22, 1999, 09/535,854, filed March 27, 2000, both of which are expressly incorporated herein by reference. For example, recent focus has been on the analysis of the relationship between genetic variation and phenotype by making use of polymorphic DNA markers. Previous work utilized short tandem repeats (STRs) as polymorphic positional markers; however, recent focus is on the use of single nucleotide polymorphisms (SNPs). Common SNPs occur at an average frequency of more than 1 per kilobase in human genomic DNA. Some SNPs, particularly those in and around coding sequences, are likely to be the direct cause of therapeutically relevant phenotypic variants. There are a number of well known polymorphisms that cause clinically important phenotypes; for example, the apoE2/3/4 variants are associated with different relative risk of Alzheimer's and other diseases (see Cordor et al., Science

261(1993). Multiplex PCR amplification of SNP loci with subsequent hybridization to oligonucleotide arrays has been shown to be an accurate and reliable method of simultaneously genotyping at least hundreds of SNPs; see Wang et al., Science, 280:1077 (1998); see also Schafer et al., Nature Biotechnology 16:33-39 (1998). The compositions of the present invention may easily be substituted for the arrays of the prior art.

In a preferred embodiment, the compositions of the invention are used to screen bioactive agents to find an agent that will bind, and preferably modify the function of, a target molecule. As above, a wide variety of different assay formats may be run, as will be appreciated by those in the art. Generally, the target analyte for which a binding partner is desired is labeled; binding of the target analyte by the bioactive agent results in the recruitment of the label to the bead, with subsequent detection.

In a preferred embodiment, the binding of the bioactive agent and the target analyte is specific; that is, the bioactive agent specifically binds to the target analyte. By "specifically bind" herein is meant that the agent binds the analyte, with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. However, as will be appreciated by those in the art, it will be possible to detect analytes using binding which is not highly specific; for example, the systems may use different binding ligands, for example an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands, similar to the manner in which "electronic noses" work. This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding, although in some embodiments, wash steps are not desired; i.e. for detecting low affinity binding partners. In some embodiments, for example in the detection of certain biomolecules, the dissociation constants of the analyte to the binding ligand will be less than about 10^{-4}-10^{-6} M^{-1}, with less than about 10^{-5} to 10^{-9} M^{-1} being preferred and less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred.

Generally, a sample containing a target analyte (whether for detection of the target analyte or screening for binding partners of the target analyte) is added to the array, under conditions suitable for binding of the target analyte to at least one of the bioactive agents, i.e. generally physiological conditions. The presence or absence of the target analyte is then detected. As will be appreciated by those in the art, this may be done in a variety of ways, generally through the use of a change in an optical signal. This change can occur via many different mechanisms. A few examples include the binding of a dye-tagged analyte to the bead, the production of a dye species on or near the beads, the destruction of an existing dye species, a change in the optical signature upon analyte interaction with dye on bead, or any other optically interrogatable event.

In a preferred embodiment, the change in optical signal occurs as a result of the binding of a target analyte that is labeled, either directly or indirectly, with a detectable label, preferably an optical label such as a fluorochrome. Thus, for example, when a proteinaceous target analyte is used, it may be

either directly labeled with a fluor, or indirectly, for example through the use of a labeled antibody. Similarly, nucleic acids are easily labeled with fluorochromes, for example during PCR amplification as is known in the art. Alternatively, upon binding of the target sequences, a hybridization indicator may be used as the label. Hybridization indicators preferentially associate with double stranded nucleic acid, usually reversibly. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only occurs in the presence of double stranded nucleic acid, only in the presence of target hybridization will the label light up. Thus, upon binding of the target analyte to a bioactive agent, there is a new optical signal generated at that site, which then may be detected.

Alternatively, in some cases, as discussed above, the target analyte such as an enzyme generates a species that is either directly or indirectly optically detectable.

Furthermore, in some embodiments, a change in the optical signature may be the basis of the optical signal. For example, the interaction of some chemical target analytes with some fluorescent dyes on the beads may alter the optical signature, thus generating a different optical signal.

As will be appreciated by those in the art, in some embodiments, the presence or absence of the target analyte may be done using changes in other optical or non-optical signals, including, but not limited to, surface enhanced Raman spectroscopy, surface plasmon resonance, radioactivity, etc.

The assays may be run under a variety of experimental conditions, as will be appreciated by those in the art. A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding. Various blocking and washing steps may be utilized as is known in the art.

In a preferred embodiment, two-color competitive hybridization assays are run. These assays can be based on traditional sandwich assays. The beads contain a capture sequence located on one side (upstream or downstream) of the SNP, to capture the target sequence. Two SNP allele-specific probes, each labeled with a different fluorophor, are hybridized to the target sequence. The genotype can be obtained from a ratio of the two signals, with the correct sequence generally exhibiting better binding. This has an advantage in that the target sequence itself need not be labeled. In addition, since the probes are competing, this means that the conditions for binding need not be optimized. Under conditions where a mismatched probe would be stably bound, a matched probe can still displace it. Therefore the competitive assay can provide better discrimination under those conditions. Because many assays are carried out in parallel, conditions cannot be optimzed for every probe

simultaneously. Therefore, a competitive assay system can be used to help compensate for non-optimal conditions for mismatch discrimination.

In a preferred embodiment, dideoxynucleotide chain-termination sequencing is done using the compositions of the invention. In this embodiment, a DNA polymerase is used to extend a primer using fluorescently labeled ddNTPs. The 3' end of the primer is located adjacent to the SNP site. In this way, the single base extension is complementary to the sequence at the SNP site. By using four different fluorophors, one for each base, the sequence of the SNP can be deduced by comparing the four base-specific signals. This may be done in several ways. In a first embodiment, the capture probe can be extended; in this approach, the probe must either be synthesized 5'-3' on the bead, or attached at the 5' end, to provide a free 3' end for polymerase extension. Alternatively, a sandwich type assay can be used; in this embodiment, the target is captured on the bead by a probe, then a primer is annealed and extended. Again, in the latter case, the target sequence need not be labeled. In addition, since sandwich assays require two specific interactions, this provides increased specificity which is particularly helpful for the analysis of complex samples.

In addition, when the target analyte and the DBL both bind to the agent, it is also possible to do detection of non-labelled target analytes via competition of decoding.

In a preferred embodiment, the methods of the invention are useful in array quality control. Prior to this invention, no methods have been described that provide a positive test of the performance of every probe on every array. Decoding of the array not only provides this test, it also does so by making use of the data generated during the decoding process itself. Therefore, no additional experimental work is required. The invention requires only a set of data analysis algorithms that can be encoded in software.

The quality control procedure can identify a wide variety of systematic and random problems in an array. For example, random specks of dust or other contaminants might cause some sensors to give an incorrect signal-this can be detected during decoding. The omission of one or more agents from multiple arrays can also be detected; that is, since the arrays formulated herein are randomly self-assembled, arrays comprising different statistical distributions of each sensor elements may be made; the invention allows actual QC on the arrays, for "sorting" arrays with good distributions from those with less than ideal distributions. These procedures also allow the determination of sites that do not comprise beads. This quality control procedure can also be used in non-random arrays.

An advantage of this quality control procedure is that it can be implemented immediately prior to the assay itself (or after, in some instances), and is a true functional test of each individual sensor. Therefore any problems that might occur between array assembly and actual use can be detected. In applications where a very high level of confidence is required, and/or there is a significant chance of

sensor failure during the experimental procedure, decoding and quality control can be conducted both before and after the actual sample analysis.

In an alternative method of quality control, a single fiber bundle of an array is dedicated to quality control. That is a dedicated bundle or subset of fibers within an array is independently analyzed for quality control. In one embodiment, the control fiber(s) are targeted or labeled with a unique code or label. At each state of hybridization, in addition to the experimental probes, the quality control probes are hybridized with the array. Upon taking the image from the first hybridization of the first stage, the intensity of the control fiber(s) is analyzed. An aberrant reading or signal from the control fiber indicates a failed or at least non-optimal hybridization step.

In a preferred embodiment, the arrays can be used to do reagent quality control. In many instances, biological macromolecules are used as reagents and must be quality controlled. For example, large sets of oligonucleotide probes may be provided as reagents. It is typically difficult to perform quality control on large numbers of different biological macromolecules. For example, when large populations of different random oligonucleotides are synthesized, it may be desirable to check that each population is represented. The approach described here can be used to do this by treating the reagents (formulated as the DBLs) as variable instead of the arrays.

In a preferred embodiment, the methods outlined herein are used in array calibration. For many applications, such as mRNA quantitation, it is desirable to have a signal that is a linear response to the concentration of the target analyte, or, alternatively, if non-linear, to determine a relationship between concentration and signal, so that the concentration of the target analyte can be estimated. For example, different probes can show differences in hybridization efficiencies, etc., and it may be desirable to choose appropriate probes under experimental conditions. Thus, for example, when expression profiling is done, and quantitation is desirable, calibration curves can be done to see how individual probes react, and then the probes that give the best response (i.e. linearity at the concentrations and conditions of interest) can be chosen for further assays. Accordingly, the present invention provides methods of creating calibration curves in parallel for multiple beads in an array. The calibration curves can be created under conditions that simulate the complexity of the sample to be analyzed. Each curve can be constructed independently of the others (e.g. for a different range of concentrations), but at the same time as all the other curves for the array.

Thus, in these embodiments, different types of experiments can be done. For example, the sequential decoding scheme can be implemented with different concentrations being used as the code "labels", rather than different fluorophores. In this way, signal as a response to concentration can be measured for each bead. This calibration can be carried out just prior to array use, so that every probe on every array is individually calibrated as needed. Alternatively, different concentrations of the decoding probe can have different labels.

It should be noted that the assay calibration methods find use in non-random arrays, as well; that is, other types of support-bound nucleic acid arrays can be calibrated using these methods as well. Thus, for example, the sequential addition of different pools of probes, wherein the concentration of the probes is varied, to biochips can allow calibration of any assay system. This type of analysis can also be done on non-random arrays for quality control, to verify the integrity and sequence of the support bound probes, and in assay development to identify good probes.

In a preferred embodiment, the methods of the invention can be used in assay development as well. Thus, for example, the methods allow the identification of good and bad probes; as is understood by those in the art, some probes do not function well because they do not hybridize well, or because they cross-hybridize with more than one sequence. These problems are easily detected during decoding. The ability to rapidly assess probe performance has the potential to greatly reduce the time and expense of assay development. Thus, probes that respond linearly with concentration, show low non-specific binding, or give signals in a particular range, can be chosen for addition to a new array for assays.

Similarly, in a preferred embodiment, the methods of the invention are useful in quantitation in assay development. Major challenges of many assays is the ability to detect differences in analyte concentrations between samples, the ability to quantitate these differences, and to measure absolute concentrations of analytes, all in the presence of a complex mixture of related analytes. An example of this problem is the quantitation of a specific mRNA in the presence of total cellular mRNA. One approach that has been developed as a basis of mRNA quantitation makes use of a multiple match and mismatch probe pairs (Lockhart et al., 1996), hereby incorporated by reference in its entirety. While this approach is simple, it requires relatively large numbers of probes. In this approach, a quantitative response to concentration is obtained by averaging the signals from a set of different probes to the gene or sequence of interest. This is necessary because only some probes respond quantitatively, and it is not possible to predict these probes with certainty. In the absence of prior knowledge, only the average response of an appropriately chosen collection of probes is quantitative. However, in the present invention, that can be applied generally to nucleic acid based assays as well as other assays. In essence, the approach is to identify the probes that respond quantitatively in a particular assay, rather than average them with other probes. This is done using the array calibration scheme outlined above, in which concentration-based codes are used. Advantages of this approach include: fewer probes are needed; the accuracy of the measurement is less dependent on the number of probes used; and that the response of the sensors is known with a high level of certainty, since each and every sequence can be tested in an efficient manner. It is important to note that probes that perform well are selected empirically, which avoids the difficulties and uncertainties of predicting probe performance, particularly in complex sequence mixtures. In contrast, in experiments described to date with ordered arrays, relatively small numbers of sequences are checked by performing quantitative spiking experiments, in which a known mRNA is added to a mixture.

In a preferred embodiment, cDNA arrays are made for RNA expression profiling. In this embodiment, individual cDNA clones are amplified (for example, using PCR) from cDNA libraries propagated in a host-vector system. Each amplified DNA is attached to a population of beads. Different populations are mixed together, to create a collection of beads representing the cDNA library. The beads are arrayed, decoded as outlined above, and used in an assay (although as outlined herein, decoding may occur after assay as well). The assay is done using RNA samples (whole cell or mRNA) that are extracted, labeled if necessary, and hybridized to the array. Comparative analysis allows the detection of differences in the expression levels of individual RNAs. Comparison to an appropriate set of calibration standards allows quantification of absolute amounts of RNA.

The cDNA array can also be used for mapping, e.g. to map deletions/insertions or copy number changes in the genome, for example from tumors or other tissue samples. This can be done by hybridizing genomic DNA. Instead of cDNAs (or ESTs, etc.), other STS (sequence tagged sites), including random genomic fragments, can also be arrayed for this purpose.

In one embodiment, the invention provides a method of single nucleotide polymorphism (SNP) genotyping. As described herein and in U.S.S.N.s 09/553,993 filed April 20, 2000, 09/556,463 filed April 21, 2000 and 60/244,119, filed October 26, 2000, all of which are expressly incorporated herein by reference, a target nucleic acid is contacted with a probe that is specific for a particular allele of a single nucleotide polymorphism. The target nucleic acid can be immobilized prior to contacting with the probe, or alternatively, the probe: target hybridization can occur in solution following by immobilization of the probe: target nucleic acid complex. Alternatively, a capture prove on a bead may be specific for a particular SNP. What is important in this invention is the nature of the probe for detecting a SNP.

There are four possibilities for the bases at a polymorphic site: -A, T, G, or C. However, it is known that most of the human SNP's are dimorphic, i.e., they only include the combinations of two of the four bases. As such, the prior art generally relied on distinguishing between two alleles that are present within a SNP. and characterized them as either "AA", "AB, or "BB".

However, according to the present invention, the probes are designed such that each of the four possible nucleotides that could be present at a particular position are included in the SNP probes. In addition, the probes are labeled with a specific color that corresponds with the nucleotide that hybridizes to the nucleotide to be interrogated. For instance, all the alleles that correspond to an A-polymorphic site are labeled with red. Similarly, all the alleles that correspond to a G-polymorphic site are labeled green, etc. As such the true identity of the polymorphism is determined. That is, if the polymorphism is of the type GT, one would not classify is as "AB" genotype. Instead, the GT code will come up directly from the genotyping routine.

An advantage of this method over the prior art is the capability of independent error checking. That is, knowing that polymorphisms are typically dimorphic, by using probes that correspond to all possibilities of polymorphisms at a sites, one obtains a check against false results, for example of a signal is detected that does not correspond with the known polymorphisms. That is, in the prior art, the resultant genotyping would be one of the following three possibilities: AA, AB, or BB. However, by using this invention, the number of possible results is increased from 3 to 10: AA, AG, AC, AT, GG, GC, GT, CC, CT, TT . Of these ten possibilities, only 3 are the possible combinations for each SNP, e.g., a T/G polymorphism can only render one of the following three possibilities: TT, TG, or GG. Therefore, in this case, if the computed genotype falls into any of the remaining 10-3=7 combinations (e.g., either of AA, AG, AC, AT, GC, CC, CT), it would be a clear indication that an error has occurred.

All references cited herein are incorporated by reference in their entirety.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1962

Examples

Example 1

Sixteen microspheres (beads) were labeled combinatorially with two different fluorophores (FAM and Cy3). In a first round of labeling, either FAM or Cy3 labeled oligonucleotides that were complementary to the oligonucleotide (IBL) on the microsphere, were hybridized with the microsphere. Labeling of oligonucleotides was performed as is well known in the art. Hybridization conditions are known in the art.

Following a first round of hybridization, the two pools of beads were divided into two pools each and each labeled either with the FAM or Cy3 labeled oligonucleotide. This process was repeated two additional times. Thus, following four successive rounds of labeling, each microsphere was labeled with a unique code (see Figure 1). The identity of each microsphere was elucidated by determining the identity of each fluorophore in succession; the terminal fluorophere was determined and then removed to allow for the identification of the next fluorophore. In this fashion, with as few as 4 decoding steps, the identity of 16 microspheres is determined.

Example 2

A decoding scheme similar to that described in Example 1 was implemented for four color decoding. In this example, beads were labeled as described in Example 1 with the exception that 4 labels were used at each stage. 4013 beads were labeled using Bod493, BodR6G, Bod564 and BodTXR labeled oligonucleotides. 128 different bead types were identified based on the successive decoding of the four colors.

Example 3

An alternative method to using multiple colors is to use ratiometric intensities as a coding scheme. A normalizing image is acquired in which every bead exhibits its "full" intensity. Subsequent decode stages generate intensity codes by hybridizing mixtures of "labeled":"unlabeled" complementary oligonucleotides. For instance, Fig. 1 depicts three different intensity shades (low, medium, and high) which can be ratioed to a stage with all complements present at a "high" shading value. An experiment using grey scale decoding on 16 different bead types is shown in Fig. 3.

Figure 3A depicts the combinatorial pooling scheme for labeling beads with different ratios of labeled oligonucleotides. A particular oligo is present at either 100% Cy3-labeled, 40% Cy3-labeled (60% unlabeled), or 10% Cy3-labeled (90% unlabeled) fraction. Decode oligos were hybridized to the array

for 2 min. at a 50 nM concentration. Subsequently, two independent normalizing images (all oligo complements are present as 100% Cy3-labeled species) were acquired, and the resulting bead intensities compared. This is depicted in Figure 3B as the normalized values are plotted against each other. Finally, to identify or decode the beads, the alpha values (ratio of bead intensity in indicated decode stage to intensity in normalization image) are plotted for three decode stages described in (A). In stage 1, only two peaks are observed in the alpha value histogram since only 16 bead types are present on the array. Three distinguishable peaks are observed in the second and third decode stages indicating the feasibility of grey scale decoding.

Physical attributes and different "levels" of the attributes can be used as codes by which to distinguish bead types from another. Thus, for an attribute to act as a robust code, it should be possible to imbue a bead with different "levels" of a particular attribute. Each "level" of an attribute should be quantitatively well separated from other "levels". The important point is to maximize the dynamic range of the attribute measurement, and minimize the spread of the measurement.

Example 4

For a given oligo sequence, several codes can be generated by varying the distance of separation of the two fluorophores . As shown in Figure 4, a complementary sequence containing FRET dyes separated by a linker of varying lengths allows multiple codes to be generated (Figure 4A). As an example, Probe A and B are hybridized to a complementary sequence immobilized on a solid support. Probe A contains dyes separated by an 8 poly T linker, whereas probe B is hybridized to a linker with a 4 poly T linker. The efficiency of energy transfer (ratio of intensities of dye2:dye1) is greater for probe B than for probe A (Figure 4B).

Example 5

The following FRET oligonucleotides (probes ET1, ET2, ET5 and ET7) were synthesized as is known in the art and labeled with Cy3 and fluorscein separated by linkers of varying length:

ET1 T**G*CACGAGAATGGAGGTATCT
ET2 C**TGTCGC*ACGAGAATGGAGGTATCT
ET5 C**TGTCGGGGCACTCATTTGTGC*ACGAGAATGGAGGTATCT
ET7 C**TGTCGGGGCACTCATTTGTCTGTCGGGGCGC*ACGAGAATGGAGGTATCT.

**is Cy3 * is fluorescein

An aliquot of each FRET oligonucleotide was assayed as is known in the art. Emission spectra were normalized relative to emission at 530nm. As can be seen in Figure 5 emission of fluorescein increases as the linker is shortened. The ratio of intensities of the two fluorophores, therefore, is characteristic of the distance between the fluorophores.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1964

Example 6

To illustrate the primer extension decoding, a two-color model is illustrated in Figure 9. To decode 16 different bead addresses using only two colors. 16 different beads are labeled by attaching 16 different unique oligos (IBLs) to each of 16 batches of beads and pooling the bead batches together. Along with the beads 16 oligos (DBLs) that are complementary to these IBLs are synthesized. Each unique IBL used in this method contains two domains. The domains are composed of a binding domain and an extension domain. The pooled bead populations are then self-assembled onto a fiber optic tip end as described herein. After array self-assembly a combinatorial color decoding process is initiated as follows: A primer extension reaction is initiated at the end of the fiber bundle tip containing the arrayed beads by simultaneously hybridizing 16 complementary oligos (DBLs) to their cognate IBL. In the first extension stage, the DBLs would have exactly the same length (n) as the binding domain of the IBL . The first stage primer extension reaction would contain 16 primer oligos, a DNA polymerase, appropriate buffer, and two fluorescently-labeled dideoxynucleotide terminators. During the extension reaction, hybridized primers are extended by a single nucleotide complementary to the first nucleotide in the extension domain of the immobilized IBL. The described extension reaction 'paints' or labels any given bead with one of two possible fluorescent terminator colors. After the reaction is over the array tip is imaged to capture the color of the beads at this stage. Beads are then immediately stripped of color or label by denaturing and washing off the first extended primer at each zip code loci. This process can be repeated through multiple stages. In subsequent stages, the primer extension reactions contain the same reagents as the previous extension reaction except that the binding complementary oligos are one base longer. These (n+1) primers hybridize with their cognate binding domain and one base into the 'extension domain'. Figure 9 illustrates a four-stage extension process (2-color) whereby each stage allows for the assignment of a unique combinatorial color-coded 'address' to each bead type.

In addition, if this primer-based extension method were to be combined with degenerate DBL-BL combinations for decoding large combinatorial libraries, for example using the CVC (constant-variable-constant domain idea), as described herein, the extension reaction would extend into one of 10 different possible nucleotide combinations in a ' variable extension region'. This would require only three extension reactions to decodify 1000 unique addresses (i.e. A = lot where L is the length of the variable region). The combination of the degenerate DBL-IBL combinations such as CVC with the primer extension method is illustrated in Figure 10.

Example 7

This method relates to fluorescent based encoding and decoding process based on the fact that fluorescent dyes have broad band absorption and emission spectra. Fewer detection channels are used than the number of encoding dyes used in the process. That is, for n detection channels, a particular dye, x, can be represented by a vector I_x in the n -dimensional space.

$$I_x \{ \frac{I}{\sum_{i}^{n} I_i} \perp I_1 I_2 I_3 .... I_n$$

where I_i is the intensity detected at channel i. Theoretically, an infinite number of vectors are possible in the n-dimensional space when n is larger or equal to 2. With an appropriate dye selection and optical filter design, for any given number of detection channels, a larger number of dyes can be used for decoding. For example, to distinguish three species, it is possible to tag the species with three different dyes, x, y, z and use only two detection channels, such that

$I_x = \{1 \quad 0\}$
$I_y = \{a \quad 1-a\} \quad 0< a <1$
$I_z = \{ 0 \quad 1\}$

Figure 11 illustrates the experimental results for this example scheme used in decoding. Three classes of beads were tagged with Bodipy-493, Bodipy-R6G and Bodipy564. Detection channel 1 consisted of a 410nm/20 excitation filter and a 540nm/20 emission filter. Detection channel 2 consisted of a 535nm/20 excitation filter and a 585nm/nm emission filter. Excited with a xenon arc lamp, the fluorescence emission of Bodipy-493 can only be detected in channel 1. Bodipy-564 emission can be detected in channel 2. Bodipy-R6G can be detected in both channels. As expected, the scatter plot (Figure 11) of the intensity in the two channels of the beads clearly indicates three classes. Within each class, the beads have the same relative intensity ratio (corresponding to the same unit vector) with a certain uncertainties.

Similar design and data analysis procedure can be used for other dyes and detection channels.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1966

CLAIMS

We claim:

1. A method of decoding an array composition comprising
a) providing an array composition comprising a population of microspheres comprising at least 500 subpopulations, wherein each subpopulation comprises a bioactive agent;
b) adding a plurality of decoding binding ligands to said population of microspheres to identify at least 500 of the bioactive agents.

2. A method according to claim 1 wherein at least one subpopulation of microspheres comprises an identifier binding ligand to which a decoding binding ligand can bind.

3. A method according to claim 1 or 2 wherein said decoding binding ligands bind to said bioactive agents.

4. A method according to claim 1, 2 or 3, wherein said decoding binding ligands are labeled.

5. A method according to claim 1, 2, 3 or 4, wherein the is bioactive agent of each subpopulation is determined.

6. A method according to claim 1,2, 3, 4 or 5, wherein said array composition further comprises a substrate with a surface comprising discrete sites, wherein said microspheres are randomly distributed on said substrate.

7. A method according to claim 1,2, 3, 4 or 5, wherein said array composition is a liquid array.

8. A method according to claim 1,2, 3, 4 or 5, wherein each subpopulation comprises a bioactive agent and does not comprise an optical signature

9. A method according to claim 1, 2, 3, 4, 5, 6, 7 or 8. wherein said bioactive agents are nucleic acids.

10. A method according to claim 1, 2, 3, 4, 5, 6, 7 or 8 wherein said bioactive agents are proteins.

11. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, wherein said population comprises at least 1000 subpopulations.

12. A method according to claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11, wherein said population comprises at least 1500 subpopulations.

13. A method of determining the presence of a target analyte in a sample comprising:
a) contacting said sample with a composition comprising a population of microspheres comprising at least 500 subpopulations, wherein each subpopulation comprises a bioactive agent;
b) adding a plurality of decoding binding ligands to said population of microspheres to identify at least 500 of the bioactive agents; and
c) determining the presence or absence of said target analyte.

14. A method according to claim 13, wherein said subpopulations are distributed on a substrate.

15. A method according to claim 14 wherein said array is a liquid array.

16. A method according to claim 15, wherein said determining is by FACS.

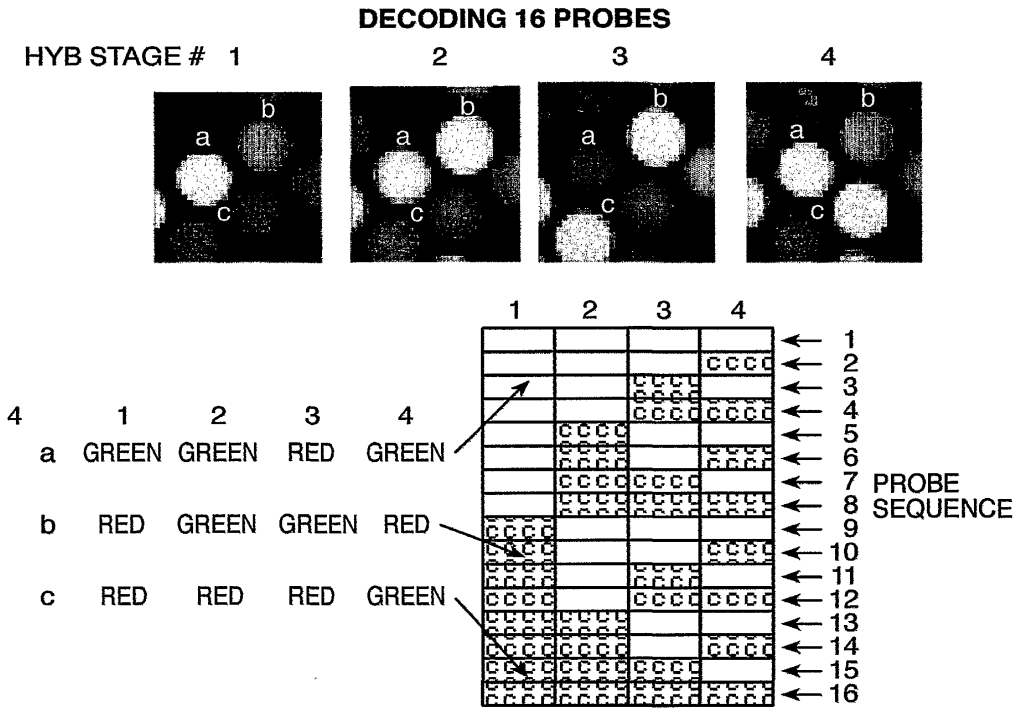


FIG._1

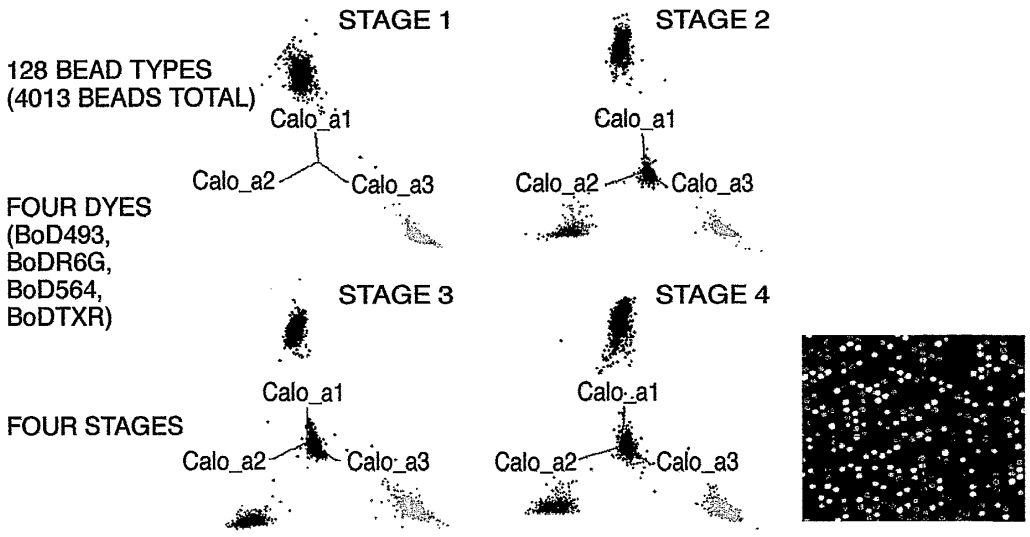


FIG._2

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1969

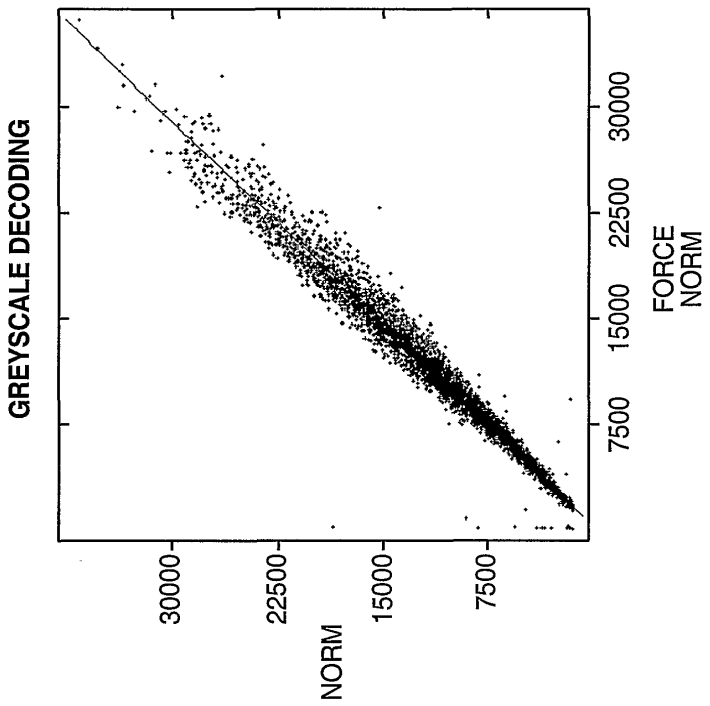


FIG._3B

GREYSCALE DECODING

CODE	S1	S2	S3
1	100	100	100
2	100	100	40
3	100	100	10
4	100	40	100
5	100	40	40
6	100	40	10
7	100	10	100
8	100	10	40
9	100	10	10
10	40	100	100
11	40	100	40
12	40	100	10
13	40	40	100
14	40	40	40
15	40	40	10
16	40	10	100
17	40	10	40
18	40	10	10
19	10	100	100
20	10	100	40
21	10	100	10
22	10	40	100
23	10	40	40
24	10	40	10
25	10	10	100
26	10	10	40
27	10	10	10

FIG._3A

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1970

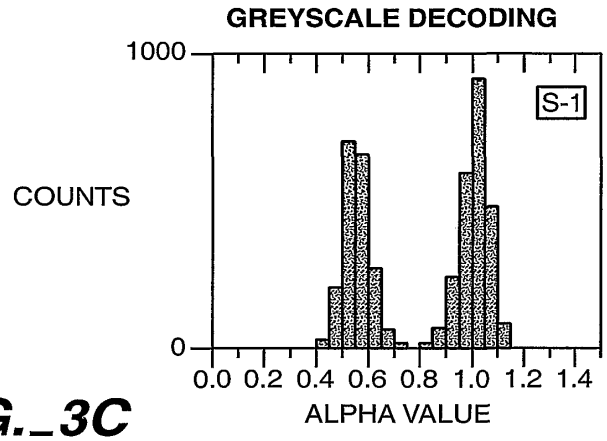


FIG._3C

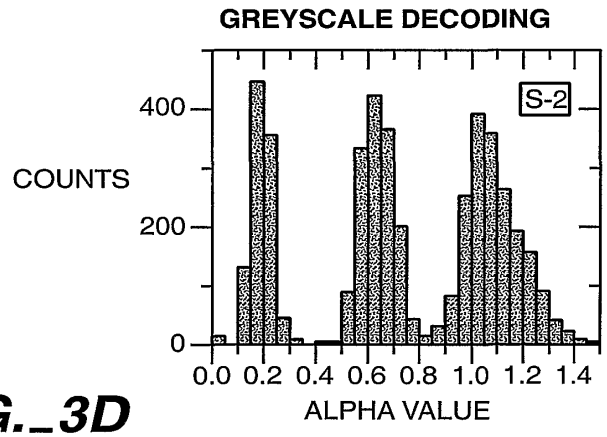


FIG._3D

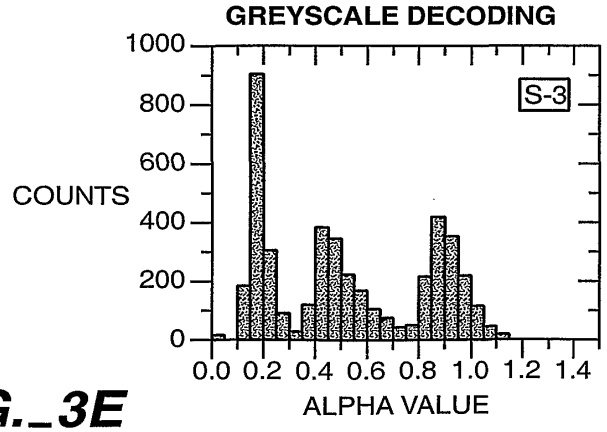


FIG._3E

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1971

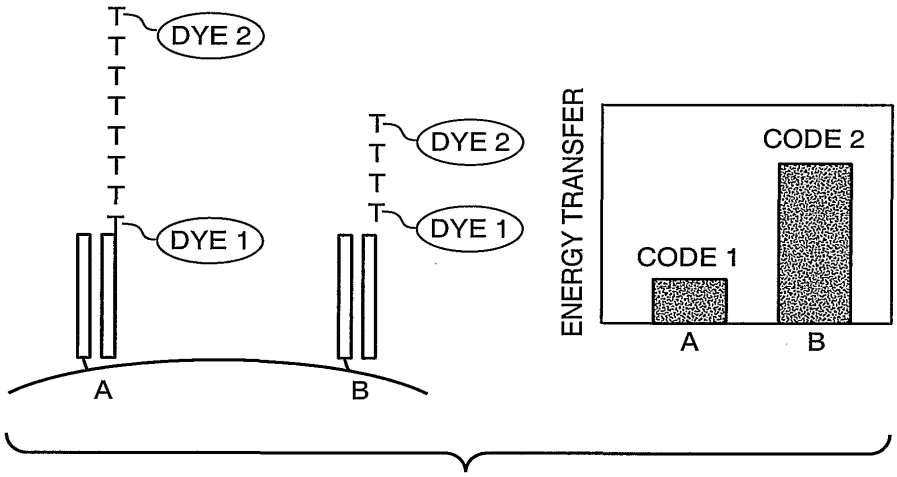


FIG._4

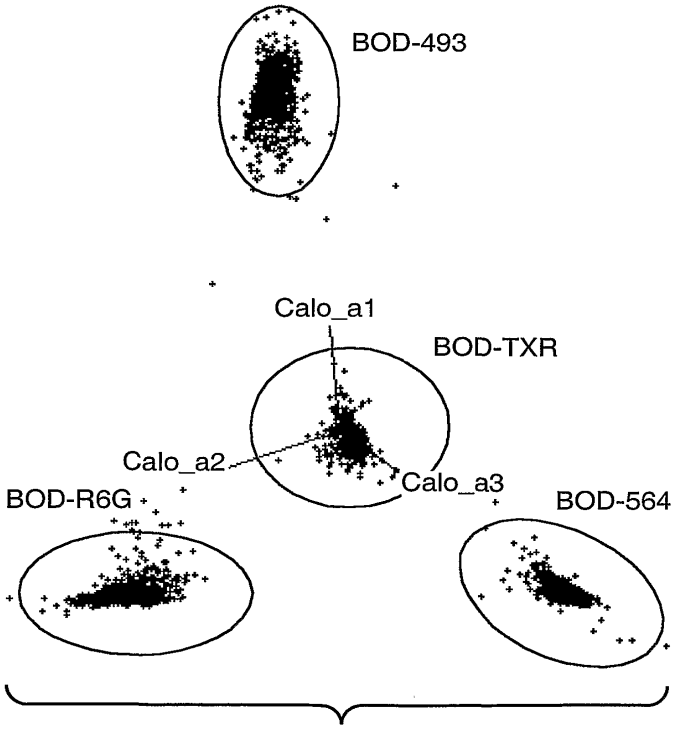
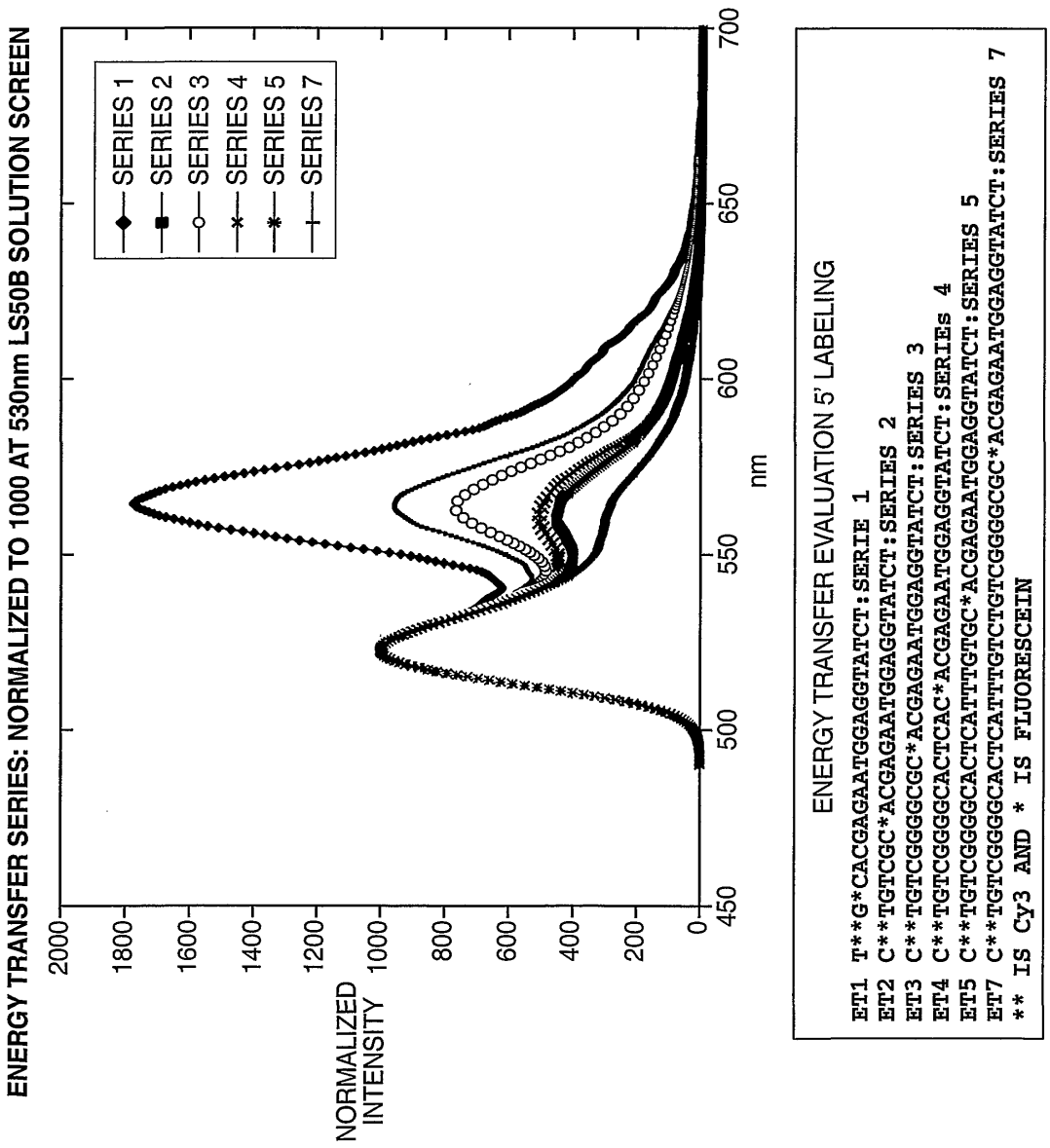


FIG._6

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1972

FIG._5



IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1973

	1 "COLOR" DECODING					
BEAD	STAGE 1	STAGE 2	STAGE 3	STAGE 4	CODE	PARITY
1	1	1	1	1	1111	EVEN
	1	1	1		1110	ODD
	1	1		1	1101	ODD
2	1	1			1100	EVEN
	1		1	1	1011	ODD
3	1		1		1010	EVEN
4	1			1	1001	EVEN
	1				1000	ODD
		1	1	1	0111	ODD
5		1	1		0110	EVEN
6		1		1	0101	EVEN
		1			0100	ODD
7			1	1	0011	EVEN
			1		0010	ODD
				1	0001	ODD
8					0000	EVEN

1001 DIGIT SUM = 2, EVEN PARITY

↓

1011 DIGIT SUM = 3, ODD PARITY

FIG._7

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1974

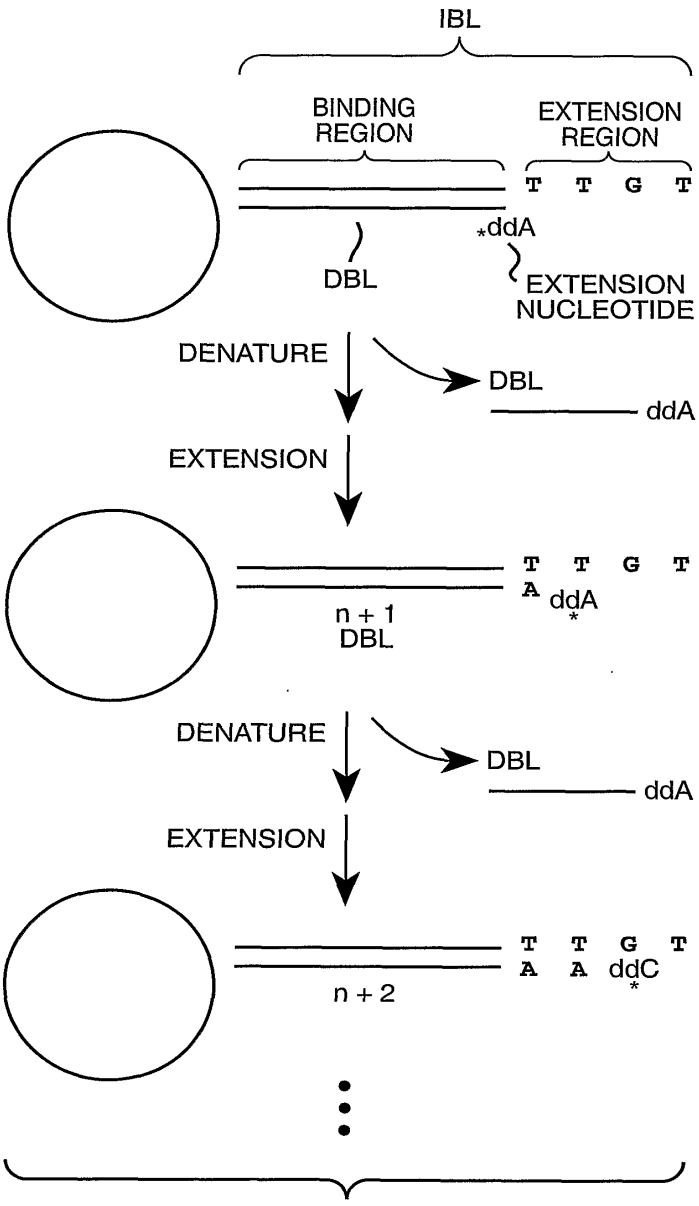


FIG._8

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1975

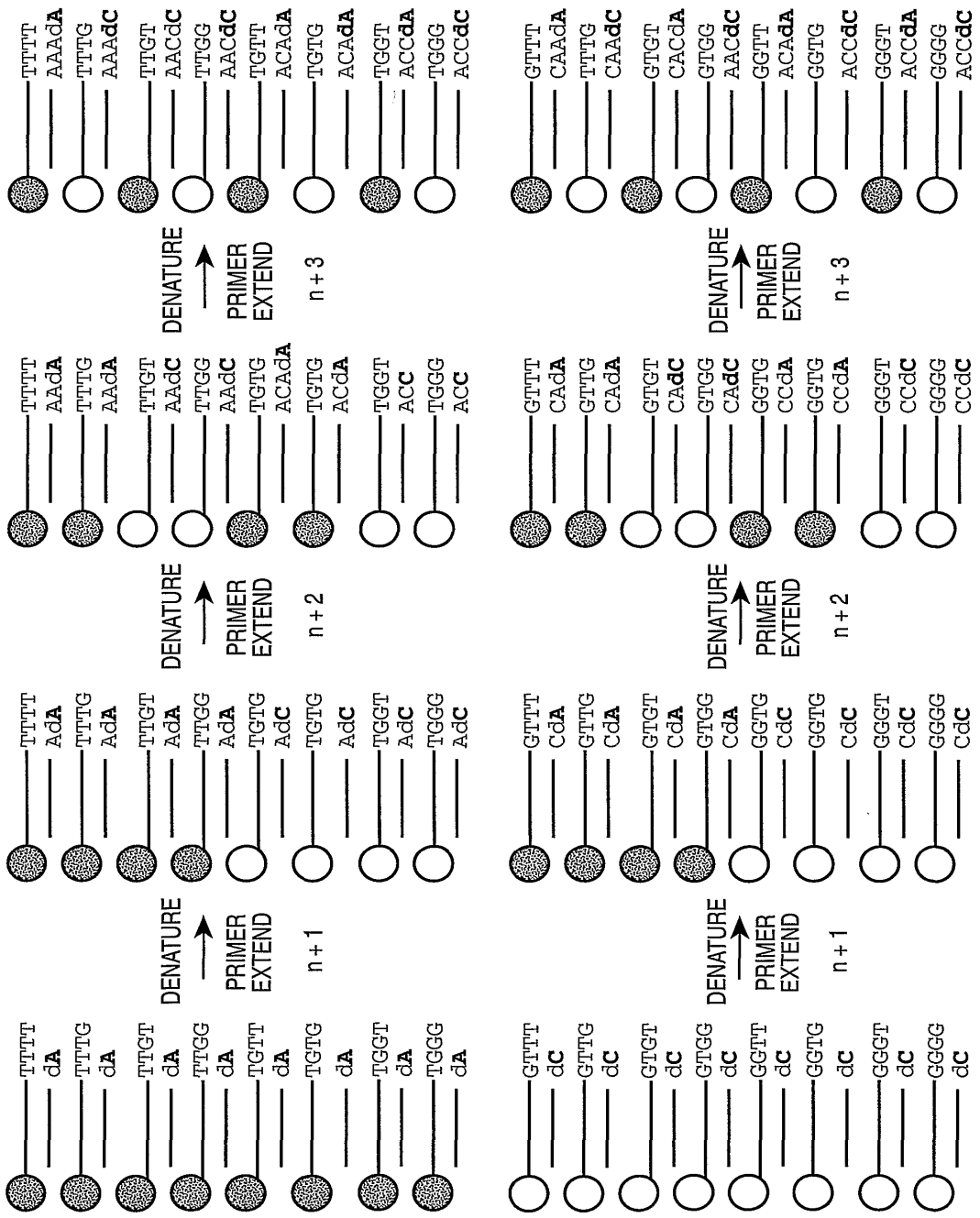


FIG._9

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1976

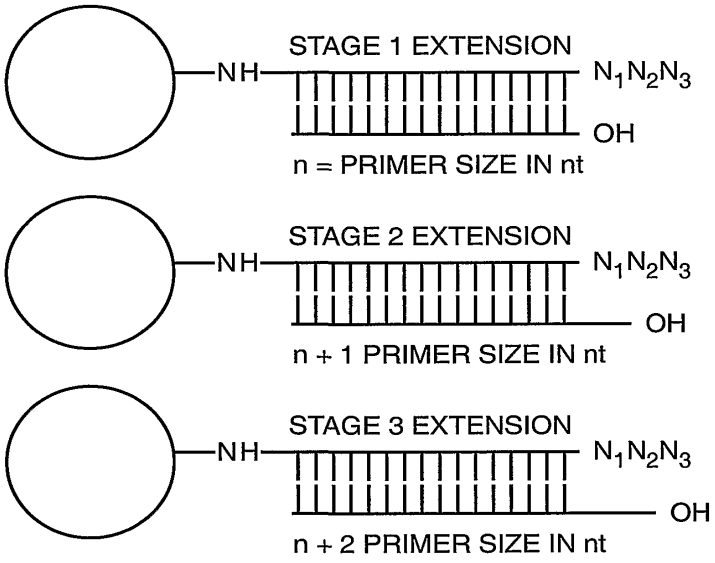


FIG._10

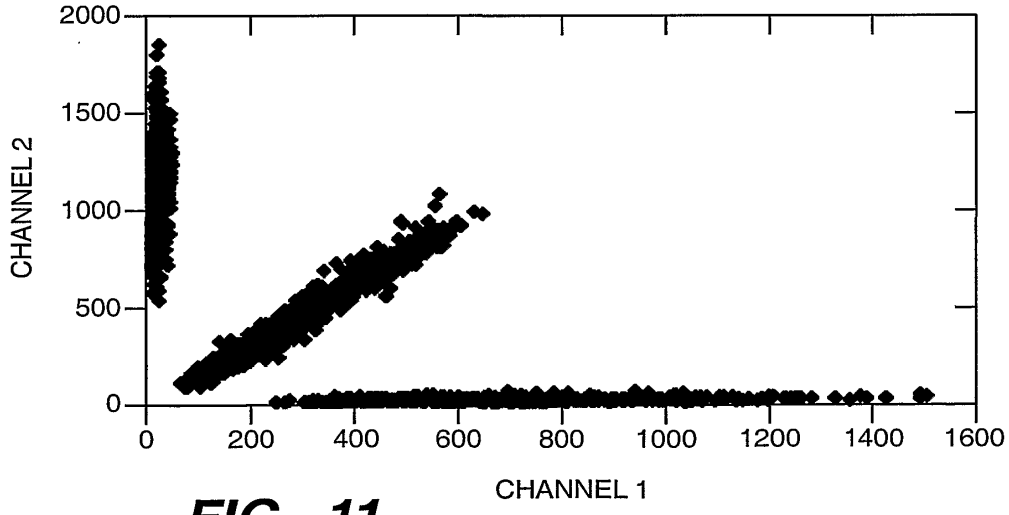


FIG._11

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1977

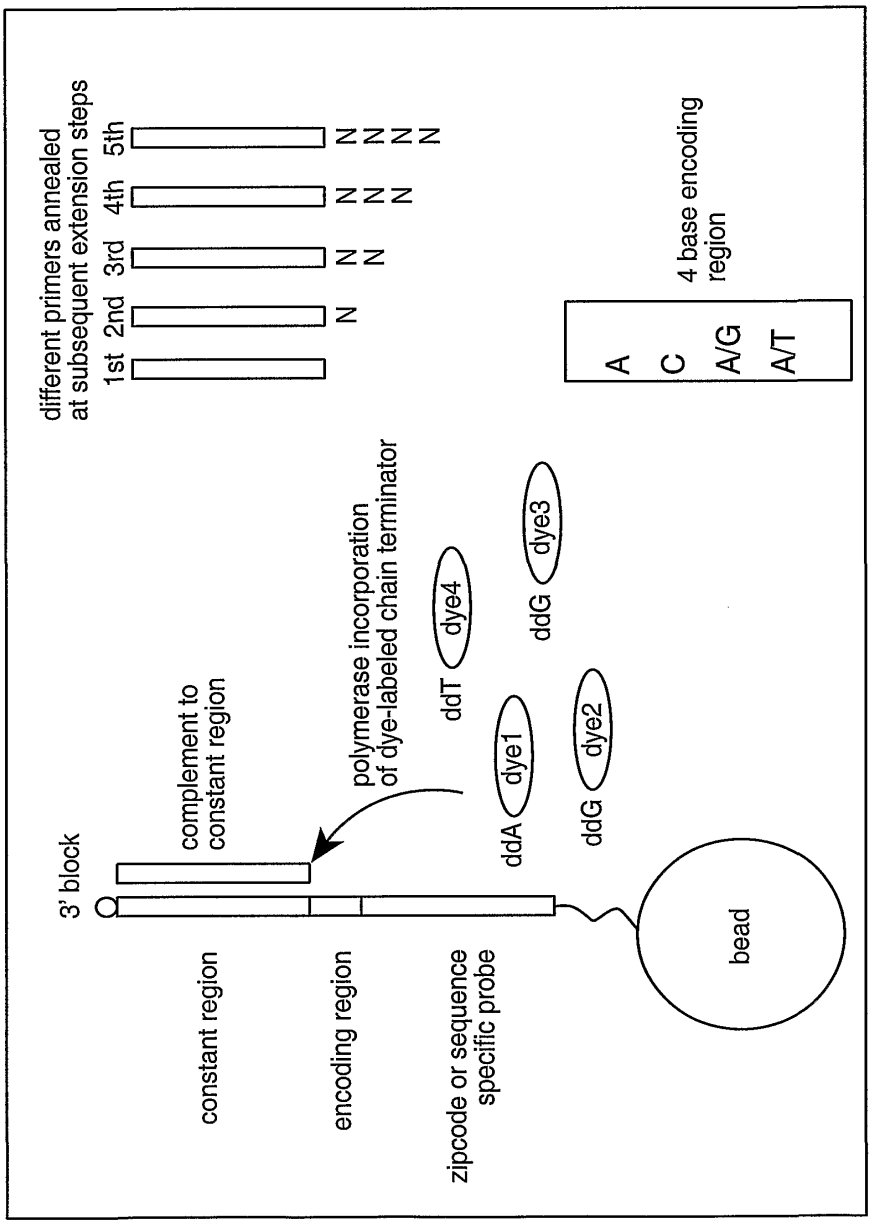


FIG._12

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1978

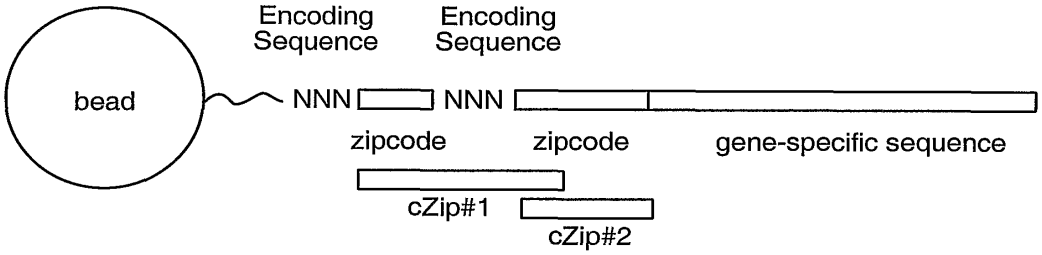


FIG._13

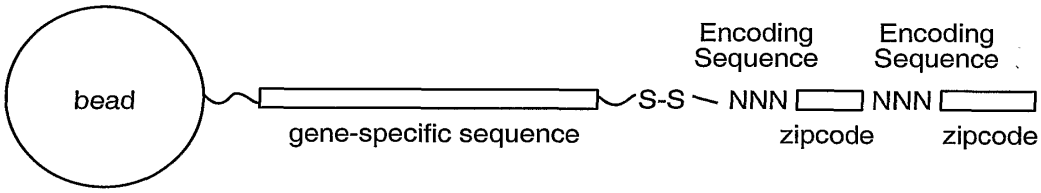


FIG._14

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1979

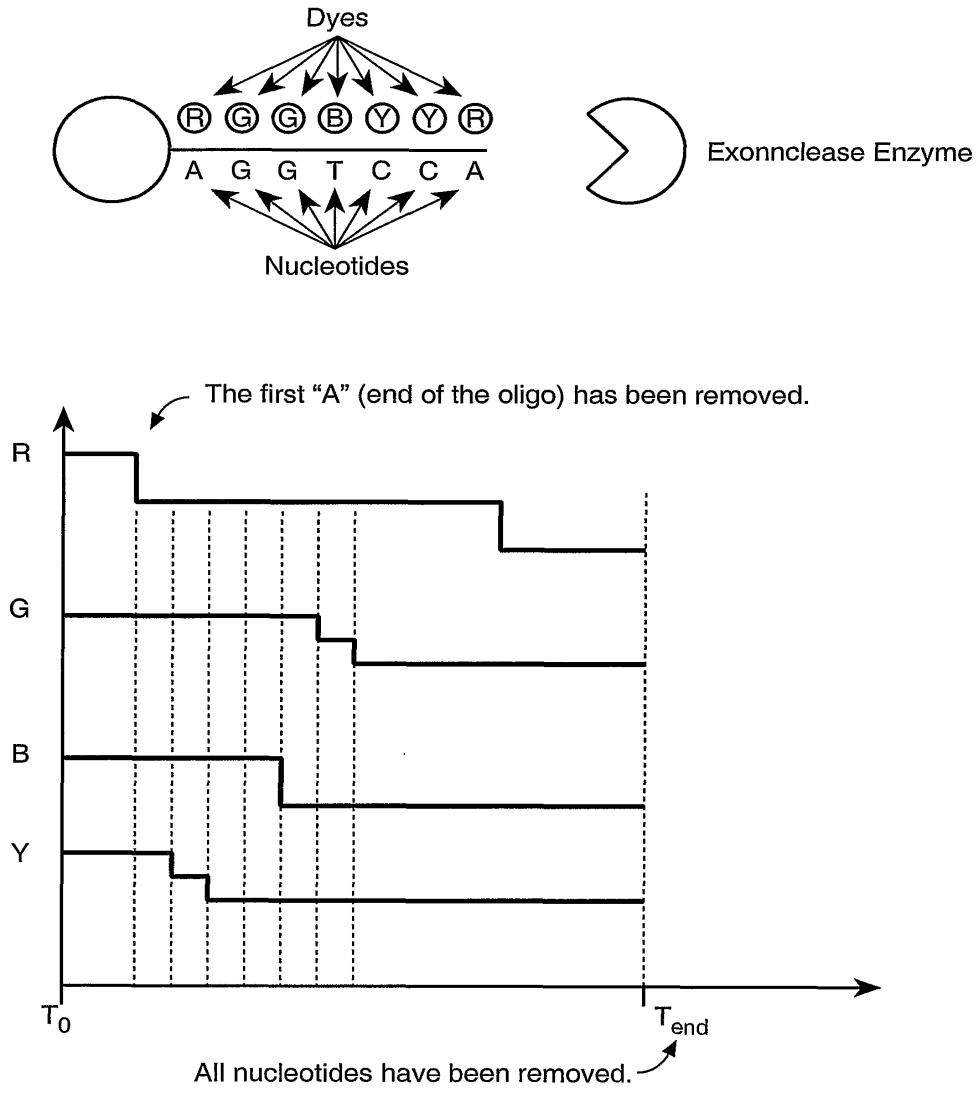


FIG._15

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1980

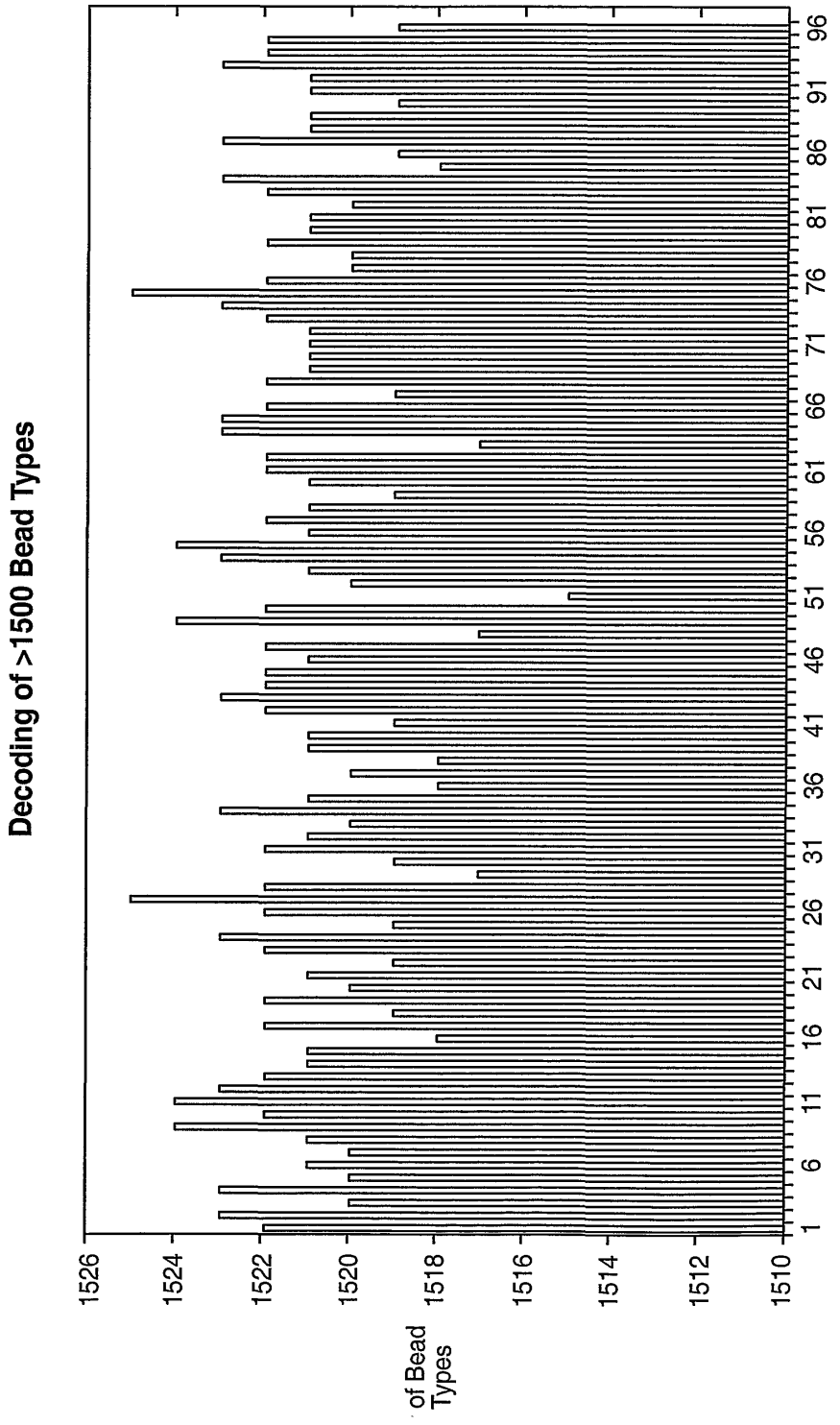


FIG._16

IPR2024-01177
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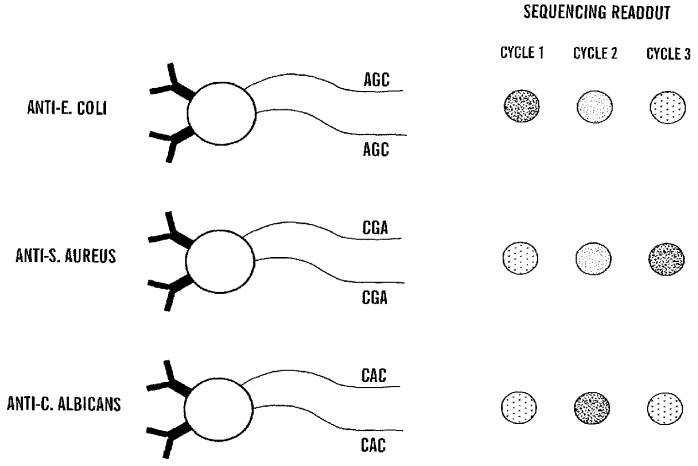
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(54) **Title**: COMPOSITIONS AND METHODS FOR ANALYTE DETECTION



(57) **Abstract**: The inventions provided herein relate to detection reagents, compositions, methods, and kits comprising the detection reagents for use in detection, identification, and/or quantification of analytes in a sample. Such detection reagents and methods described herein allow multiplexing of many more labeled species in the same procedure than conventional methods, in which multiplexing is limited by the number of available and practically usable colors.

WO 2013/096851 A1

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1982

COMPOSITIONS AND METHODS FOR ANALYTE DETECTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Application No. 61/579,265 filed December 22, 2011, the content of which is incorporated herein by reference in its entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under HG005550 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD OF THE DISCLOSURE

[0003] The inventions provided herein relate to detection molecules or detection reagents, compositions, methods and kits for detection, identification, and/or quantification of analytes in a sample.

BACKGROUND

[0004] The need for multiplexing techniques in biology is often driven by the fact that test samples are precious and those analyzing them either do not know in advance precisely what to look for or must extract the most information from any single sample. Hence, it is desirable for clinicians and researcher to subject each sample to a large set of probes.

[0005] Optical readout is common in biology and can be very effective. However, it is typically limited to a relatively small number of available fluorophores or chromophores (which are referred to collectively as colors). In practice, multiplexing by fluorescence is often limited to 4 or 5 colors, which by traditional methods implies that at most 4 or 5 probes can be detected in a single sample.

[0006] The common approach to improving multiplexing in optical methods is to increase the number of available colors. To this end, quantum dots have been developed to provide a larger range of colors. However, in reality, it is difficult to use more than 6 quantum dot colors simultaneously. Another approach is to use mixtures or ratio of fluorophores as new colors. Such methods have extended multiplexing to hundreds of analytes, but due to the size of the labels (e.g., microbeads), the technology has thus far been limited to flow-cytometry based

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1983

analyses. Yet another approach involves nanostrings, which are essentially short strings of strung-up fluorophores creating visible colorful barcodes. Unfortunately, nanostring readout requires very high-resolution imaging and a special flow apparatus. Further, the nanostrings can only be used in a sample where the probes' targets are sparse, or the barcodes will overlap and create a blur.

[0007] A simple workaround for the limited number of colors (e.g., 4 or 5 colors) in optical readouts is to repeat the probing of the same sample with multiple small sets of different probes. For example, the assay can involve probing the sample with 4 different antibodies at a time and imaging after every assay. If the test requires probing the sample with a total of 64 antibodies, the 4-probe procedure would have to be repeated 16 times using the sample. As such, the order of detecting different target analytes in a single sample may need to be prioritized, because some target analytes in the sample can degrade during successive probings. Accordingly, there is still a strong need for accurate and sensitive methods with a high throughput for detection, identification, and/or quantification of target molecules in a sample, e.g., complex mixtures.

SUMMARY

[0008] Embodiments provided herein are based on, at least in part, the development of a multiplexed biological assay and readout, in which a multitude of detection reagents comprising one or more probes and/or probe types are applied to a sample, allowing the detection reagents to bind target molecules or analytes, which can then be optically identified in a temporally-sequential manner. In some embodiments, the multitude of detection reagents comprising one or more probes and/or probe types can be applied to a sample simultanesouly. Accordingly, provided herein are methods, compositions (e.g., detection reagents) and kits for detecting multiple analytes in a sample.

[0009] Accordingly, one aspect provided herein relates to a method for detecting a plurality of analytes in a sample. Exemplary analytes include, without limitations, antigens, receptors, proteins, peptides, nucleic acids, sugars, lipid, carbohydrates, glycans, glycoproteins, oligonucleotides, cells, viruses, and any combinations thereof. In some embodiments, the nucleic acids can include, e.g., cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof. A sample amenable to the methods described herein can be a sample from any sources, e.g., but not limited to biological samples, e.g., collected from

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1984

organisms, animals or subjects, environmental samples, food, food byproduct, soil, archaeological samples, extraterrestrial samples, or any combinations thereof. For example, a sample can be a protein sample immobilized on a solid support including, e.g., a blotting membrane. In alternative embodiments, a sample can comprise one or more cells, one or more tissues, one or more fluids, or any combinations thereof. In some embodiments, the sample can comprise a tissue sample. In some embodiments, the sample can comprise a fluid sample. In some embodiments, a sample can comprise blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof. In some embodiments, a sample can comprise a biopsy, a surgically removed tissue, a swap, or any combinations thereof.

[0010] The method described herein comprises: (a) contacting the sample with a plurality of detection reagents as described herein, wherein each subpopulation of the detection reagents can target at least one different analyte; and (b) detecting in a temporally-sequential manner said plurality of the pre-determined subsequences of said detection reagents, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents. In some embodiments, the temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent can be unique for each subpopulation of the detection reagents.

[0011] In some embodiments, a detection reagent described herein can target at least two distinct analytes. In some embodiments, a first subpopulation of the detection reagents can target at least one analyte different from that of a second subpopulation of the detection reagents. Accordingly, in some embodiments, the readout of the detection reagents can be distinct but overlapping.

[0012] In some embodiments, the method can further comprise processing the sample before contacting with the plurality of detection reagents described herein.

[0013] In some embodiments, the method can further comprise removing any unbound detection reagents before detection of the pre-determined subsequences in a temporally-sequential manner.

[0014] In some embodiments, the method can further comprise comparing the temporal order

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1985

of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample. In some embodiments, the method can further comprise measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents. In some embodiments, the intensity of the signal signatures generated from each subpopulation of the detection reagents can indicate an amount of the analyte. In some embodiments, the relative intensity of the signal signatures can be used in identification of each subpopulation of the detection reagents. Thus, the intensity of the signal signatures can be used as part of a coding scheme of the detection reagents described herein. The comparing and intensity measuring steps can be performed by a computer-implemented software or algorithm.

[0015] Each signal signature corresponding to individual pre-determined subsequences of the detection reagent are detected in a temporally-sequential manner. In some embodiments, the detection method can include sequencing, e.g., which can be performed via any methods known in the art, including but not limited to, ligation, hybridization, synthesis, amplification, single-base extension, or any methods known in the art. In certain embodiments, the detection method can include hybridizing a decoder probe with the corresponding subsequence, wherein the decoder probe can comprise a detectable label.

[0016] In particular embodiments, the detection method can comprise: (a) hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes can comprise a detectable label, each detectable label producing a signal signature; (b) detecting said different signal signature produced by the hybridization of said set of decoder probes; (c) optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and (d) repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent. In some embodiments, removal of the different signal signature produced by the hybridization can be performed by washing, heating, photo-bleaching, displacement (e.g., displacement of decoder probes with another reagent or nucleic acid sequence), cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation, or any combinations thereof.

[0017] In some embodiments, each decoder probe in the set can independently have a

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1986

subsequence of the detection reagents.

[0018] In some embodiments, each subpopulation of the decoder probes can comprise a different detectable label, each different detectable label producing a different signal signature.

[0019] In some embodiments, each subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the subsequence of the detection reagents. In some embodiments, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the same subsequence of the detection reagents. In some embodiments, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to distinct subsequences of the detection reagents.

[0020] In some embodiments involving decoder probes for detection purposes, the detectable label associated with each subpopulation of the decoder probes can comprise an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule or protein, a quantum dot, a colorimetric reagent, a chromogenic molecule or protein, a Raman label, a chromophore, and any combinations thereof. In some embodiments, the detectable label or optical label can be a fluorescent molecule or protein.

[0021] Types of signal signature(s) can vary upon different embodiments of detection reagents and/or decoder probes described herein. By way of example, the detection reagents and/or decoder probes can comprise an optical molecule or label, thus producing optical signatures. Examples of optical signatures can include, without limitations, signatures of fluorescent color (e.g., emission spectra under one or more excitation spectra), visible light, no-color or no-light, color (e.g., color defined by a visible light wavelength), Raman signatures, and any combinations thereof. In some embodiments, an optical signature can comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors or no-lights, one or more colors, one or more Raman signatures, or any combinations thereof. For example, in one embodiment, an optical signature can comprise a plurality (e.g., at least 2 or more) of fluorescent colors (e.g., fluorescent dyes). In these embodiments, the optical signatures can be detected by optical imaging or spectroscopy.

[0022] The spatial movement limit of an analyte in a sample allowed for a temporal detection of the detection reagents to occur can vary depending on a number of factors, including, but not

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1987

limited to, presence of any distinguishable features within a field of detection, magnification used in detection (e.g., magnification of the microscope lens), density of the analytes in a sample, and any combinations thereof. In some embodiments, there can be no limit in the spatial movement of an analyte in a sample during a temporal detection of the detection reagents, for example, provided that the analyte stay within the field of detection and there is at least one same distinguishable feature in each image taken during a temporal detection so that the images can be aligned to each other based on the same distinguishable feature. In some embodiments where there is no such distinguishable feature, the spatial movement of an analyte in a sample can be less than 100 μm, including less than 50 μm, less than 25 μm, less than 10 μm, less than 1 μm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. In some embodiments, the spatial movement of an analyte in a sample can be less than 1000 nm, including less than 500 nm, less than 250 nm, less than 100 nm, less than 50 nm, less than 10 nm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. More importantly, the spatial movement limit of an analyte in a sample during a temporal detection is determined by the ability of matching distinguishable features between images taken during a temporal detection, which can be affected by imaging conditions. In some embodiments, the analyte can be fixed on a solid substrate or support.

[0023] In one aspect, embodiments provided herein relate to a detection reagent, which can be used in a multiplexing assay. The detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner; wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

[0024] In some embodiments, the probe reagent and the nucleic acid label can be conjugated together by at least one linker. The linker can be monovalent or multivalent. Exemplary linkers include, but are not limited to, a bond, a linker molecule, and/or a particle, for example, selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof. In some embodiments, the linker can be a nanoparticle. Examples of linker molecules can include, but are not limited to, a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 1988