SNPs and indels with a quality score above the threshold are identified as putative polymorphisms. For SSR mining we use the MISA (MIcroSAtellite identification) tool (http://pgrc.ipk-gatersleben.de/misa). This tool identifies di-, tri-, tetranucleotide and compound SSR motifs with predefined criteria and summarizes occurrences of these SSRs.

The polymorphism mining and quality assignment process is shown in Figure 4B

RESULTS

The table below summarizes the results of the combined analysis of sequences obtained from 2 454 sequence runs for the combined pepper samples and 2 runs for the combined maize samples.

	Pepper	Maize
Total number of reads	457178	492145
Number of trimmed reads	399623	411008
Number singletons	105253	313280
Number of contigs	31863	14588
Number of reads in contigs	294370	97728
Total number of sequences containing SSRs	611	202
Number of different SSR-containing sequences	104	65
Number of different SSR motifs (di, tri, tetra and compound)	49	40
Number SNPs with Q score ≥ 0.3 *	1636	782
Number of indels *	4090	943

* both with selection against neighboring SNPs, at least 12 bp flanking sequence and not occurring in homopolymer sequences larger than 3 nucleotides.

Example 4. Single nucleotide polymorphism (SNP) discovery in pepper.

DNA isolation

Genomic DNA was isolated from the two parental lines of a pepper recombinant inbred (RIL) population and 10 RIL progeny. The parental lines are PSP11 and PI201234. Genomic DNA was isolated from leaf material of individual seedlings using a modified CTAB procedure described by Stuart and Via (Stuart,C.N., Jr and Via,L.E. (1993) A rapid CTAB DNA isolation technique useful for RAPD fingeprinting and other PCR applications. *Biotechniques*, **14**, 748-750). DNA samples were

diluted to a concentration of 100 ng/µl in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at -20 °C.

AFLP template preparation using tagged AFLP primers

AFLP templates of the pepper parental lines PSP11 and PI201234 were prepared using the restriction endonuclease combination *Eco*RI/*Mse*I as described by Zabeau & Vos, 1993: Selective restriction fragment amplification; a general method for DNA fingerprinting. EP 0534858-A1, B1; US patent 6045994) and Vos et al (Vos,P., Hogers,R., Bleeker, M., Reijans, M., van de Lee, T., Hornes,M., Frijters,A., Pot,J., Peleman,J., Kuiper,M. *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucl. Acids Res.*, **21**, 4407-4414).

Specifically, restriction of genomic DNA with EcoRI and MseI was carried out as follows:

DNA Restriction

DNA	100-500ng
EcoRI	5 units
MseI	2 units
5xRLbuffer	8µl
MilliQ water to	40µl

Incubation was for 1 hour at 37°C. After the enzyme restriction, enzymes were inactivated by incubation for 10 minutes at 80 °C.

Ligation of adapters

10mM ATP	1µl
T4 DNA ligase	1µl
EcoRI adaptor (5pmol/µl)	1µl
MseI adaptor (50pmol/µl)	1µl
5xRLbuffer.	2µl
MilliQ water to	40µl

Incubation was for 3 hours at 37°C.

Selective AFLP amplification

Following restriction-ligation, the restriction/ligation reaction was diluted 10-fold with $T_{10}E_{0.1}$ and 5µl diluted mix was used as a template in a selective amplification step. Note that since a +1/+2 selective amplification was intended, first a +1/+1 selective pre-amplification step (with standard AFLP primers) was performed. Reaction conditions of the +1/+1 (+A/+C) amplification were as follows.

Restriction-Ligation mix (10-fold diluted)	5µl
EcoRI-primer +1 (50ng/µl):	0.6µl
MseI- primer +1 (50ng/µl)	0.6µl
dNTPs (20mM)	0.2µl
Taq.polymerase (5U/µl Amplitaq, PE)	0.08µl
10XPCRbuffer	2.0µl
MilliQ water to	20µl

Primers sequences were:

EcoRI+1: 5'- AGACTGCGTACCAATTCA -3' [SEQ ID 9] and

MseI+1: 5'- GATGAGTCCTGAGTAAC -3'[SEQ ID 10]

PCR amplifications were performed using a PE9700 with a gold or silver block using the following conditions: 20 times (30 s at 94°C, 60 s at 56°C and 120 s at 72°C).

The quality of the generated +1/+1 preamplification products was checked on a 1% agarose gel using a 100 basepair ladder and a 1Kb ladder to check the fragment length distribution. Following +1/+1 selective amplification, the reaction was diluted 20-fold with $T_{10}E_{0.1}$ and 5µl diluted mix is used as a template in the +1/+2 selective amplification step using tagged AFLP primers.

Finally, +1/+2 (A/+CA) selective AFLP amplifications were performed:

+1/+1 selective amplification product (20-fold diluted)	5.0µl
KRS EcoRI-primer +A (50ng/µl)	1.5µl
KRS MseI- primer + CA (50ng/µl)	1.5µl
dNTPs (20mM)	0.5µl
Taq polymerase (5U/µl Amplitaq, Perkin Elmer)	0.2µl
10X PCR buffer	5.0µl
MQ to	50µl

Tagged AFLP primers sequences were:

PSP11:

O5F212: EcoRI+1: 5'-CGTCAGACTGCGTACCAATTCA-3' [SEQ ID 1] and

O5F214: MseI+2: 5'-TGGTGATGAGTCCTGAGTAACA-3'[SEQ ID 2]

PI201234:

05F213: EcoRI+1: 5'-CAAGAGACTGCGTACCAATTCA-3' [SEQ ID 3] and

05F215: MseI+1: 5'-AGCCGATGAGTCCTGAGTAACA-3'[SEQ ID 4]

Note that these primers contain 4 bp tags (underlined above) at their 5 prime ends to distinguish amplification products originating from the respective pepper lines at the end of the sequencing process.

Schematic representation of pepper AFLP +1/+2 amplification products after amplification with AFLP primers containing 4 bp 5 prime tag sequences.

```
            EcoRI tag                                        MseI tag
PSP 11: 5'-CGTC ---------------------------------- ACCA-3'
          3'-GCAG---------------------------------- TGGT-5'
PI201234  5'-CAAG ---------------------------------- GGCT-3'
          3'-GTTC ---------------------------------- CCGA-5'
```

PCR amplifications (24 per sample) were performed using a PE9700 with a gold or silver block using the following conditions: 30 times (30 s at 94 °C + 60 s at 56 °C + 120 s at 72°C).

The quality of the generated amplification products was checked on a 1% agarose gel using a 100 basepair ladder and a 1Kb ladder to check the fragment length distribution.

AFLP reaction purification and quantification.

After pooling two 50 microliter +1/+2 selective AFLP reactions per pepper sample, the resulting 12 100µl AFLP reaction products were purified using the QIAquick PCR Purification Kit (QIAGEN), following the QIAquick® Spin handbook (Page 18). On each column a maximum of 100µl product was loaded. Amplified products were eluted in $T_{10}E_{0.1}$. The quality of the purified products is checked on a 1% agarose gel and concentrations were measured on the Nanodrop (Figure 2).

Nanodrop concentration measurements were used to adjust the final concentration of each purified PCR product to 300 nanograms per microliter. Five micrograms purified amplified product of PSP11 and 5 microgram of PI201234 were mixed to generate 10 microgram template material for preparation of the 454 sequencing library.

Sequence library preparation and high-throughput sequencing

Mixed amplification products from both pepper lines were subjected to high-throughput sequencing using 454 Life Sciences sequencing technology as described by Margulies et al., (Margulies et al., *Nature* 437, pp. 376-380 and Online Supplements). Specifically, the AFLP PCR products were first end-polished and subsequently ligated to adaptors to facilitate emulsion-PCR amplification and subsequent fragment sequencing as described by Margulies and co-workers. 454 adaptor sequences, emulsion PCR primers, sequence-primers and sequence run conditions were all as described by Margulies and co-workers. The linear order of functional elements in an emulsion-PCR fragment amplified on Sepharose beads in the 454 sequencing process was as follows as exemplified in figure 1A:

454 PCR adaptor - 454 sequence adaptor - 4 bp AFLP primer tag 1 - AFLP primer sequence 1 including selective nucleotide(s) - AFLP fragment internal sequence - AFLP primer sequence 2 including selective nucleotide(s), 4 bp AFLP primers tag 2 - 454 sequence adaptor - 454 PCR adaptor - Sepharose bead

Two high-throughput 454 sequence runs were performed by 454 Life Sciences (Branford, CT; United States of America).

454 sequence run data-processing.

Sequence data resulting from 2 454 sequence runs were processed using a bio-informatics pipeline (Keygene N.V.). Specifically, raw 454 basecalled sequence reads were converted in FASTA format and inspected for the presence of tagged AFLP adaptor sequences using a BLAST algorithm. Upon high-confidence matches to the known tagged AFLP primer sequences, sequences were trimmed, restriction endonuclease sites restored and assigned the appropriate tags (sample 1 EcoRI (ES1), sample 1 MseI (MS1), sample 2 EcoRI (ES2) or sample 2 MseI (MS2), respectively). Next, all trimmed sequences larger than 33 bases were clustered using a megaBLAST procedure based on overall sequence homologies. Next, clusters were assembled into one or more contigs and/or singletons per cluster, using a CAP3 multiple alignment algorithm. Contigs containing more than one sequence were inspected for the sequence mismatches, representing putative polymorphisms. Sequence mismatches were assigned quality scores based on the following criteria:

* the numbers of reads in a contig
* the observed allele distribution

The above two criteria form the basis for the so called Q score assigned to each putative SNP/indel. Q scores range from 0 to 1; a Q score of 0.3 can only be reached in case both alleles are observed at least twice.

* location in homopolymers of a certain length (adjustable; default setting to avoid polymorphism located in homopolymers of 3 bases or longer).
* number of contigs in cluster.
* distance to nearest neighboring sequence mismatches (adjustable; important for certain types of genotyping assays probing flanking sequences)
* the level of association of observed alleles with sample 1 or sample 2; in case of a consistent, perfect association between the

alleles of a putative polymorphism and samples 1 and 2, the polymorphism (SNP) is indicated as an "elite" putative polymorphism (SNP). An elite polymorphism is thought to have a high probability of being located in a unique or low-copy genome sequence in case two homozygous lines have been used in the discovery process. Conversely, a weak association of a polymorphism with sample origin bears a high risk of having discovered false polymorphisms arising from alignment of non-allelic sequences in a contig.

Sequences containing SSR motifs were identified using the MISA search tool (MIcroSAtellelite identification tool; available from http://pgrc.ipk-gatersleben.de/misa/

Overall statistics of the run is shown in the Table below.

Table. Overall statistics of a 454 sequence run for SNP discovery in pepper.

Enzyme combination	Run
Trimming	
All reads	254308
Fault	5293 (2 %)
Correct	249015 (98%)
Concatamers	2156 (8.5 %)
Mixed tags	1120 (0.4 %)
Correct reads	
Trimmed one end	240817 (97%)
Trimmed both ends	8198 (3 %)
Number of reads sample 1	136990 (55%)
Number of reads sample 2	112025 (45 %)
Clustering	
Number of contigs	21918
Reads in contigs	190861
Average number reads per contig	8.7
SNP mining	
SNPs with Q score ≥ 0.3 *	1483
Indel with Q score ≥ 0.3 *	3300
SSR mining	
Total number of SSR motifs identified	359
Number of reads containing one or more SSR motifs	353
Number of SSR motif with unit size 1	0

(homopolymer)	
Number of SSR motif with unit size 2	102
Number of SSR motif with unit size 3	240
Number of SSR motif with unit size 4	17

* SNP / indel mining criteria were as follows:

No neighbouring polymorphisms with Q score larger than 0.1 within 12 bases on either side, not present in homopolymers of 3 or more bases. Mining criteria did not take into account consistent association with sample 1 and 2, i.e. the SNPs and indels are not necessarily elite putative SNPs/indels

An example of a multiple alignment containing an elite putative single nucleotide polymorphism is shown in Figure 7.

Example 5. SNP validation by PCR amplification and Sanger sequencing

In order to validate the putative A/G SNP identified in example 1, a sequence tagged site (STS) assay for this SNP was designed using flanking PCR primers. PCR primer sequences were as follows:

Primer_1.2f: 5'- AAACCCAAACTCCCCCAATC-3', [SEQ ID 37] and

Primer_1.2r:5'- AGCGGATAACAATTTCACACAGGACATCAGTAGTCACACTGGTA CAAAAATAGAGCAAAACAGTAGTG -3' [SEQ ID 38]

Note that primer 1.2r contained an M13 sequence primer binding site and length stuffer at its 5 prime end. PCR amplification was carried out using +A/+CA AFLP amplification products of PSP11 and PI210234 prepared as described in example 4 as template. PCR conditions were as follows:

For 1 PCR reaction the following components were mixed:

5 µl 1/10 diluted AFLP mixture (app. 10 ng/µl)
5 µl 1pmol/µl primer 1.2f (diluted directly from a 500 µM stock)
5 µl 1pmol/µl primer 1.2r (diluted directly from a 500 µM stock)

5 µl PCR mix
- 2 µl 10 x PCR buffer
- 1 µl 5 mM dNTPs
- 1.5 µl 25 mM $MgCl_2$
- 0.5 µl H_2O

5 µl Enzyme mix
- 0.5 µl 10 x PCR buffer (Applied Biosystems)
- 0.1 µl 5U/µl AmpliTaq DNA polymerase (Applied Biosystems)

- 4.4 µl H_2O

The following PCR profile was used:

Cycle 1	2';	94° C	
Cycle 2-34	20'';	94° C	
	30'';	56° C	
	2'30'';	72° C	
Cycle 35	7';	72° C	
	∞;	4° C	

PCR products were cloned into vector pCR2.1 (TA Cloning kit ; Invitrogen) using the TA Cloning method and transformed into INVαF' competent *E. coli* cells. Transformants were subjected to blue/white screening. Three independent white transformants each for PSP11 and PI-201234 were selected and grown O/N in liquid selective medium for plasmid isolation.

Plasmids were isolated using the QIAprep Spin Miniprep kit (QIAGEN). Subsequently, the inserts of these plasmids were sequenced according to the protocol below and resolved on the MegaBACE 1000 (Amersham). Obtained sequences were inspected on the presence of the SNP allele. Two independent plasmids containing the PI-201234 insert and 1 plasmid containing the PSP11 insert contained the expected consensus sequence flanking the SNP. Sequence derived from the PSP11 fragment contained the expected A (underlined) allele and sequence derived from PI-201234 fragment contained the expected G allele (double underlined):

PSP11 (sequence 1): (5'-3')

AAACCCAAACTCCCCCAATCGATTTCAAACCTAGAACAATGTTGGTTTTGGTGCTAACTTCAA CCCCACTACTGTTTTGCTCTATTTTTGT [SEQ ID 39]

PI-201234 (sequence 1): (5'-3')

AAACCCAAACTCCCCCAATCGATTTCAAACCTAGAACAGTGTTGGTTTTGGTGCTAACTTCAA CCCCACTACTGTTTTGCTCTATTTTTG [SEQ ID 40]

PI-201234 (sequence 2): (5'-3')

AAACCCAAACTCCCCCAATCGATTTCAAACCTAGAACAGTGTTGGTTTTGGTGCTAACTTCAA CCCCACTACTGTTTTGCTCTATTTTTG [SEQ ID 41]

This result indicates that the putative pepper A/G SNP represents a true genetic polymorphism detectable using the designed STS assay.

Example 6: SNP validation by SNPWave detection.

In order to validate the putative A/G SNP identified in example 1, SNPWave ligation probes sets were defined for both alleles of this SNP using the consensus sequence. Sequence of the ligation probes were as follows:

SNPWave probe sequences (5'-3'):

06A162 GATGAGTCCTGAGTAACCCAATCGATTTCAAACCTAGAACAA (42 bases) [SEQ ID 42]

06A163 GATGAGTCCTGAGTAACCACCAATCGATTTCAAACCTAGAACAG (44 bases) [SEQ ID 43]

06A164 Phosphate-TGTTGGTTTTGGTGCTAACTTCAACCAACATCTGGAATTGGTACGCAGTC (52 bases) [SEQ ID 44]

Note the allele specific probes 06A162 and 06A163 for the A and G alleles, respectively, differ by 2 bases in size, such that upon ligation to the common locus-specific probe 06A164, ligation product sizes of 94 (42+54) and 96 (44+52) bases result.

SNPWave ligation and PCR reactions were carried as described by Van Eijk and co-workers (M. J. T. van Eijk, J. L.N. Broekhof, H. J.A. van der Poel, R. C. J. Hogers, H. Schneiders, J. Kamerbeek, E. Verstege, J. W. van Aart, H. Geerlings, J. B. Buntjer, A. J. van Oeveren, and P. Vos. (2004). SNPWave™: a flexible multiplexed SNP genotyping technology. Nucleic Acids Research 32: e47), using 100 ng genomic DNA of pepper lines PSP11 and PI201234 and 8 RIL offspring as starting material. Sequences of the PCR primers were:

93L01FAM (E00k): 5-GACTGCGTACCAATTC-3' [SEQ ID 45]

93E40 (M00k): 5-GATGAGTCCTGAGTAA-3' [SEQ ID 46]

Following PCR amplification, PCR product purification and detection on the MegaBACE1000 was as described by van Eijk and co-workers (vide supra). A pseudo-gel image of the amplification products obtained from PSP11, PI201234 and 8 RIL offspring is shown in Figure 8B.

The SNPWave results demonstrate clearly that the A/G SNP is detected by the SNPWave assay, resulting in 92 bp products (=AA homozygous genotype) for P1 (PSP11) and RIL offspring 1, 2, 3, 4, 6 and 7), and in 94 bp products (=GG homozygous genotype) for P2 (PI201233) and RIL offspring 5 and 8.

Example 7: Strategies for enriching AFLP fragment libraries for low-copy sequences.

This example describes several enrichment methods to target low-copy of unique genome sequences in order to increase the yield of elite polymorphisms such as described in example 4. The methods can be divided into four categories:

1) methods aimed at preparing high-quality genomic DNA, excluding chloroplast sequences.

Here it is proposed to prepare nuclear DNA instead of whole genomic DNA as described in Example 4, to exclude co-isolation of abundant chloroplast DNA,which may result in reduced number of plant genomic DNA sequences, depending on the restriction endonucleases and selective AFLP primers used in the fragment library preparation process. A protocol for isolation of highly pure tomato nuclear DNA has been described by Peterson, DG., Boehm, K.S. & Stack S. M. (1997). Isolation of Milligram Quantities of Nuclear DNA From Tomato (*Lycopersicon esculentum*), A Plant Containing High Levels of Polyphenolic Compounds. Plant Molecular Biology Reporter 15 (2), pages 148-153.

2) methods aimed at using restriction endonucleases in the AFLP template preparation process which are expected to yield elevated levels of low-copy sequences.

Here it is proposed to use certain restriction endonucleases in the AFLP template preparation process, which are expected to target low-copy or unique genome sequences, resulting in fragment libraries enriched for polymorphisms with increased ability to be convertible into genotyping assays. An examples of a restriction endonuclease targeting low-copy sequence in plant genomes is PstI. Other methylation sensitive restriction endonucleases may also target low-copy or unique genome sequences preferentially.

3) methods aimed a selectively removing highly duplicated sequences based on re-annealing kinetics of repeat sequences versus low-copy sequences.

Here it is proposed to selectively remove highly duplicated (repeat) sequences from either the total genomic DNA sample or from the (cDNA-)AFLP template material prior to selective amplification.

3a) High-C_0t DNA preparation is a commonly used technique to enrich slowly annealing low-copy sequences from a complex plant

genomic DNA mixture (Yuan et al. 2003; High-Cot sequence analysis of the maize genome. *Plant J.* 34: 249-255). It is suggested to take High- C_0t instead of total genomic DNA as starting material to enrich for polymorphisms located in low-copy sequences.

3b) An alternative to laborious high-C_0t preparation may be incubate denatured and re-annealing dsDNA with a novel nuclease from the Kamchatka crab, which cleaves short, perfectly matched DNA duplexes at a higher rate than nonperfectly matched DNA duplexes, as described by Zhulidov and co-workers (2004; Simple cDNA normalization using Kamchatka crab duplex-specific nuclease. *Nucleic Acids Research* 32, e37) and Shagin and co-workers (2006; a novel method for SNP detection using a new duplex-specific nuclease from crab hepatopancreas. *Genome Research* 12: 1935-1942). Specifically, it is proposed to incubate AFLP restriction /ligation mixtures with this endonuclease to deplete the mixture of highly duplicated sequences, followed by selective AFLP amplification of the remaining low-copy or unique genome sequences.

3c) Methyl filtration is a method to enrich for hypomethylated genomic DNA fragments using the restriction endonuclease McrBC which cuts methylated DNA in the sequence [A/G]C, where the C is methylated (see Pablo D. Rabinowicz , Robert Citek, Muhammad A. Budiman, Andrew Nunberg, Joseph A. Bedell, Nathan Lakey, Andrew L. O'Shaughnessy, Lidia U. Nascimento, W. Richard McCombie and Robert A. Martienssen. Differential methylation of genes and repeats in land plants. Genome Research 15:1431-1440, 2005). McrBC may be used to enrich the low-copy sequence fraction of a genome as starting material for polymorphism discovery.

4) <u>the use of cDNA as opposed to genomic DNA in order to target gene sequences.</u>

Finally, here it is proposed to use oligodT-primed cDNA as opposed to genomic DNA as starting material for polymorphism discovery, optionally in combination with the use the Crab duplex-specific nuclease described in 3b above for normalization. Note that the use of oligodT primed cDNA also excludes chloroplast sequences. Alternatively, cDNA-AFLP templates instead of oligodT primed cDNA is used to facilitate amplification of the remaining low-copy sequences in analogy to AFLP (see also 3b above).

Example 8: Strategy for simple-sequence repeat enrichment

This example describes the proposed strategy for discovery of Simple Sequence repeats sequences, in analogy to SNP discovery described in Example 4.

Specifically, Restriction-ligation of genomic DNA of two or more samples is performed, e.g. using restriction endonucleases PstI/MseI. Selective AFLP amplification is performed as described in Example 4. Next fragments containing the selected SSR motifs are enriched by one of two methods:

1) Southern blot hybridization onto filters containing oligonucleotides matching the intended SSR motifs (e.g. $(CA)_{15}$ in case of enrichment for CA/GT repeats), followed by amplification of bound fragments in a similar fashion as described by Armour and co-workers (Armour, J., Sismani, C., Patsalis, P., and Cross, G. (2000). Measurement of locus copy number by hybridization with amplifiable probes. Nucleic Acids Research vol 28, no. 2, pp. 605-609) or by

2) enrichment using biotinylated capture oligonucleotide hybridization probes to capture (AFLP) fragments in solution as described by Kijas and co-workers (Kijas, J.M,. Fowler, J.C., Garbett C.A., and Thomas, M.R., (1994). Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques, vol. 16, pp. 656-662.

Next, the SSR-motif enriched AFLP fragments are amplified using the same AFLP primers are used in the preamplification step, to generate a sequence library. An aliqout of the amplified fragments are T/A cloned and 96 clones are sequences to estimate the fraction of positive clones (clones containing the intended SSR motif, e.g. CA/GT motifs longer than 5 repeat units. Another aliquot of the enriched AFLP fragment mixture is detected by polyacrylamide gel electrophoresis (PAGE), optionally after further selective amplification to obtain a readable fingerprint, in order to visually inspect whether SSR containing fragments are enriched. Following successful completion of these control steps, the sequence libraries are subjected to high-throughput 454 sequencing.

The above strategy for *de novo* SSR discovery is schematically depicted in Figure 8A, and can be adapted for other sequence motifs by substituting the capture oligonucleotide sequences accordingly.

Example 9. Strategy for avoiding mixed tags.

Mixed tags refers to the observation that besides the expected tagged AFLP primer combination per sample, a low fraction of sequences are observed which contain a sample 1 tag at one end, and a sample 2 tag a the other end (See also the table 1 in example 4). Schematically, the configuration of sequences containing mixed tags is depicted here-in below.

Schematic representation of the expected sample tag combinations.

```
        EcoRI tag                                          MseI tag
PSP 11:  5'-CGTC ---------------------------------------- ACCA-3'
         3'-GCAG---------------------------------------- TGGT-5'

PI-201234 5'-CAAG ---------------------------------------- GGCT-3'
          3'-GTTC ---------------------------------------- CCGA-5'
```

Schematic representation of the mixed tags.

```
        EcoRI tag                                          MseI tag
         5'-CGTC ---------------------------------------- GGCT-3'
         3'-GCAG---------------------------------------- CCGA-5'

         5'-CAAG ---------------------------------------- ACCA-3'
         3'-GTTC ----------------------------------------  TGGT-5'
```

The observation of mixed tags precludes correct assignment of the sequence to either PSP11 or PI-201234.

An example of a mixed tag sequence observed in the pepper sequence run described in Example 4 is shown in Figure 5A. An overview of the configuration of observed fragments containing expected tags and mixed tags is shown in panel 2 of Figure 5A.

The proposed molecular explanation for mixed tags is that during the sequence library preparation step, DNA fragments are made blunt by using T4 DNA polymerase or Klenow enzyme to remove 3 prime protruding ends, prior to adaptor ligation (Margulies et al., 2005). While this may work well when a single DNA sample is processed, in

case of using a mixture of two or more samples differently tagged DNA samples, fill in by the polymerase results in incorporation of the wrong tag sequence in case when a heteroduplex has been formed between the complementary strands derived from different samples (Figure 5B panel 3 mixed tags) The solution has been found to pool samples after the purification step that followed adaptor ligation in the 454 sequence library construction step as shown in Figure 5C panel 4.

Example 10. Strategy for avoiding mixed tags and concatamers using an improved design for 454 sequence library preparation.

Besides the observation of low frequencies of sequence reads containing mixed tags as described in Example 9, a low frequency of sequence reads observed from concatenated AFLP fragments have been observed.

An example of a sequence read derived from a concatamer is shown in Figure 6A Panel 1. Schematically, the configuration of sequences containing expected tags and concatamers is shown in Figure 6A Panel 2.

The proposed molecular explanation for the occurrence of concatenated AFLP fragments is that during the 454 sequence library preparation step, DNA fragments are made blunt using T4 DNA polymerase or Klenow enzyme to remove 3 prime protruding ends, prior to adaptor ligation (Margulies et al., 2005). As a result, blunt end sample DNA fragments are in competition with the adaptors during the ligation step and may be ligated to each other prior to being ligated to adaptors. This phenomenon is in fact independent of whether a single DNA sample or a mixture of multiple (tagged) samples are included in the library preparation step, and may therefore also occur during the conventional sequencing as described by Margulies and co-workers. In case of the using multiple tagged samples as described in Example 4, concatamers complicate correct assignment of sequence reads to samples based on the tag information and are therefore to be avoided.

The proposed solution to the formation of concatamers (and mixed tags) is to replace blunt-end adaptor ligation with ligation of adaptors containing a 3 prime T overhang, in analogy to T/A cloning of PCR products, as shown in Figure 6B Panel 3. Conveniently, these modified 3'prime T overhang-containing adaptors are proposed to

contain a C overhang at the opposite 3'end (which will not be ligated to the sample DNA fragment, to prevent blunt-end concatamer formation of adaptor sequences (see Figure 6B Panel 3). The resulting adapted workflow in the sequence library construction process when using the modified adaptor approach is shown schematically in Figure 6C Panel 4.

IDT Exhibit 1020.10130

Claims

1. Method for identifying one or more polymorphisms, said method comprising the steps of:

a) providing a first nucleic acid sample of interest;
b) performing a complexity reduction on the first nucleic acid sample of interest to provide a first library of the first nucleic acid sample;
c) consecutively or simultaneously performing steps a) and b) with a second or further nucleic acid sample of interest to obtain a second or further library of the second or further nucleic acid sample of interest;
d) sequencing of at least a portion of the first library and of the second or further library;
e) aligning the sequences obtained in step d);
f) determining one or more polymorphisms between the first nucleic acid sample and second or further nucleic acid sample in the alignment of step e);
g) using the one or more polymorphisms determined in step f) to design detection probes;
h) providing a test sample nucleic acid of interest;
i) performing the complexity reduction of step b) on the test sample nucleic acid of interest to provide a test library of the test sample nucleic acid;
j) subjecting the test library to high-throughput screening to identify the presence, absence or amount of the polymorphisms determined in step f) using the detection probes designed in step g).

2. Method according to claim 1, step b) further comprising the step of tagging of the library to obtain a tagged library, and said method further comprising step c1) of combining the first tagged library and second or further tagged library to obtain a combined library.

3. Method according to claim 2, wherein the tagging of the first nucleic acid sample of interest and the second or further nucleic acid sample of interest is performed using different tags for each sample.

4. Method according to any of the preceding claims, wherein the complexity reduction is performed by

- digesting the nucleic acid sample with at least one restriction endonuclease to fragment it into restriction fragments;
- ligating the restriction fragments obtained with at least one double-stranded synthetic oligonucleotide adaptor having one end compatible with one or both ends of the restriction fragments to produce adapter-ligated restriction fragments;
- contacting said adapter-ligated restriction fragments with one or more oligonucleotide primers under hybridizing conditions; and
- amplifying said adapter-ligated restriction fragments by elongation of the one or more oligonucleotide primers,
- wherein at least one of the one or more oligonucleotide primers include a nucleotide sequence having the same nucleotide sequence as the terminal parts of the strands at the ends of said adapter-ligated restriction fragments, including the nucleotides involved in the formation of the target sequence for said restriction endonuclease and including at least part of the nucleotides present in the adaptors, wherein, optionally, at least one of said primers includes at its 3' end a selected sequence comprising at least one nucleotide located immediately adjacent to the nucleotides involved in the formation of the target sequence for said restriction endonuclease.

5. Method according to claim 4, wherein the adaptor and/or the primer comprises a tag.

6. Method according to claim 5, wherein the tag is an identifier sequence.

7. Method according to claim 4, wherein at least one of the primers is phosphorylated.

8. Method according to any of the preceding claims, wherein sequencing is performed on a solid support such as a bead.

9. Method according to any of the preceding claims wherein sequencing is based on dideoxy chain-terminating sequencing.

10. Method according to claim 8, wherein the sequencing comprises the steps of:

- annealing adapter-ligated fragments to beads, each bead annealing with a single adapter-ligated fragment;
- emulsifying the beads in water-in-oil microreactors, each water-in-oil microreactor comprising a single bead;
- loading the beads in wells, each well comprising a single bead; and
- generating a pyrophosphate signal.

11. Method according to claim 10, wherein, preceding the annealing step, sequencing adaptors are ligated to fragments within the first tagged library and the second tagged library or combination library.

12. Method according to claim 11, wherein the sequencing adaptors carry a 3'-T overhang.

13. Method according to any of the preceding claims, wherein high-throughput screening is performed by immobilization of the probes designed in step h) onto an array, followed by contacting of the array comprising probes with a test library under hybridizing conditions.

14. Method for identifying one or more polymorphisms, said method comprising the steps of:

a) providing a plurality of nucleic acid samples of interest;
b) performing a complexity reduction on each of the samples to provide a plurality of libraries of the nucleic acid samples, wherein the complexity reduction is performed by
- digesting each nucleic acid sample with at least one restriction endonuclease to fragment it into restriction fragments;

- ligating the restriction fragments obtained with at least one double-stranded, synthetic oligonucleotide adaptor having one end compatible with one or both ends of the restriction fragments to produce adapter-ligated restriction fragments;
- contacting said adapter-ligated restriction fragments with one or more phosporylated oligonucleotide primers under hybridising conditions; and
- amplifying said adapter-ligated restriction fragments by elongation of the one or more oligonucleotide primers, wherein at least one of the one or more oligonucleotide primers include a nucleotide sequence having the same nucleotide sequence as the terminal parts of the strands at the ends of said adapter-ligated restriction fragments, including the nucleotides involved in the formation of the target sequence for said restriction endonuclease and including at least part of the nucleotides present in the adaptors, wherein, optionally, at least one of said primers includes at its 3′ end a selected sequence comprising at least one nucleotide located immediately adjacent to the nucleotides involved in the formation of the target sequence for said restriction endonuclease and wherein the adaptor and/or the primer contain a tag;

c) combining said libraries to a combined library;

d) ligating sequencing adapters capable of annealing to beads to the amplified adapter-capped fragments in the combined library, using an sequencing adapter carrying a 3′-T overhang and subjecting the bead-annealed fragments to emulsion polymerisation;

e) sequencing of at least a portion of the combined library;

f) aligning the sequences from each sample obtained in step e);

g) determining one or more polymorphisms between the plurality of nucleic acid samples in the alignment of step f);

h) using the one or more polymorphisms determined in step g) to design detection probes;

i) providing a test sample nucleic acid of interest;

j) performing the complexity reduction of step b) on the test sample nucleic acid of interest to provide a test library of the test sample nucleic acid;

k) subjecting the test library to high-throughput screening to identify the presence, absence or amount of the polymorphisms determined in step g) using the detection probes designed in step h).

15. Use of the method of claims 1-11 for screening enriched microsatellite libraries, performing transcript profiling cDNA-AFLP (digital Northern), sequencing of complex genomes, Expressed Sequence Tag library sequencing (on whole cDNA or cDNA-AFLP), microRNA discovery (sequencing of small insert libraries), Bacterial Artificial Chromosome (contig) sequencing, Bulked Segregant analysis approach in combination with AFLP/cDNA-AFLP, routine detection of AFLP fragments (Marker-assisted back-crosses).

IDT Exhibit 1020.10135

Figure 1

Primer set I used for preamplification of PSP-11

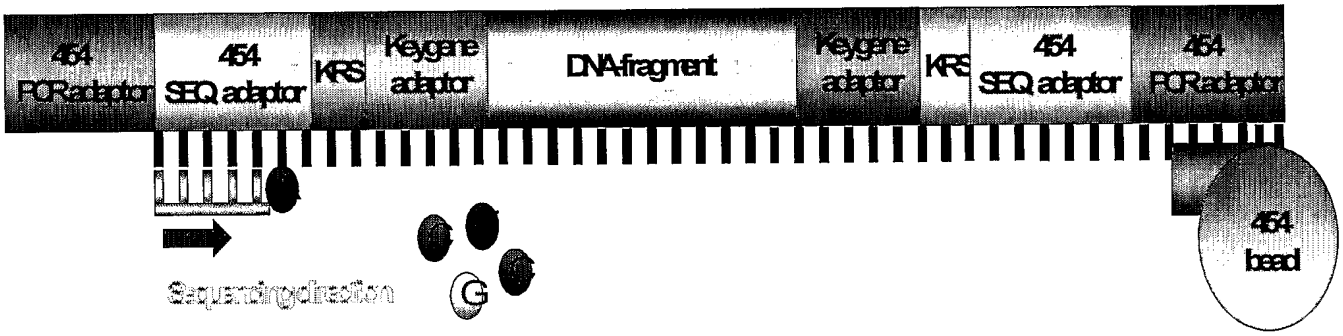
E01LKRS1 5'-CGTCAGACTGCGTACCAATTCA-3'

M15KKRS1 5'-TGGTGATGAGTCCTGAGTAACA-3'

Primer set II used for preamplification of PI20234

E01LKRS2 5'-CAAGAGACTGCGTACCAATTCA-3'

M15KKRS2 5'-AGCCGATGAGTCCTGAGTAACA-3'



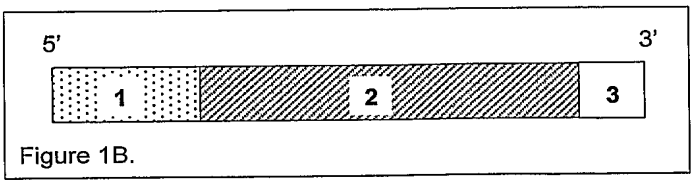
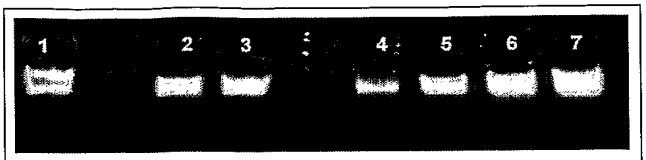


Figure 1B.

Figure 2

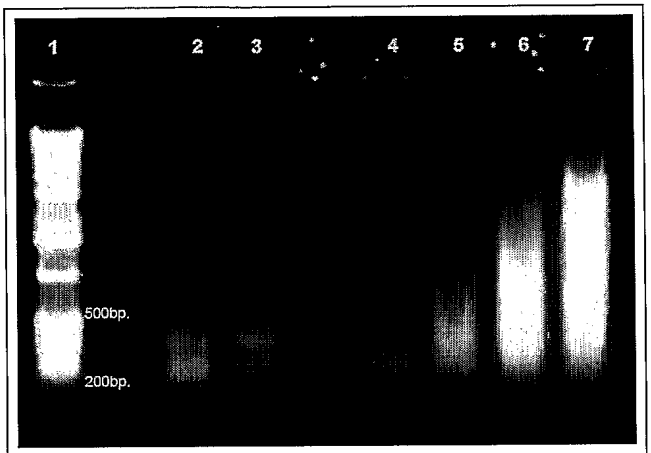
DNA quality control on a 1% agarose gel



1: 1kb ladder 2: sample 1 3:sample 2

4: 50ng DNA 5: 100ng DNA 6: 250ng DNA 7: 500ng DNA

Figure 2A. Short gel electrophoresis



1: 1kb ladder 2: sample 1 3:sample 2

4: 50ng DNA 5: 100ng DNA 6: 250ng DNA 7: 500ng DNA

Figure 2B. Long gel electrophoresis

DNA concentration measured with the Nanodrop

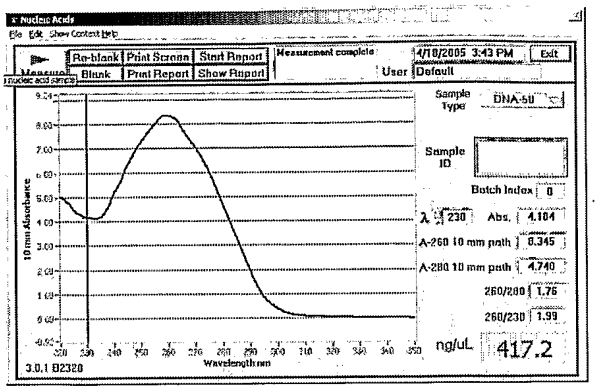


Figure 2C. Concentration sample 1.

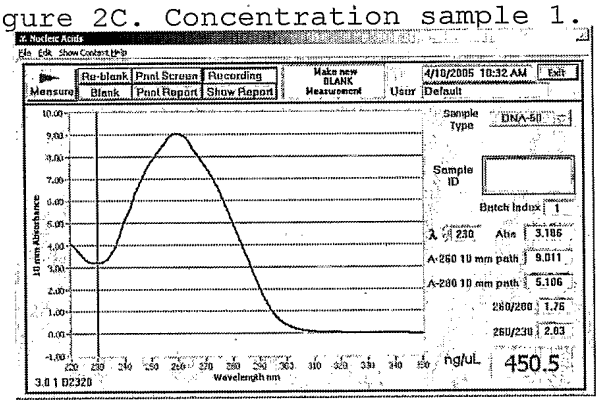


Figure 2D Concentration sample 2

Figure 3

DNA quality control on a 1% agarose gel

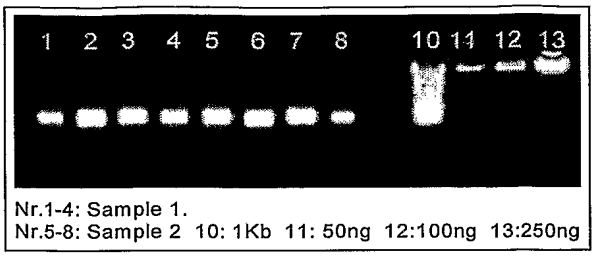


Figure 3A. Short gel electrophoresis

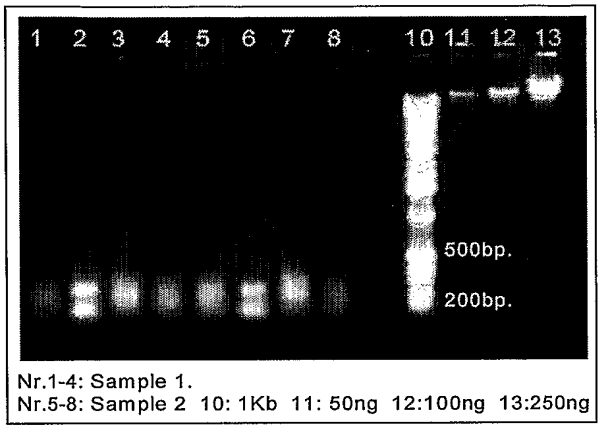


Figure 3B. Long gel electrophoresis

DNA concentrations measured on the Nanodrop

Nr.	Sample ID	ng/uL	A260	260/280	260/230	Constant
1	P1.1	22.61	0.452	1.5	1.81	50
2	P1.2	19.08	0.382	1.67	2.49	50
3	P1.3	18.05	0.361	1.63	2.35	50
4	P1.4	15.19	0.304	1.71	2.1	50

Nr.	Sample ID	ng/uL	A260	260/280	260/230	Constant
5	P2.1	17.5	0.35	1.66	2.01	50
6	P2.2	16.67	0.333	1.96	2	50
7	P2.3	22.03	0.441	1.81	2.28	50
8	P2.4	9.8	0.196	1.78	1.98	50

Figure 4A. Sequence data processing pipeline

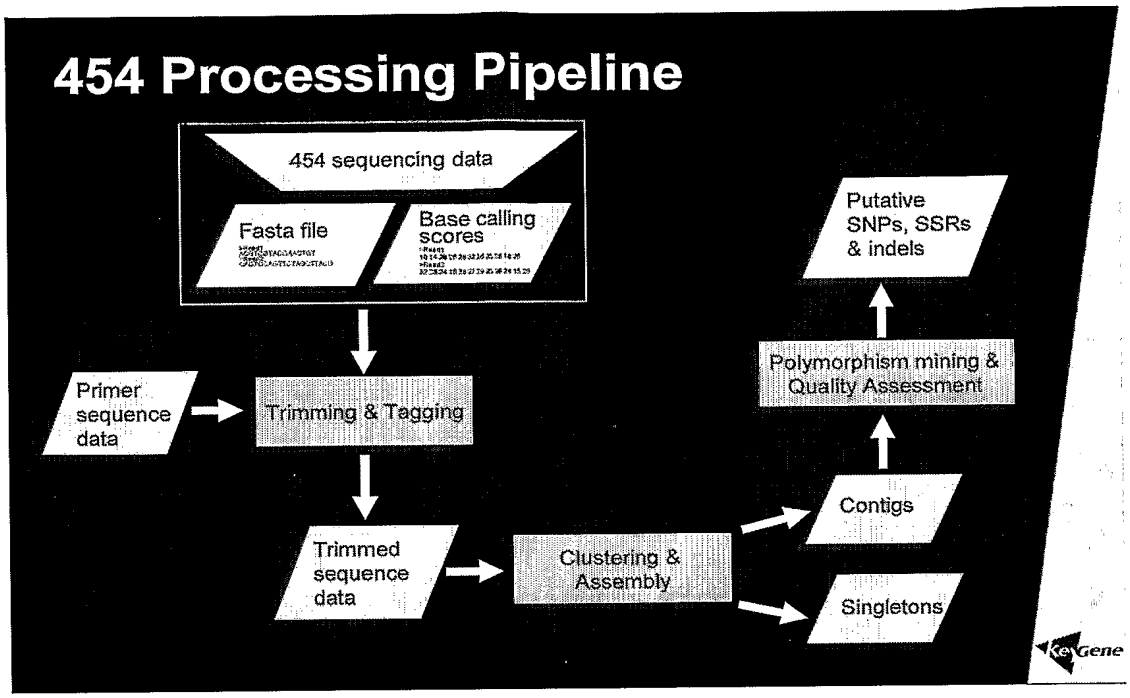


Figure 4B. Polymorphism mining and quality assignment process

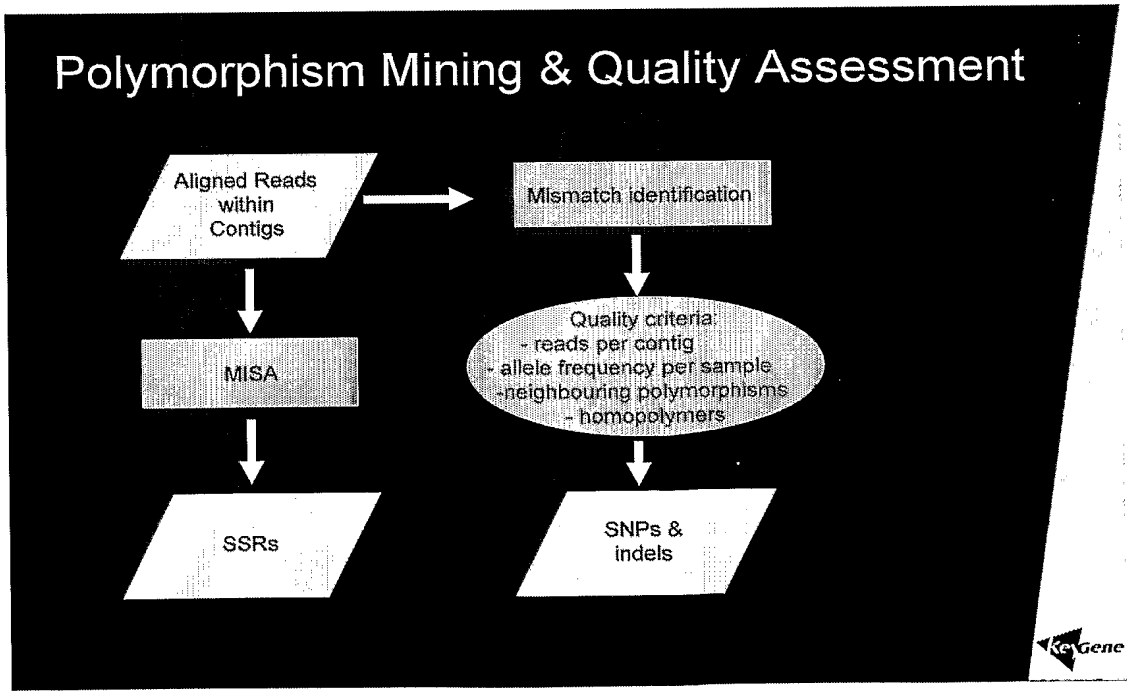


FIG 5A

Panel 1: Example of a mixed tag

CAAGAGACTGCGTACCAATTCAACTTTGAGGTGAAAGATCGAAGGTTGCA
CAAGAGACTGCGTACCAATTCA (ES2)

AACACCAAGTGGCCGACCATCTCTTGCGTGTTACTCAGGACTCATCACCAC
(MS1) TGTTACTCAGGACTCATCACCA

Panel 2: Overview of observed fragments containing expected tags and mixed tags

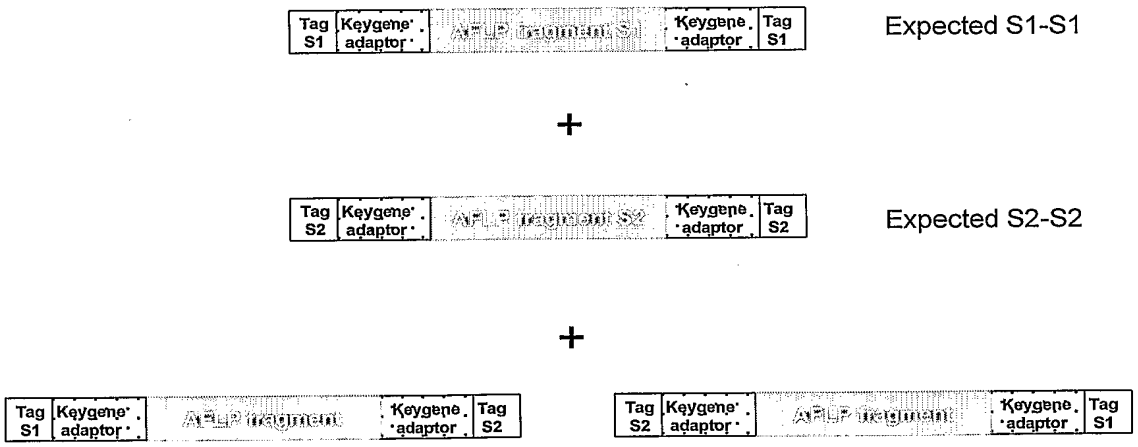


FIG 5B

Panel 3: Hypothesized cause for generation of mixed tags

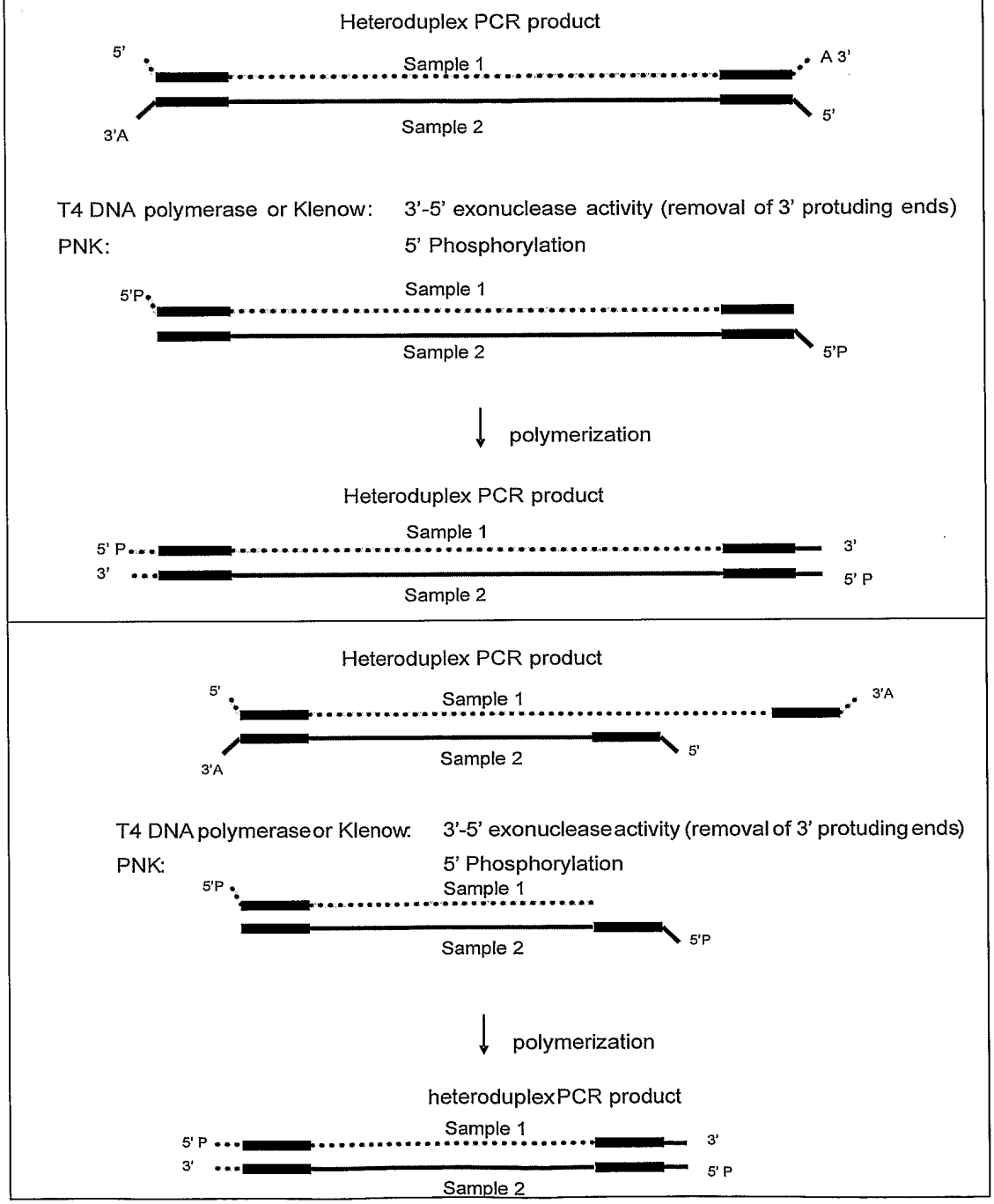


FIG 5C

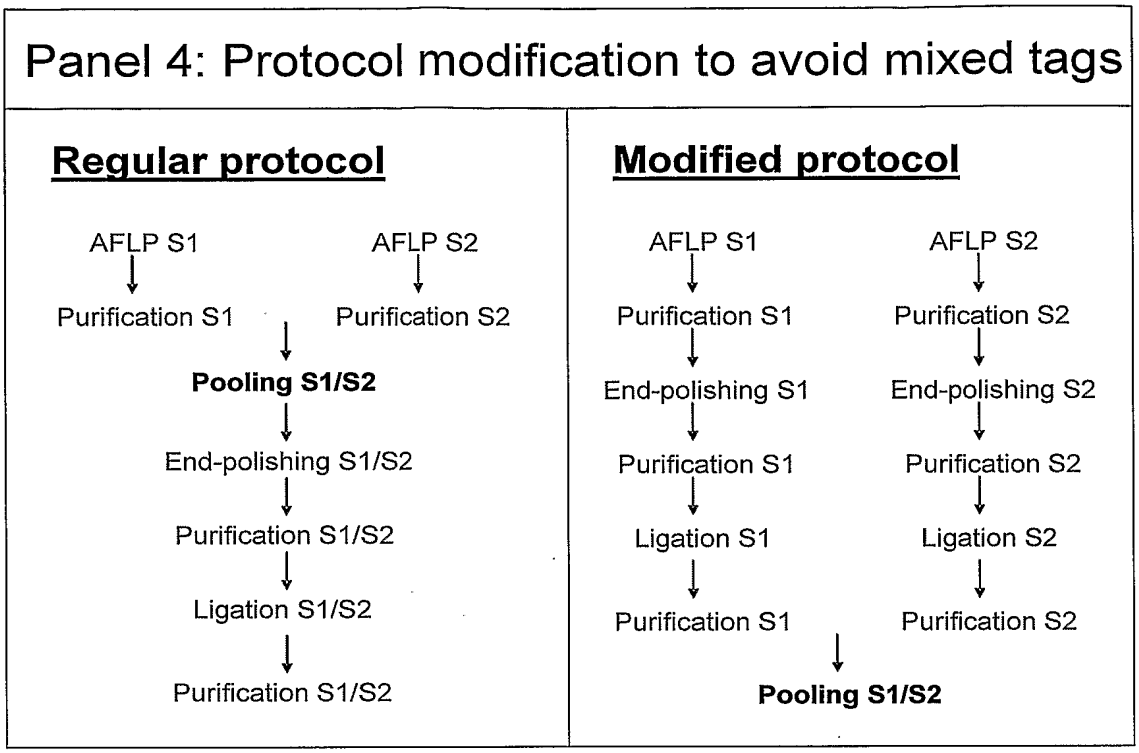

Panel 4: Protocol modification to avoid mixed tags
Regular protocol
AFLP S1
AFLP S2
Purification S1
Purification S2
Pooling S1/S2
End-polishing S1/S2
Purification S1/S2
Ligation S1/S2
Purification S1/S2
Modified protocol
AFLP S1
AFLP S2
Purification S1
Purification S2
End-polishing S1
End-polishing S2
Purification S1
Purification S2
Ligation S1
Ligation S2
Purification S1
Purification S2
Pooling S1/S2

FIG 6A

Panel 1: Example of a concatamer

TGGTGATGAGTCCTGAGTAACGGGCCTTTCTTTGTACACT
TGGTGATGAGTCCTGAGTAACA (MS1)

TGAATTGGTACGCAGTCTGACG|AGCCGATGAGTCCTGAGTAACA
TGAATTGGTACGCAGTCTGACG|AGCCGATGAGTCCTGAGTAACA
(ES1) | (MS2)

TAAAGGTAAAGCGTGAATTGGTACG
TGAATTGGTACGCAGTCTCTTG (ES2)

Panel 2: Overview of observed fragments containing expected tags and concatamers

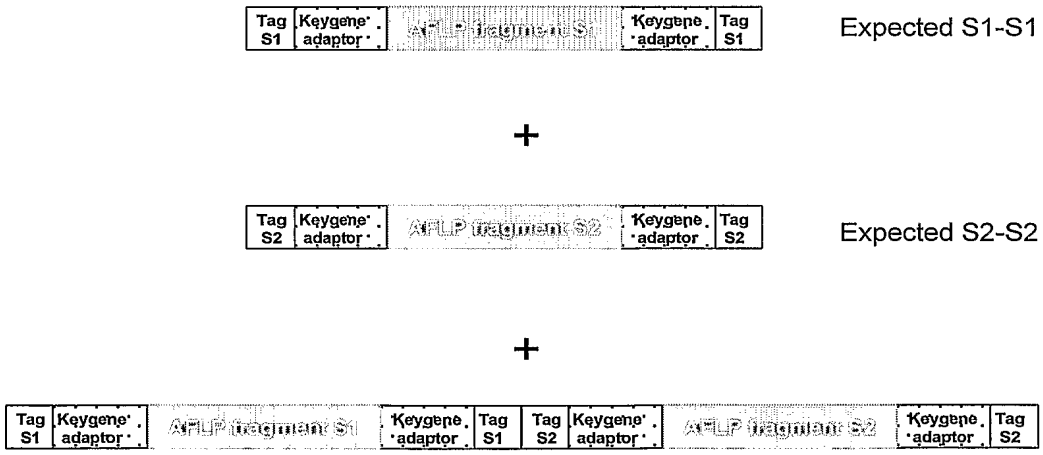


FIG 6B

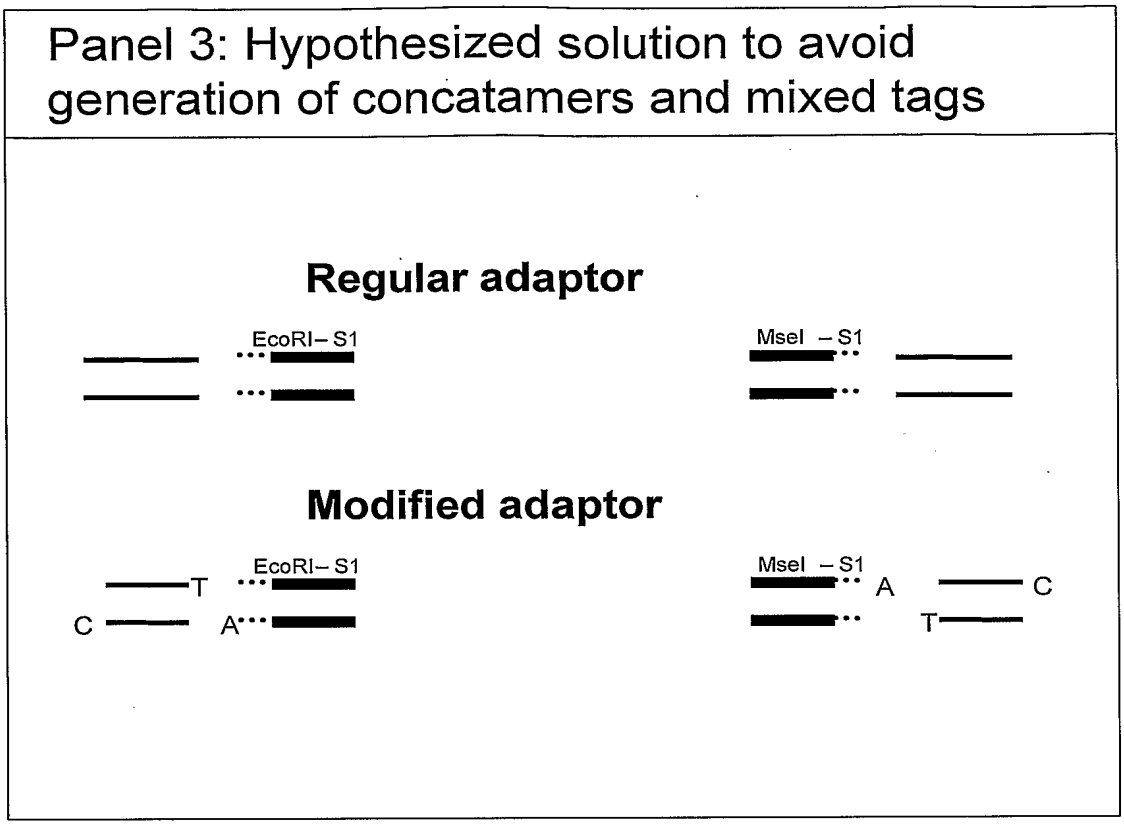

Panel 3: Hypothesized solution to avoid generation of concatamers and mixed tags
Regular adaptor
EcoRI– S1
MseI – S1
Modified adaptor
EcoRI– S1
T
C
A
MseI – S1
A
C
T

FIG 6C

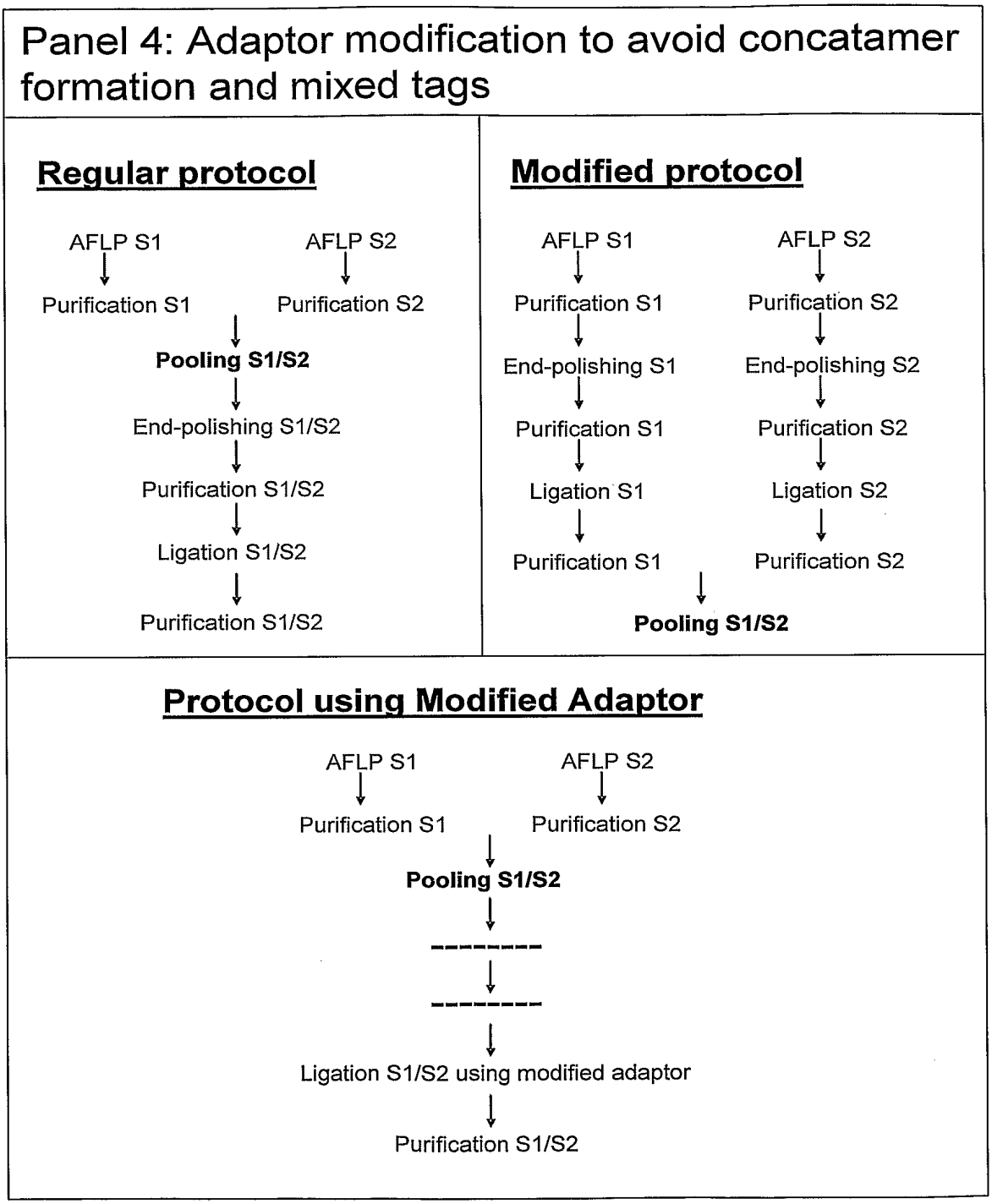

Panel 4: Adaptor modification to avoid concatamer formation and mixed tags
Regular protocol
AFLP S1
AFLP S2
Purification S1
Purification S2
Pooling S1/S2
End-polishing S1/S2
Purification S1/S2
Ligation S1/S2
Purification S1/S2
Modified protocol
AFLP S1
AFLP S2
Purification S1
Purification S2
End-polishing S1
End-polishing S2
Purification S1
Purification S2
Ligation S1
Ligation S2
Purification S1
Purification S2
Pooling S1/S2
Protocol using Modified Adaptor
AFLP S1
AFLP S2
Purification S1
Purification S2
Pooling S1/S2
Ligation S1/S2 using modified adaptor
Purification S1/S2

FIG 7

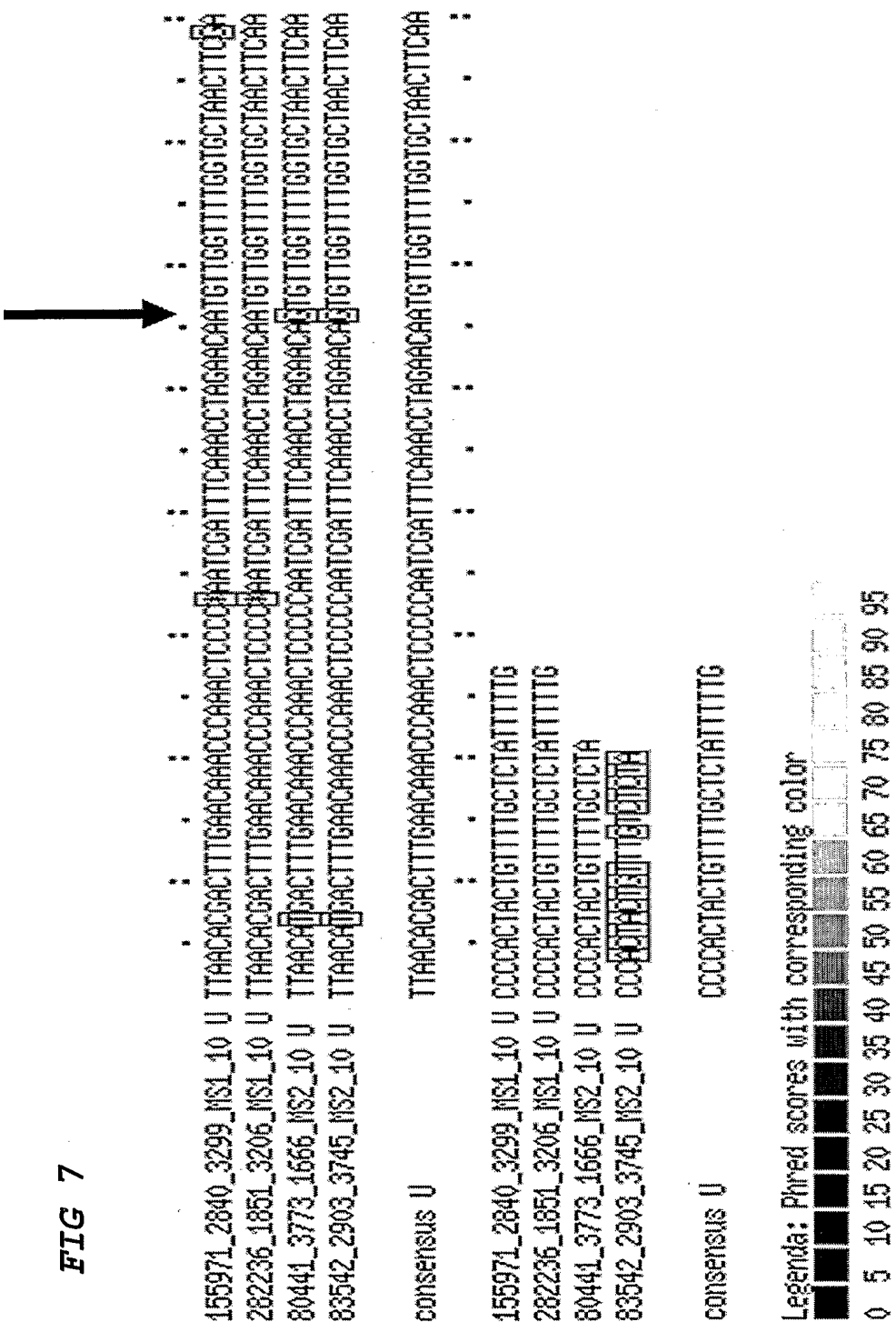

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consensus U
155971_2840_3299_MS1_10 U
282236_1851_3206_MS1_10 U
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consensus U
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FIG 8A.

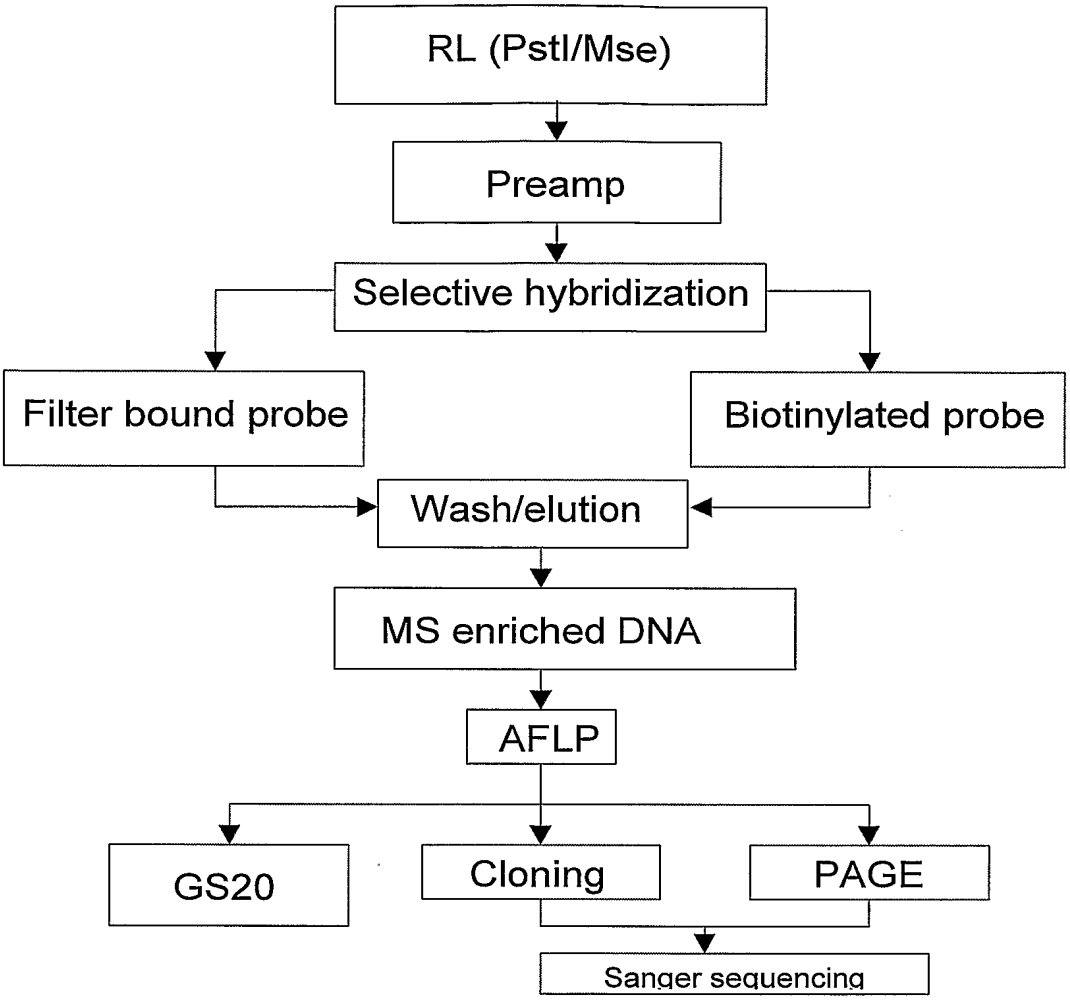
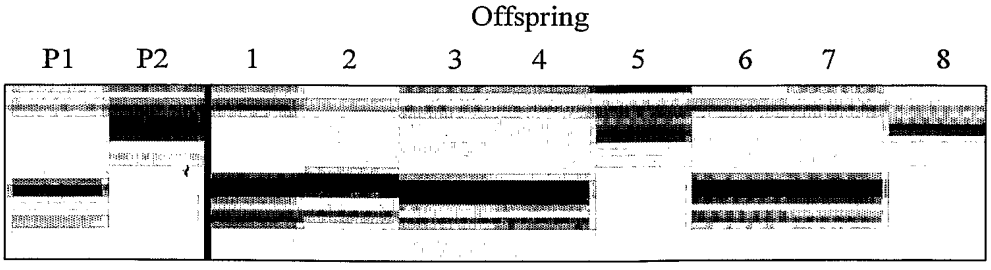

RL (PstI/Mse)
Preamp
Selective hybridization
Filter bound probe
Biotinylated probe
Wash/elution
MS enriched DNA
AFLP
GS20
Cloning
PAGE
Sanger sequencing

FIG 8B


Offspring
P1
P2
1
2
3
4
5
6
7
8

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2006/000311

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

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)
C12Q

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 practical, search terms used)
EPO-Internal, BIOSIS, 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	JORDAN BARBARA ET AL: "Genome complexity reduction for SNP genotyping analysis" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 99, no. 5, 5 March 2002 (2002-03-05), pages 2942-2947, XP002398229 ISSN: 0027-8424 page 2943, column 1, paragraph 2 page 2946, column 1, paragraph 3	1-14
X	WO 2004/022758 A (PLANT BIOSCIENCE LIMITED; ZHU, JIAHUI) 18 March 2004 (2004-03-18) page 16	1-14

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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

"E" earlier document 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)

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"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
11 September 2006	02/10/2006

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Aslund, Fredrik

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALTSHULER D ET AL: "An SNP map of the human genome generated by reduced representation shotgun sequencing" NATURE, NATURE PUBLISHING GROUP, LONDON, GB, vol. 407, 28 September 2000 (2000-09-28), pages 513-516, XP002955779 ISSN: 0028-0836 page 515 - page 516	1-14
X	WO 00/78945 A (STRATAGENE; SORGE, JOSEPH, A; DAVIS, RONALD, W) 28 December 2000 (2000-12-28) page 33 - page 38 page 56	1-14
A	NICOD JEAN-CLAUDE ET AL: "SNPs by AFLP (SBA): a rapid SNP isolation strategy for non-model organisms." NUCLEIC ACIDS RESEARCH. 1 MAR 2003, vol. 31, no. 5, 1 March 2003 (2003-03-01), page e19, XP002398230 ISSN: 1362-4962	
A	GUPTA P K ET AL: "Single nucleotide polymorphisms: A new paradigm for molecular marker technology and DNA polymorphism detection with emphasis on their use in plants" CURRENT SCIENCE, vol. 80, no. 4, 25 February 2001 (2001-02-25), pages 524-535, XP002972388 ISSN: 0011-3891	
A	DONG S ET AL: "FLEXIBLE USE OF HIGH-DENSITY OLIGONUCLEOTIDE ARRAYS FOR SINGLE-NUCLEOTIDE POLYMORPHISM DISCOVERY AND VALIDATION" GENOME RESEARCH, COLD SPRING HARBOR LABORATORY PRESS, WOODBURY, NY, US, vol. 11, no. 8, 2001, pages 1418-1424, XP001039177 ISSN: 1088-9051 page 1419, column 1 page 1421, column 1	
P,X	EP 1 574 585 A (PLANT RESEARCH INTERNATIONAL B.V) 14 September 2005 (2005-09-14) the whole document	1-14

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2006/000311

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004022758 A	18-03-2004	AU 2003260790 A1	29-03-2004
		CA 2496517 A1	18-03-2004
		EP 1546345 A1	29-06-2005
WO 0078945 A	28-12-2000	AU 5763900 A	09-01-2001
		US 2002119448 A1	29-08-2002
		US 2004018512 A1	29-01-2004
EP 1574585 A	14-09-2005	WO 2005087945 A1	22-09-2005

IDT Exhibit 1020.10161

Espacenet

Bibliographic data: WO2005065321 (A2) — 2005-07-21

METHODS FOR ANALYSIS OF NUCLEIC ACID METHYLATION STATUS AND METHODS FOR FRAGMENTATION, LABELING AND IMMOBILIZATION OF NUCLEIC ACIDS

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Classification: **- international:** ***C12P19/34; C12Q1/68***
- cooperative: **C12P19/34; C12Q1/6827** more

Application number: WO2004US43710 20041229 Global Dossier

Priority number (s): US20030533381P 20031229

Also published as: WO2005065321 (A3) AU2004311882 (A1) CA2552007 (A1) EP1711591 (A2) EP1711591 (A4) more

Abstract of WO2005065321 (A2)

The invention relates to methods for analysis of nucleic acid methylation status, and fragmentation and/or labeling and/or immobilization of nucleic acids. More particularly, the invention relates to methods for fragmentation and/or labeling and/or immobilization of nucleic acids comprising labeling and/or cleavage and/or immobilization at abasic sites.

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 July 2005 (21.07.2005)

PCT

(10) International Publication Number
WO 2005/065321 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2004/043710

(22) International Filing Date:
29 December 2004 (29.12.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/533,381 29 December 2003 (29.12.2003) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2005/065321 A2

(54) Title: METHODS FOR ANALYSIS OF NUCLEIC ACID METHYLATION STATUS AND METHODS FOR FRAGMENTATION, LABELING AND IMMOBILIZATION OF NUCLEIC ACIDS

(57) Abstract: The invention relates to methods for analysis of nucleic acid methylation status, and fragmentation and/or labeling and/or immobilization of nucleic acids. More particularly, the invention relates to methods for fragmentation and/or labeling and/or immobilization of nucleic acids comprising labeling and/or cleavage and/or immobilization at abasic sites.

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METHODS FOR ANALYSIS OF NUCLEIC ACID METHYLATION STATUS AND METHODS FOR FRAGMENTATION, LABELING AND IMMOBILIZATION OF NUCLEIC ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/533,381, filed on December 29, 2003, the disclosure of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0001] The invention relates to methods for analysis of nucleic acid methylation status, and methods for fragmentation and/or labeling and/or immobilization of nucleic acids. More particularly, the invention relates to methods for fragmentation and/or labeling and/or immobilization of nucleic acids comprising labeling and/or cleavage and/or immobilization at abasic sites.

BACKGROUND

[0002] Methylation of DNA is involved in both normal and abnormal cellular processes. For example, DNA methylation has been implicated in X-inactivation, genomic imprinting, and differential gene expression (such as by upregulation or silencing of genetic loci). DNA methylation plays a role in gene inactivation, cell differentiation, tumorigenesis, X-chromosome inactivation, and is required for mammalian development (Li, et al., Cell 69:915-926, 1992; Okano et al., Cell 99:247-57, 1999). In bacteria, methylation of cytosine and adenine residues plays a role in the regulation of DNA replication and DNA repair. DNA methylation has also been associated with increased risk of cancer, as well as cancer development itself.

[0003] Methylation of DNA is carried out by methylases (also known as methyltransferases). These enzymes are generally sequence-specific, and they can methylate both nucleic acid strands (in the case of DNA). Replication of these strands yields a hemi-methylated state which is recognized by a class of maintenance methylases capable of restoring full methylation to both strands.

[0004] Methylation can occur at all nucleotide residues, although in mammalian species, DNA methylation commonly occurs at cytosine residues, and more commonly at cytosine residues that lie next to a guanosine residue, i.e., at cytosine residues of a CG dinucleotide. CG dinucleotides in "CpG islands" remain methylation-free. CpG islands are rich in CG sites and are often found near coding regions within the genome (i.e., genes). About half of the genes in the human genome are associated with CpG islands. Importantly, the vast majority of CpG islands in the genome remain unmethylated in normal adult cells and tissues. Methylation of CpG islands is normally seen only on the inactive X-chromosome in females and at imprinted genes where it functions in the stable silencing of such genes. Strict control over the levels and distribution of DNA methylation are essential to normal animal development.

[0005] Alteration in DNA methylation is one manifestation of the genome instability characteristic of human tumors. A hallmark of human carcinogenesis is the loss of normal constraints on cell growth resulting from genetic alterations in the genes that control cell growth. The consequences of such mutations include the activation of positive growth signals and the inactivation of growth inhibitory signals. Identification of gene targets which when methylated lead to the loss of normal cell responses would be valuable. This would facilitate the diagnosis and treatment of disorders associated with abnormal methylation and any downstream events resulting therefrom.

[0006] The level of methylation of a nucleic acid can be determined using a number of techniques available in the art. Some methods of analysis involve the use of the chemical regent, bisulfite. Other methods for methylation analysis include methylation-sensitive restriction analysis, methylation-specific polymerase chain reaction (MSP), sequencing of bisulfite-modified DNA, Ms-SnuPE, and COBRA.

[0007] There is a serious need for improved methods for analyzing nucleic acid methylation status.

[0008] Fragmentation and labeling of nucleic acids are important for the analysis of nucleic acid sequences. For example, fragmentation and/or labeling are commonly required for detection of sequences by binding of a sample nucleic acid to complementary sequences immobilized on a surface, for example, on a microarray. Cleavage of sample nucleic acid into small fragments (e.g., 50-100 base pairs) facilitates diffusion of nucleic acid onto the

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surface, and may facilitate hybridization. It is known, for example, that steric and charge hindrance effects increase with the size of nucleic acids that are hybridized. Moreover, cleavage of sample nucleic acids into small fragments may ensure that two sequences of interest in the sample do not appear to bind to the same template nucleic acid simply by virtue of their proximity on the test nucleic acid. Cleavage of nucleic acids also facilitates detection of hybridized nucleic acid when, as in many detection methods, the size of the signal is proportional to the size of the bound fragment and thus, control of fragment size is desirable. Labeling of nucleic acids is necessary in many methods of nucleic acid analysis because there are presently few techniques for direct detection of unlabeled nucleic acid with the requisite sensitivity for analysis on chips. Methods for fragmenting and/or labeling nucleic acids are known in the art. *See, e.g.*, U.S. Patent Nos. 5,082,830; 4,996,143; 5,688,648; 6,326,142; and PCT Publication No. WO 02/090584, and references cited therein.

[0009] Immobilization of nucleic acids to create, for example, microarrays or tagged analytes, is useful for, e.g., detection and analysis of nucleic acids and tagged analytes. Methods for immobilizing nucleic acids are known in the art. *See, e.g.*, U.S. Patent Nos. 5,667,979; 6,077,674; 6,280,935; and references cited therein.

[0010] There is a serious need for improved methods for labeling and/or fragmenting and/or immobilizing nucleic acids to a surface (such as a microarray).

[0011] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0012] In one aspect, the invention provides methods for labeling and fragmenting a polynucleotide comprising a methylated nucleotide comprising: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an agent capable of cleaving a base portion of the methylated nucleotide (i.e., cleaving a base portion of the methylated nucleotide), whereby an abasic site is generated; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the

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abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated. In some embodiments, the agent capable of cleaving the base portion of the methylated nucleotide is selected from the group consisting of an enzyme, a chemical agent, and acidic conditions. In some embodiments, cleavage of the phosphodiester backbone of a polynucleotide is performed with an agent selected from the group consisting of an enzyme, a chemical agent, acidic conditions, basic conditions, and heat. Two or more of the steps described above may be performed simultaneously, or the steps may be performed sequentially. For example, steps (a), (b), and (c) may be performed simultaneously, steps (a) and (b) may be performed simultaneously, or steps (b) and (c) may be performed simultaneously. When the steps are performed sequentially, step (b) may be performed before step (c) or step (c) may be performed before step (b).

[0013] In another aspect, the invention provides methods for labeling a polynucleotide comprising a methylated nucleotide, comprising: (a) cleaving a base portion of the methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated; and (b) labeling at the abasic site, whereby a labeled polynucleotide is generated.

[0014] In another aspect, the invention provides a method for fragmenting a polynucleotide comprising a methylated nucleotide, comprising: (a) cleaving a base portion of the methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide whereby an abasic site is generated; and (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site, whereby polynucleotide fragments are generated.

[0015] In another aspect, the invention provides methods for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, comprising: (a) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising a methylated nucleotide; and (ii) an agent capable of specifically cleaving a base portion of a methylated nucleotide; wherein the incubation is under conditions that permit cleavage of the base portion of the methylated nucleotide, whereby a polynucleotide comprising an abasic site is generated; (b) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising an abasic site; and (ii) an agent capable of effecting

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(generally, specific) cleavage of a phosphodiester backbone at the abasic site; wherein the incubation is under conditions that permit cleavage of the phosphodiester backbone at the abasic site; whereby fragments of the polynucleotide are generated; (c) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising an abasic site; and (ii) an agent capable of labeling the abasic site; wherein the incubation is under conditions that permit labeling at the abasic site; whereby labeled fragments are generated.

[0016] In one embodiment, the invention provides a method for fragmenting a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated. In another embodiment, the invention provides a method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, and labeling at the abasic site, whereby a labeled polynucleotide fragment is generated. In another embodiment, the invention provides a method for labeling a polynucleotide comprising a methylated nucleotide, said method comprising labeling at the abasic site of a polynucleotide comprising an abasic site, whereby a labeled polynucleotide is generated, wherein the abasic site is generated by cleaving a base portion of a methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated. In another embodiment, the invention provides a method for labeling a polynucleotide fragment, said method comprising labeling at an abasic site of a polynucleotide fragment comprising the abasic site, whereby a labeled polynucleotide fragment is generated, wherein the abasic site is generated by cleaving a base portion of a methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, and wherein the polynucleotide fragment is generated by cleaving the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site.

[0017] In one aspect, the invention provides methods for labeling and fragmenting a polynucleotide comprising a methylated nucleotide, said methods comprising: (a)

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contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an agent (such as an enzyme) capable of cleaving a base portion of an unmethylated nucleotide (i.e., cleaving a base portion of the unmethylated nucleotide), whereby an abasic site is generated, wherein the agent (such as an enzyme) is not capable of cleaving a methylated nucleotide; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated. In some embodiments, the agent capable of cleaving a base portion of an unmethylated nucleotide comprises an enzyme. In one embodiment, the unmethylated nucleotide is cytosine and the enzyme is cytosine deaminase in conjunction with uracil DNA glycosylase. In some embodiments, cleavage of a phosphodiester backbone of a polynucleotide is performed with an agent selected from the group consisting of an enzyme, a chemical agent, acidic conditions, basic conditions, and heat. Two or more of the steps described above may be performed simultaneously, or the steps may be performed sequentially. For example, steps (a), (b), and (c) may be performed simultaneously, steps (a) and (b) may be performed simultaneously, or steps (b) and (c) may be performed simultaneously. When the steps are performed sequentially, step (b) may be performed before step (c) or step (c) may be performed before step (b).

[0018] In another aspect, the invention provides a method for producing a labeled polynucleotide comprising a methylated nucleotide, comprising: (a) cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving the base portion of a methylated nucleotide; and (b) labeling at the abasic site, whereby a labeled polynucleotide is generated.

[0019] In another aspect, the invention provides a method for fragmenting a polynucleotide comprising a methylated nucleotide, comprising: (a) cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving the base portion of a methylated nucleotide; and (b) cleaving the

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backbone of the polynucleotide comprising the abasic site, whereby polynucleotide fragments are generated.

[0020] In another aspect, the invention provides method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, comprising: (a) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising a methylated nucleotide; and (ii) an agent capable of cleaving a base portion of a unmethylated nucleotide; wherein the incubation is under conditions that permit cleavage of the base portion of the unmethylated nucleotide, wherein the agent is not capable of cleaving a methylated nucleotide, whereby a polynucleotide comprising an abasic site is generated; (b) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising an abasic site; and (ii) an agent capable of effecting (generally, specific) cleavage of a phosphodiester backbone at the abasic site; wherein the incubation is under conditions that permit cleavage of the phosphodiester backbone at the abasic site; whereby fragments of the polynucleotide are generated; (c) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising an abasic site; and (ii) an agent capable of labeling the abasic site; wherein the incubation is under conditions that permit labeling at the abasic site; whereby labeled fragments are generated.

[0021] In one embodiment, the invention provides a method for fragmenting a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving a methylated nucleotide. In another embodiment, the invention provides a method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving a methylated nucleotide, and labeling at the abasic site, whereby a labeled polynucleotide fragment is generated. In another embodiment, the invention

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provides a method for labeling a polynucleotide comprising a methylated nucleotide, said method comprising labeling at the abasic site of a polynucleotide comprising an abasic site, whereby a labeled polynucleotide is generated, wherein the abasic site is generated by cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving a methylated nucleotide. In another embodiment, the invention provides a method for labeling a polynucleotide fragment, said method comprising labeling at an abasic site of a polynucleotide fragment comprising the abasic site, whereby a labeled polynucleotide fragment is generated, wherein the abasic site is generated by cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, and wherein the polynucleotide fragment is generated by cleaving the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site.

[0022] In another aspect, the invention provides methods using the labeled and/or fragmented methylated polynucleotides generated using the methods for labeling and/or fragmenting a methylated polynucleotide described herein for , e.g., detection of methylation (including presence and/or absence and/or quantity or level of methylation), identification of methylated polynucleotide sequences, isolation of methylated polynucleotide sequences, characterization of methylated polynucleotide sequences, and other applications as described herein. In one embodiment, the invention provides a method for characterizing a methylated polynucleotide, comprising detecting a polynucleotide fragment or a labeled polynucleotide fragment produced by any of the methods described herein, wherein detection of the polynucleotide fragment correlates with presence, absence, sequence, or amount of the methylated polynucleotide.

[0023] In another aspect, the invention provides methods for labeling and fragmenting a polynucleotide comprising a canonical nucleotide, said methods comprising: (a) cleavage of a base portion of a canonical nucleotide present in a polynucleotide comprising the canonical nucleotide with an agent (such as an enzyme) capable of cleaving a base portion of the canonical nucleotide (whereby an abasic site is generated); (b) cleavage of the phosphodiester backbone at the abasic site, and (c) labeling at the abasic site, whereby labeled polynucleotide fragments are generated. In some embodiments, the agent capable

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of cleaving a base portion of a canonical nucleotide comprises an enzyme. In one embodiment, the canonical nucleotide is cytosine and the enzyme comprises cytosine deaminase in conjunction with uracil DNA glycosylase. In some embodiment, cleavage of the phosphodiester backbone of a polynucleotide is performed with an agent selected from the group consisting of an enzyme, a chemical agent, acidic conditions, basic conditions, and heat. Two or more of the steps described above may be performed simultaneously, or the steps may be performed sequentially. For example, steps (a), (b), and (c) may be performed simultaneously, steps (a) and (b) may be performed simultaneously, or steps (b) and (c) may be performed simultaneously. When the steps are performed sequentially, step (b) may be performed before step (c) or step (c) may be performed before step (b).

[0024] In another aspect, the invention provides methods for fragmenting and labeling a polynucleotide comprising a canonical nucleotide: (a) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising a canonical nucleotide; and (ii) an agent capable of specifically cleaving a base portion of a canonical nucleotide; wherein the incubation is under conditions that permit cleavage of the base portion of the canonical nucleotide, whereby a polynucleotide comprising an abasic site is generated; (b) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising an abasic site; and (ii) an agent capable of effecting (generally, specific) cleavage of a phosphodiester backbone at the abasic site; wherein the incubation is under conditions that permit cleavage of the phosphodiester backbone at the abasic site; whereby fragments of the polynucleotide are generated; (c) incubating a reaction mixture, said reaction mixture comprising: (i) a polynucleotide comprising an abasic site; and (ii) an agent capable of labeling the abasic site; wherein the incubation is under conditions that permit labeling at the abasic site; whereby labeled fragments are generated.

[0025] In another aspect, the invention provides methods for labeling a polynucleotide comprising a canonical nucleotide, said methods comprising (a) cleavage of a base portion of a canonical nucleotide present in a polynucleotide comprising the canonical nucleotide with an agent (such as an enzyme) capable of cleaving a base portion of the canonical nucleotide (whereby an abasic site is generated); and (b) labeling at the site of incorporation of the canonical nucleotide (i.e., at the abasic site), whereby a labeled polynucleotide(s) is generated.

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[0026] In another aspect, the invention provides methods for fragmenting a polynucleotide comprising a canonical nucleotide, said methods comprising: (a) cleavage of a base portion of a canonical nucleotide present in a polynucleotide comprising the canonical nucleotide with an agent (such as an enzyme) capable of cleaving a base portion of the canonical nucleotide (whereby an abasic site is generated); and (b) cleavage of the phosphodiester backbone at the abasic site, whereby polynucleotide fragments are generated.

[0027] In one embodiment, the invention provides a method for fragmenting a polynucleotide comprising a canonical nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a canonical nucleotide with an agent capable of cleaving the base portion of the canonical nucleotide, whereby an abasic site is generated. In another embodiment, the invention provides a method for fragmenting and labeling a polynucleotide comprising a canonical nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a canonical nucleotide with an agent capable of cleaving the base portion of the canonical nucleotide, whereby an abasic site is generated, and labeling at the abasic site, whereby a labeled polynucleotide fragment is generated. In another embodiment, the invention provides a method for labeling a polynucleotide comprising a canonical nucleotide, said method comprising labeling at the abasic site of a polynucleotide comprising an abasic site, whereby a labeled polynucleotide is generated, wherein the abasic site is generated by cleaving a base portion of a canonical nucleotide with an agent capable of cleaving the base portion of the canonical nucleotide, whereby an abasic site is generated. In another embodiment, the invention provides a method for labeling a polynucleotide fragment, said method comprising labeling at an abasic site of a polynucleotide fragment comprising the abasic site, whereby a labeled polynucleotide fragment is generated, wherein the abasic site is generated by cleaving a base portion of a canonical nucleotide with an agent capable of cleaving the base portion of a canonical nucleotide, and wherein the polynucleotide fragment is generated by cleaving the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site.

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[0028] In some embodiments, the polynucleotide comprising the canonical nucleotide (that is to be cleaved by the agent that cleaves a base portion of the canonical nucleotide) can be single stranded, double-stranded or partially double stranded. In some embodiments, the polynucleotide comprises a cDNA. In other embodiments, the polynucleotide comprises RNA, mRNA, genomic DNA, or synthetic DNA. In other embodiments, the polynucleotide comprises a cDNA library, a subtractive hybridization library, or a genomic library.

[0029] In some embodiments, the invention provides methods wherein the cleavage of the base portion of the cleavable canonical nucleotide is under the same conditions (using the same agent) as the cleavage of the backbone at the abasic site, and labeling at the abasic site. In some embodiments, the reaction conditions are acidic reaction conditions (such as pH 3, pH 3.5 or pH 4). In still other embodiments, labeling and fragmentation (including cleavage of the base portion of the canonical nucleotide and cleavage of the phosphodiester backbone at an abasic site) are under the same conditions (using the same agent). In still other embodiments, the reaction conditions are acidic reaction conditions (such as pH 3, pH 3.5 or pH 4).

[0030] The methods of the invention involving cleavage of a cleavable canonical nucleotide include methods using the labeled polynucleotide fragments and labeled polynucleotides produced by the methods of the invention (so-called "applications"). The invention provides methods to characterize (for example, detect presence or absence of and/or quantify) a sequence of interest by analyzing the labeled and/or fragmented products by detection/quantification methods such as those based on array technologies or solution phase technologies. In some embodiments, the invention provides methods of detecting the presence or absence of mutations.

[0031] In other embodiments, the invention provides methods of producing a hybridization probe or target, hybridization using the hybridization probes or targets; detection using the hybridization probes or targets; characterizing and/or quantitating nucleic acid, preparing a subtractive hybridization probe, comparative genomic hybridization, and determining a gene expression profile, using the labeled and/or fragmented nucleic acids generated by the methods of the invention. Any of the methods described herein may be used to generated labeled polynucleotides and/or polynucleotide fragments which may be

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used as hybridization probes or targets. Labeled or unlabeled targets produced by a method of the invention may be hybridized to a probe.

[0032] The invention also provides methods for the generation of polynucleotides, or fragments thereof, immobilized to a substrate (surface), *e.g.*, hybridization probes. In some embodiments, the immobilized polynucleotide, or immobilized polynucleotide fragment (in embodiments involving fragmentation) is labeled according to the labeling methods described herein. These methods are suitable for, for example, the production of microarrays or tagged analytes.

[0033] As is evident to one skilled in the art, aspects that refer to combining and incubating the resultant mixture also encompasses method embodiments which comprise incubating the various mixtures (in various combinations and/or subcombinations) so that the desired products are formed. The reaction mixtures may be combined (thus reducing the number of incubations) in any way, with one or more reaction mixtures above combined. It is understood that any combination of these incubation steps, and any single incubation step, to the extent that the incubation is performed as part of any of the methods described herein, fall within the scope of the invention.

[0034] One or more steps may be combined and/or performed sequentially (often in any order, as long as the requisite product(s) are able to be formed), and, as is evident, the invention includes various combinations of the steps described herein. It is also evident, and is described herein, that the invention encompasses methods in which the initial, or first, step is any of the steps described herein. Methods of the invention encompass embodiments in which later, "downstream" steps are an initial step.

[0035] The invention also provides compositions, kits, complexes, reaction mixtures and systems comprising various components (and various combinations of the components) used in the methods described herein. In one aspect, the invention provides a composition comprising an agent capable of cleaving a base portion of a methylated nucleotide to produce an abasic site on a polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide. In one embodiment, the composition further comprises an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site. In another aspect, the invention provides a composition comprising an agent capable of cleaving a base portion of a canonical nucleotide to produce an abasic site on a

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polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide. In one embodiment, the composition further comprises an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site on a polynucleotide. In another aspect, the invention provides a composition comprising a population of labeled and/or fragmented polynucleotides produced by any of the methods described herein. Compositions of the invention may also optionally further comprise a composite primer comprising a DNA portion and a 5' RNA portion.

[0036] In another aspect, the invention provides a kit comprising an agent capable of cleaving a base portion of a methylated nucleotide to produce an abasic site on a polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide. In one embodiment, the kit comprises an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site. In another aspect, the invention provides a kit comprising an agent capable of cleaving a base portion of a canonical nucleotide to produce an abasic site on a polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide. In one embodiment, the kit comprises an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site. Kits of the invention may also optionally further comprise a composite primer comprising a DNA portion and a 5' RNA portion.

DESCRIPTION OF THE FIGURE

[0037] FIGURE 1: is a photograph of a gel showing the labeled and fragmented polynucleotide generated by acid-catalyzed cleavage fragmentation and labeling of cDNA.

MODES FOR CARRYING OUT THE INVENTION

Methods of the invention

I. Methods for methylation analysis

[0038] The invention provides methods and kits for analyzing DNA methylation (including detecting and/or identifying methylated DNA sequences). In one aspect, the methods comprise use of an agent (such as an enzyme) that cleaves a base portion from a

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methylated nucleotide (such as 5-methylcytosine), whereby an abasic site is generated; cleavage of the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and labeling at the abasic site, whereby labeled polynucleotide fragments are generated. In another aspect, the methods comprise use of an enzyme (such as cytosine deaminase in conjunction with uracil N glycosylase (UNG)) that cleaves a base portion from an unmethylated nucleotide, whereby an abasic site is generated, wherein the enzyme is not capable of cleaving a methylated nucleotide; cleavage of the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and labeling at the abasic site, whereby labeled polynucleotide fragments are generated.

[0039] Generally, the polynucleotide comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated nucleotide) is fragmented and labeled at the abasic site (which is generated by cleavage of a base portion of the methylated nucleotide). Thus, the methods of the invention are useful for, e.g., detecting methylation (including presence and/or absence and/or quantity or level of methylation), identifying methylated polynucleotide sequences, isolating methylated polynucleotide sequences, identifying and/or isolating methylatable polynucleotide sequences, and other applications as described herein. The methods of the invention generate labeled polynucleotide fragments which are useful for, e.g., hybridization to a microarray and other uses described herein.

[0040] In one aspect, the methods involve the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an agent capable of cleaving a base portion of the methylated nucleotide (i.e., cleaving a base portion of the methylated nucleotide), whereby an abasic site is generated; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated.

[0041] In another aspect, the methods involve the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an agent capable of cleaving a base portion of an unmethylated nucleotide (i.e., cleaving a base portion of the unmethylated nucleotide), whereby an abasic site is generated, wherein the agent is not capable of cleaving a methylated nucleotide; (b) cleaving

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the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated.

[0042] In another aspect, the methods comprise the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an enzyme capable of cleaving a base portion of an unmethylated nucleotide (i.e., cleaving a base portion of the unmethylated nucleotide), whereby an abasic site is generated, wherein the enzyme is not capable of cleaving a methylated nucleotide; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated. In some embodiments, the enzyme is cytosine deaminase in conjunction with uracil DNA glycosylase.

[0043] In another aspect, the invention provides methods using the labeled and/or fragmented methylated polynucleotides, for, e.g., detection of methylation (including presence and/or absence and/or quantity or level of methylation), identification of methylated polynucleotide sequences, isolation of methylated polynucleotide sequences, characterization of methylated polynucleotide sequences, and other applications as described herein.

II. Methods for labeling and fragmenting a polynucleotide comprising a cleavable canonical nucleotide, and methods for labeling a polynucleotide comprising a cleavable canonical nucleotide

[0044] The invention provides novel methods and kits for labeling and fragmenting a polynucleotide, and novel methods and kits for labeling a polynucleotide. These methods are suitable for, for example, generation of labeled polynucleotides, or labeled polynucleotide fragments, for use as hybridization targets. Generally, the polynucleotide is labeled at an abasic site present in the polynucleotide, and fragmented at an abasic site present in the polynucleotide (in embodiments involving fragmentation). The abasic site present in the polynucleotide is generally prepared by cleavage of a base portion of a

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cleavable canonical (interchangeably termed "canonical") nucleotide present in the polynucleotide.

[0045] Thus, in one aspect, the invention provides methods for labeling and fragmenting a polynucleotide. The methods generally comprise cleavage of a base portion of a canonical nucleotide present in a polynucleotide comprising the canonical nucleotide with an agent (such as an enzyme) capable of cleaving a base portion of the canonical nucleotide (whereby an abasic site is generated); cleavage of the phosphodiester backbone at the abasic site, and labeling at the abasic site, whereby labeled polynucleotide fragments are generated. In another aspect, the invention provides methods for labeling a polynucleotide. The methods generally comprise cleavage of a base portion of a canonical nucleotide present in a polynucleotide comprising the canonical nucleotide with an agent (such as an enzyme) capable of cleaving a base portion of the canonical nucleotide (whereby an abasic site is generated); and labeling at the site of incorporation of the canonical nucleotide (i.e., at the abasic site), whereby a labeled polynucleotide(s) is generated.

[0046] The polynucleotide comprising the canonical nucleotide (that is to be cleaved by the agent that cleaves a base portion of the canonical nucleotide) can be single stranded, double-stranded or partially double stranded. In some embodiments, the polynucleotide comprises a cDNA. In other embodiments, the polynucleotide comprises RNA, mRNA, genomic DNA, or synthetic DNA. In other embodiments, the polynucleotide comprises a cDNA library, a subtractive hybridization library, or a genomic library.

[0047] It is understood that a polynucleotide comprising a canonical nucleotide can be a multiplicity (from small to very large) of different polynucleotide molecules. Such populations can be related in sequence (e.g., members of a gene family or superfamily) or extremely diverse in sequence (e.g., generated from all mRNA, generated from all genomic DNA, etc.). Polynucleotides can also correspond to single sequences (which can be part or all of a known gene, including, e.g., a coding region, genomic region, gene locus, etc.).

[0048] A base portion of the canonical nucleotide is cleaved by an agent (such as an enzyme) capable of cleaving a base portion of a canonical nucleotide. Such agents are known in the art and described herein. In some embodiments, the agent capable of cleaving a base portion of a canonical nucleotide is cytosine deaminase (generally in conjunction with UNG), acidic conditions or treatment with an alkylating agent. In some embodiments, the

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agent is an enzyme. In other embodiments, the agent is a chemical agent. In still other embodiments, the agent is reaction conditions (e.g., acidic conditions).

[0049] In some embodiments, the invention provides methods wherein the cleavage of the base portion of the cleavable canonical nucleotide is under the same conditions (using the same agent) as the cleavage of the backbone at the abasic site, and labeling at the abasic site. In some embodiments, the reaction conditions are acidic reaction conditions (such as pH 3, pH 3.5 or pH 4). In still other embodiments, labeling and fragmentation (including cleavage of the base portion of the canonical nucleotide and cleavage of the phosphodiester backbone at an abasic site) are under the same conditions (using the same agent). In still other embodiments, the reaction conditions are acidic reaction conditions (such as pH 3, pH 3.5 or pH 4).

[0050] The methods of the invention involving cleavage of a cleavable canonical nucleotide include methods using the labeled polynucleotide fragments and labeled polynucleotides produced by the methods of the invention (so-called "applications"). The invention provides methods to characterize (for example, detect presence or absence of and/or quantify) a sequence of interest by analyzing the labeled and/or fragmented products by detection/quantification methods such as those based on array technologies or solution phase technologies. In some embodiments, the invention provides methods of detecting the presence or absence of mutations. The invention provides methods of detection whrein detection correlates with presence, absence, sequence, and/or amount of polynucleotide. These detection methods apply to all methods described herein (based on cleavage of methylated nucleotides, unmethylated nucleotides, as well as canonical nucleotides).

[0051] In other embodiments, the invention provides methods of producing a hybridization target, hybridization to a hybridization probe; detection of the target hybridized to the probe; characterizing and/or quantitating nucleic acid, preparing a subtractive hybridization probe, comparative genomic hybridization, and determining a gene expression profile, using the labeled and/or fragmented nucleic acids generated by the methods of the invention.

[0052] The invention also provides methods for the generation of polynucleotides, or fragments thereof, immobilized to a substrate (surface). In some embodiments, the immobilized polynucleotide, or immobilized polynucleotide fragment (in embodiments

involving fragmentation) is labeled according to the labeling methods described herein. These methods are suitable for, for example, the production of microarrays or tagged analytes.

[0053] The methods of the invention include methods using the immobilized polynucleotides, or immobilized polynucleotide fragments produced by the methods of the invention (so-called "applications"). In some embodiments, the invention provides methods of detecting nucleic acid sequence mutations.

[0054] The invention also provides methods to characterize (for example, detect presence or absence of and/or quantify) a sequence of interest using the immobilized polynucleotides, or fragments thereof.

[0055] In another embodiment, the invention provides methods of determining a gene expression profile, using the immobilized polynucleotides, or fragments thereof, generated by the methods of the invention.

General Techniques

[0056] The practice of the invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

[0057] Primers, oligonucleotides and polynucleotides employed in the invention can be generated using standard techniques known in the art.

Definitions

[0058] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA. The nucleotides can be deoxyribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate

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that can be incorporated into a polymer by DNA polymerase. In embodiments involving methylation analysis, nucleotides include methylated nucleotides. A polynucleotide may comprise modified (altered) nucleotides, such as, for example, modification to the nucleotide structure and or modification to the phosphodiester backbone. As discussed herein modified nucleotide can be a canonical (cleavable) nucleotides, though, generally, a canonical nucleotide according to the methods for cleavage of a canonical nucleotide includes an unmodified base portion (i.e., unmodified adenine, cytosine, guanine and thymine base). It is understood, however, that modified nucleotides that are not (canonical) cleavable nucleotide under the reaction conditions used in the methods of the invention, if present, generally should not affect the ability of the polynucleotide to undergo cleavage of a base portion of the cleavable canonical nucleotide, such that an abasic site is generated, and/or cleavage of a phosphodiester backbone at an abasic site, such that fragments are generated, and/or immobilization of a polynucleotide (or fragment thereof) to a substrate, as described herein. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). It is understood that internucleotide modifications may, e.g., alter the efficiency and/or kinetics of cleavage of the phosphodiester backbone (as when, for example a phosphodiester backbone is cleaved at an abasic site, as described herein). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to

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additional nucleotides. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including DNA. It is understood, however, that modified nucleotides and/or internucleotide linkages, if present, generally should not affect the ability of the polynucleotide to undergo cleavage of a base portion of a canonical nucleotide, such that an abasic site is generated, and/or the ability of a polynucleotide to undergo cleavage of a phosphodiester backbone at an abasic site, such that fragments are generated, and/or the ability of a polynucleotide to be immobilized at an abasic site (such as an abasic site at an end of a polynucleotide and/or an abasic site that is not at an end of a polynucleotide) to a surface, as described herein.

[0059] "Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

[0060] A "primer," as used herein, refers to a nucleotide sequence (a polynucleotide), generally with a free 3'-OH group, that hybridizes with a template sequence (such as a template RNA, or a primer extension product) and is capable of promoting polymerization of a polynucleotide complementary to the template. A "primer" can be, for

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example, an oligonucleotide. It can also be, for example, a sequence of the template (such as a primer extension product or a fragment of an RNA template created following cleavage of a template RNA in an RNA-DNA complex by RNase H or other agent) that is hybridized to a sequence in the template itself (for example, as a hairpin loop), and that is capable of promoting nucleotide polymerization by DNA polymerase. Thus, a primer can be an exogenous (e.g., added) primer or an endogenous (e.g., template fragment) primer.

[0061] A "complex" is an assembly of components. A complex may or may not be stable and may be directly or indirectly detected. For example, as is described herein, given certain components of a reaction, and the type of product(s) of the reaction, existence of a complex can be inferred. For purposes of this invention, a complex is generally an intermediate with respect to the final polynucleotide fragments, labeled polynucleotide, labeled polynucleotide fragments, and/or immobilized polynucleotide or fragment thereof.

[0062] A "fragment" of a polynucleotide or oligonucleotide is a contiguous sequence of 2 or more bases. In other embodiments, a fragment (also termed "region" or "portion") is any of about 3, about 5, about 10, about 15, about 20, about 25, about 30 about 35 about 40, about 50, about 65, about 75, about 85, about 100, about 125, about 150, about 175, about 200, about 225, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650 or more nucleotides in length. In some embodiments, the fragments can be at least about 3, about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 50, about 65, about 75, about 85, about 100, about 125, about 150, about 175, about 200, about 225, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650 or more nucleotides in length. In other embodiments, the fragments can be less than about 3, about 5, about 10, about 15, about 20, about 25, about 30 about 35 about 40, about 50, about 65, about 75, about 85, about 100, about 125, about 150, about 175, about 200, about 225, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650 or more nucleotides in length. In some embodiments, these fragment lengths represent an average size in the population of fragments generated using the methods of the invention.

[0063] A "reaction mixture" is an assemblage of components, which, under suitable conditions, react to form a complex (which may be an intermediate) and/or a product(s).

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[0064] "A", "an" and "the", and the like, unless otherwise indicated include plural forms. "A" fragment means one or more fragments. "A" methylated nucleotide means one or more methylated nucleotides. "An" enzyme means one or more than one enzyme.

[0065] "Comprising" means including in accordance with well-established principles of patent law (*i.e.*, open language).

[0066] Conditions that "allow" an event to occur or conditions that are "suitable" for an event to occur, such as polynucleotide synthesis, cleavage of a base portion of a canonical nucleotide, cleavage of a base portion of a methylated nucleotide, cleavage of a phosphodiester backbone at an abasic site, and the like, or "suitable" conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event. Such conditions, known in the art and described herein, depend upon, for example, the nature of the polynucleotide sequence, temperature, and buffer conditions. These conditions also depend on what event is desired, such as polynucleotide synthesis, cleavage of a base portion of a canonical nucleotide, cleavage of a base portion of a methylated nucleotide, cleavage of a phosphodiester backbone at an abasic site, labeling an abasic site, immobilizing a polynucleotide fragment or a polynucleotide, etc.

[0067] "Microarray" and "array," as used interchangeably herein, comprise a surface with an array, preferably ordered array, of putative binding (e.g., by hybridization) sites for a biochemical sample (target) which often has undetermined characteristics.

[0068] The term "3'" generally refers to a region or position in a polynucleotide or oligonucleotide 3' (downstream) from another region or position in the same polynucleotide or oligonucleotide. "3'" may also refer to the 3' end of a polynucleotide or oligonucleotide.

[0069] The term "5'" generally refers to a region or position in a polynucleotide or oligonucleotide 5' (upstream) from another region or position in the same polynucleotide or oligonucleotide. "5'" may also refer to the 5' end of a polynucleotide or oligonucleotide.

[0070] The terms "3'-DNA portion," "3'-DNA region," "3'-RNA portion," and "3'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 3' end of the polynucleotide or oligonucleotide, and may or may not include the 3' most nucleotide(s) or moieties attached to the 3' most nucleotide of the same polynucleotide or oligonucleotide. The 3' most nucleotide(s) can be preferably from about 1

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to about 50, more preferably from about 10 to about 40, even more preferably from about 20 to about 30 nucleotides. In some embodiments, a 3' portion can be any of at least about 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, 30, or 40 nucleotides. In some embodiments, a 3' portion can be about 1 to about 20 nucleotides, about 5 to about 20 nucleotides, or about 5 to about 50 nucleotides.

[0071] The terms "5'-DNA portion," "5'-DNA region," "5'-RNA portion," and "5'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 5' end of the polynucleotide or oligonucleotide, and may or may not include the 5' most nucleotide(s) or moieties attached to the 5' most nucleotide of the same polynucleotide or oligonucleotide. The 5' most nucleotide(s) can be preferably from about 1 to about 50, more preferably from about 10 to about 40, even more preferably from about 20 to about 30 nucleotides. In some embodiments a 5' portion can be at least about any of 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, 30, or 40 nucleotides. In some embodiments, a 5' portion can be about 1 to about 20 nucleotides, about 5 to about 20 nucleotides, or about 5 to about 50 nucleotides.

[0072] As used herein in the context of methods for labeling and/or fragmenting a canonical nucleotide, "canonical" nucleotide means a nucleotide comprising one of the four common nucleic acid bases adenine, cytosine, guanine and thymine that are commonly found in DNA. The term also encompasses the respective deoxyribonucleosides, deoxyribonucleotides or 2'-deoxyribonucleoside-5'-triphosphates that contain one of the four common nucleic acid bases adenine, cytosine, guanine and thymine.

[0073] The term "analyte" as used herein refers to a substance to be detected or assayed by the method of the present invention, for example, a compound whose properties, location, quantity and/or identity is desired to be characterized. Typical analytes may include, but are not limited to proteins, peptides, nucleic acid segments, cells, microorganisms and fragments and products thereof, organic molecules, inorganic molecules, or any substance for which immobilization sites for binding partner(s) can be developed. As this disclosure clearly conveys, an analyte is a substrate.

[0074] As used herein, an "abasic site" refers to the site of a canonical nucleotide following treatment with an agent capable of effecting cleavage of a base portion of the canonical nucleotide (in embodiments relating to labeling and/or fragmentation of a polynucleotide comprising a canonical nucleotide), or the site of the methylated nucleotide

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following treatment with an agent capable of effecting cleavage of a base portion of the methylated nucleotide (in embodiments relating to labeling and/or fragmentation of a polynucleotide comprising a methylated nucleotide) or the site of the unmethylated nucleotide following treatment with an agent capable of effecting cleavage of a base portion of the methylated nucleotide (in embodiments relating to labeling and/or fragmentation of a polynucleotide comprising a methylated nucleotide, said methods comprising cleavage of a base portion of a unmethylated nucleotide. An abasic site (interchangeably termed "AP site") can comprise a hemiacetal ring and/or an aldehyde moiety, and lacks a base portion of the canonical nucleotide. As used herein, "abasic site" encompasses, in embodiments relating to labeling and/or fragmentation of a polynucleotide comprising a canonical nucleotide, any chemical structure remaining following treatment of a canonical nucleotide (present in a polynucleotide chain) with an agent (e.g., an enzyme, chemical reagent, heat, acidic conditions, and/or basic conditions) capable of effecting cleavage of a base portion of a canonical nucleotide, and, in embodiments relating to labeling and/or fragmentation of a polynucleotide comprising a methylated nucleotide, any chemical structure remaining following treatment of a methylated nucleotide (present in a polynucleotide chain) (or, in some embodiments, treatment of an unmethylated nucleotide) with an agent (e.g., an enzyme, chemical reagent, heat, acidic conditions, and/or basic conditions) capable of effecting cleavage of a base portion of a methylated nucleotide (or, in some embodiments, an unmethylated nucleotide). An abasic site may also occur in a natural polynucleotide, indicating damage to the polynucleotide.

[0075] As used herein, "labeling at an abasic site" means association of a label with, in embodiments relating to labeling and/or fragmentation of a polynucleotide comprising a canonical nucleotide, any chemical structure remaining following removal of a base portion (including the entire base) of a canonical nucleotide (present in a polynucleotide chain) by treatment with an agent (e.g., an enzyme, or heat)) capable of effecting cleavage of a base portion of a canonical nucleotide, or, in embodiments relating to labeling and/or fragmentation of a polynucleotide comprising a methylated nucleotide, treatment with an agent capable of effecting cleavage of a base portion of a methylated nucleotide (or, in some embodiments, an unmethylated nucleotide). In one embodiment, a reactive aldehyde form of a hemiacetal ring in an abasic site is labeled. In other embodiments involving cleavage of

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a base portion of a canonical nucleotide, the label associates with a chemical structure remaining following treatment of a canonical nucleotide (present in a polynucleotide chain) with an agent (e.g., an enzyme, or heat or basic conditions) capable of effecting cleavage of a base portion of a canonical nucleotide and treatment of polynucleotide comprising an abasic site with an agent capable of effecting cleavage of the backbone at the abasic site (as described herein), or, in embodiments involving cleavage of a base portion of a methylated nucleotide, the label associates with a chemical structure remaining following treatment of a methylated nucleotide (present in a polynucleotide chain) (in some embodiments, an unmethylated nucleotide) with an agent (e.g., an enzyme, or heat or basic conditions) capable of effecting cleavage of a base portion of a methylated nucleotide (in some embodiments, an unmethylated nucleotide) and treatment of polynucleotide comprising an abasic site with an agent capable of effecting cleavage of the backbone at the abasic site (as described herein).

[0076] As used herein, cleavage of a backbone (e.g. phosphodiester backbone) "at" an abasic site means cleavage of the phosphodiester linkage 3' to the abasic site or 5' to the abasic site, or both. As the disclosure herein conveys, "at" an abasic site refers to proximate or near location (such as immediately 3' or immediately 5'). In still other embodiments, more complex forms of cleavage are possible, for example, cleavage such that cleavage of the phosphodiester backbone and cleavage of (a portion of) the abasic site results.

[0077] As used herein, a "label" (interchangeably called a "detectable moiety") refers to a moiety that is associated or covalently linked with a polynucleotide (interchangeably called "labeling"). The labeled polynucleotide may be directly or indirectly detected, generally through a detectable signal. The detectable moiety (label) can be covalently attached (or associated) either directly or indirectly through a non-interfering linkage group with other moieties capable of specifically associating with one or more sites to be labeled. The detectable moiety (label) may be covalently linked or non-covalently associated as well as directly or indirectly associated.

Methods for labeling and/or fragmenting a polynucleotide comprising a methylated nucleotide

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[0078] The following are examples of the methods of the invention. It is understood that various other embodiments may be practiced, given the general description provided herein. For example, reference to using an agent capable of cleaving a base portion of the methylated nucleotide means that any of the agents capable of cleaving a base portion of the methylated nucleotide described herein may be used.

A. Methods for labeling and fragmenting a polynucleotide comprising a methylated nucleotide and analyzing DNA methylation

[0079] The invention provides methods for analyzing DNA methylation (including detecting and/or identifying methylated DNA sequences). Generally, the methods comprise use of an agent (such as an enzyme) that cleaves a base portion from a methylated nucleotide (such as 5-methylcytosine), whereby an abasic site is generated; cleavage of the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and labeling at the abasic site, whereby labeled polynucleotide fragments are generated. Generally, the polynucleotide comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated nucleotide) is fragmented and labeled at the abasic site (which is generated by cleavage of a base portion of the methylated nucleotide). Thus, the methods of the invention are useful for, e.g., detecting methylation (including presence and/or absence and/or quantity or level of methylation), identifying methylated polynucleotide sequences, isolating methylated polynucleotide sequences, identifying and/or isolating methylatable polynucleotide sequences, and other applications as described herein. The methods of the invention generate labeled polynucleotide fragments which are useful for, e.g., hybridization to a microarray and other uses described herein.

[0080] In one aspect, the methods involve the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an agent capable of cleaving a base portion of the methylated nucleotide (i.e., cleaving a base portion of the methylated nucleotide), whereby an abasic site is generated; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent

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capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated.

[0081] Generally, native methylation status is analyzed (i.e., methylation status or pattern as exists in vivo, or substantially similar to the in vivo status or pattern), wherein the polynucleotide is directly harvested and/or isolated from a biological sample (such as a tissue or cell culture), though other embodiments are contemplated (including embodiments comprising detection and/or identification of methylatable sequences) in which the polynucleotide is from any source (i.e., genomic DNA, or a source other than genomic DNA) and the polynucleotide is methylated prior to labeling and fragmenting as described above). Thus, methylation status may be the result of in vivo processes, or of experimental manipulations (e.g., deliberate exposure to a putative DNA damaging agent, or a putative demethylating agent). The methods of the invention (i.e., the methods comprising cleavage of a base portion of a methylated nucleotide; cleavage of the backbone at the abasic site and/or labeling at the abasic site) specifically exclude methods comprising synthesis of a polynucleotide comprising a non-canonical nucleotide as disclosed in co-pending co-owned U.S. Patent Application Serial No. 10/441,663 (publication no. 2004/0005614). Thus, the polynucleotide comprising a methylated nucleotide (or suspected of comprising a methylated nucleotide), as that term is used herein, does not encompass (specifically excludes) a polynucleotide comprising a non-canonical nucleotide, wherein the polynucleotide was generated by a method comprising synthesizing a polynucleotide comprising a non-canonical nucleotide from a template (i.e., such that the non-canonical nucleotide is incorporated during template-dependent synthesis of the polynucleotide comprising a non-canonical nucleotide.)

[0082] The polynucleotide to be analyzed for methylation status (e.g., the polynucleotide suspected of comprising a methylated nucleotide and/or the polynucleotide comprising a methylated nucleotide) may be any polynucleotide from which labeled polynucleotide fragments are desired to be produced, including double-stranded, partially double-stranded, and single-stranded nucleic acids from any source in purified or unpurified form, which can be DNA (dsDNA and ssDNA) or RNA, including tRNA, mRNA, rRNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, the genomes of biological material such as plants, animals,

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humans, and fragments thereof (including fragments comprising hypermethylated CpG islands, centromeric regions, and other hypermethylated regions), though other embodiments are contemplated, as described herein. The polynucleotide can be known or unknown and may contain more than one sequence of interest, each of which may be the same or different from each other. The polynucleotide can be a sub-population of nucleic acids, for example, a subtractive hybridization probe, total genomic DNA, or restriction fragments.

[0083] Obtaining and purifying nucleic acids use standard techniques in the art. Generally, the polynucleotide is DNA, though, as noted herein, the polynucleotide can comprise altered and/or modified nucleotides, internucleotide linkages, ribonucleotides, etc. As generally used herein, it is understood that "DNA" applies to polynucleotide embodiments. The polynucleotide suspected of comprising a methylated nucleotide can be processed or modified prior to analysis, for example, cleavage using restriction enzymes or other means known in the art, such as shearing, and using chemical or enzymatic methylation or demethylation as known in the art.

[0084] For simplicity, the polynucleotide comprising (or suspected of comprising) a methylated nucleotide is described as a single nucleic acid. It is understood that the polynucleotide is generally a population of polynucleotides (from a few to a multiplicity to a very large multiplicity of polynucleotides). It is further understood that a polynucleotide comprising a methylated nucleotide can be a multiplicity (from small to very large) of different polynucleotide molecules. Such populations can be related in sequence (e.g., member of a gene family or superfamily) or extremely diverse in sequence (such as all genomic DNA). Polynucleotides can also correspond to single sequence (which can be part or all of a known gene, for example a coding region, genomic portion, etc.). In some embodiments, the polynucleotide comprises a gene, a gene locus, a specific CpG dinucleotide, a CpG island, and/or a centromeric region.

[0085] For simplicity, individual steps of the labeling and fragmentation method are discussed herein. It is understood, however, that the steps may be performed simultaneously and/or in varied order, as discussed herein.

[0086] It is further understood that for convenience, methods involving cleavage of a base portion of a methylated nucleotide, and methods involving cleavage of a base portion of a non-methylated nucleotide (wherein the agent that cleaves a base portion of a

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nonmethylated nucleotide (interchangeably termed "unmethylated" nucleotide) is generally not capable of cleaving the base portion of a methylated nucleotide) (described below) are described separately. It is understood that the methods may be combined, performed separately, and/or performed sequentially.

1. Cleaving a base portion of a methylated nucleotide to create an abasic site

[0087] In aspects involving cleavage of a base portion of a methylated nucleotide, the polynucleotide comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated nucleotide) is treated with an agent, such as an enzyme, capable of generally, specifically, or selectively cleaving a base portion of the methylated deoxyribonucleoside to create an abasic site. As used herein, "abasic site" encompasses any chemical structure remaining following removal of a base portion (including the entire base) of a methylated nucleotide with an agent capable of cleaving a base portion of a methylated nucleotide, e.g., by treatment of a methylated nucleotide (present in a polynucleotide chain) with an agent (e.g., an enzyme) capable of effecting cleavage of a base portion of a methylated nucleotide. In some embodiments, the agent (such as an enzyme) catalyzes hydrolysis of the bond between the base portion of the methylated nucleotide and a sugar in the methylated nucleotide to generate an abasic site comprising a hemiacetal ring and lacking the base (interchangeably called "AP" site), though other cleavage products are contemplated for use in the methods of the invention. Generally, the methods involving cleavage of a base portion of a methylated nucleotide are suitable for use with polynucleotides comprising a low frequency of methylated nucleotides (i.e., generally, not hypermethylated regions, such a CpG islands and the like), though other uses are contemplated.

[0088] The polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide is treated with an agent, such as an enzyme, capable of generally, specifically, or selectively cleaving a base portion of the methylated deoxyribonucleoside to create an abasic site. As used herein, "abasic site" encompasses any chemical structure remaining following removal of a base portion (including the entire base) of a methylated nucleotide with an agent capable of cleaving a base portion of a methylated nucleotide, e.g., by treatment of a methylated nucleotide (present in a polynucleotide chain)

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with an agent (e.g., an enzyme, acidic conditions, or a chemical reagent) capable of effecting cleavage of a base portion of a methylated nucleotide. In some embodiments, the agent (such as an enzyme) catalyzes hydrolysis of the bond between the base portion of the methylated nucleotide and a sugar in the canonical nucleotide to generate an abasic site comprising a hemiacetal ring and lacking the base (interchangeably called "AP" site), though other cleavage products are contemplated for use in the methods of the invention.

[0089] In some embodiments, the methylated nucleotide is 5-methylcytosine. In some embodiments, 5-methylcytosine is present as a methylated CpG dinucleotide. The CpG dinucleotide may be fully or hemi-methylated. In other embodiments, the methylated nucleotide is 3-methyladenine. In other embodiments, the methylated nucleotide is 7-methyladenine and/or 3-methylguanine.

[0090] Suitable agents and reaction conditions for cleavage of base portions of methylated nucleotides are known in the art, and include: 5-methylcytosine DNA glycosylase (5-MCDG), which cleaves the base portion of 5-methylcytosine (5-MeC) from the DNA backbone (Wolffe et al., Proc. Nat. Acad. Sci. USA 96:5894-5896, 1999); 3-methyladenosine-DNA glycosylase I, which cleaves the base portion of 3-methyl adenosine from the DNA backbone (see, e.g. Hollis et al (2000) Mutation Res. 460: 201-210); and/or 3-methyladenosine DNA glycosylase II, which cleaves the base portion of 3-methyladenosine, 7-methylguanine, 7-methyladenosine, and/ 3-methylguanine from the DNA backbone. *See* McCarthy et al (1984) EMBO J. 3:545-550. Multifunctional and mono-functional forms of 5-MCDG have been described. *See* Zhu et al., Proc. Natl. Acad. Sci. USA 98:5031-6, 2001; Zhu et al., Nuc. Acid Res. 28:4157-4165, 2000; and Nedderrnann et al., J.B.C. 271:12767-74, 1996 (describing bifunctional 5-MCDG; Vairapandi & Duker, Oncogene 13:933-938, 1996; Vairapandi et al., J. Cell. Biochem. 79:249-260, 2000 (describing mono-functional enzyme comprising 5-MCDG activity). In some embodiments, 5-MCDG preferentially cleaves fully methylated polynucleotide sites (e.g., CpG dinucleotides), and in other embodiments, 5-MCDG preferentially cleaves a hemi-methylated polynucleotide. For example, mono-functional human 5-methylcytosine DNA glycosylase cleaves DNA specifically at fully methylated CpG sites, and is relatively inactive on hemimethylated DNA (Vairapandi & Duker, supra; Vairapandi et al., supra). By contrast, chick embryo 5-methylcytosine-DNA glycosylase has greater activity directed to

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hemimethylated methylation sites. In some embodiments, the activity of 5-MCDG is potentiated (increased or enhanced) with accessory factors, such as recombinant CpG-rich RNA, ATP, RNA helicase enzyme, and proliferating cell nuclear antigen (PCNA). See U.S. Patent Publication No. 20020197639 A1. One or more agents may be used. In some embodiments, the one or more agents cleave a base portion of the same methylated nucleotide. In other embodiments, the one or more agents cleave a base portion of different methylated nucleotides. Treatment with two or more agents may be sequential or simultaneous.

[0091] As is evident, in some embodiments, dUTP is generated as an intermediate and cleavage of a base portion of dUTP is necessary to generate the abasic site. Methods for cleaving a base portion of dUTP are known in the art. *See, e.g.*, Lindahl, PNAS (1974) 71(9):3649-3653; Jendrisak, U.S. Patent No. 6,190,865 B1; U.S. Patent No. 5,035,996; U.S. Patent No. 5,418,149; Sartori et al (2002) EMBO J 21:3182-3191. Thus, in some embodiments, an agent that cleaves a base portion of a methylated nucleotides (such as an enzyme, such as 5-MCDG) is used in conjunction with UNG to generate an abasic site from the methylated nucleotide. As used herein, "in conjunction" encompasses simultaneous treatment (e.g., when 5-MCDG and UNG cleavage occurs in the same reaction mixture) and/or treatment at different times (e.g., when 5-MCDG and UNG treatment is conducted sequentially).

[0092] In some embodiments, the agent that cleaves the base portion of the methylated nucleotide is the same agent that cleaves a phosphodiester backbone at the abasic site.

[0093] In some embodiments, cleavage of the base portion of the methylated nucleotides is general, specific or selective cleavage (in the sense that the agent (such as an enzyme) capable of cleaving a base portion of a methylated nucleotide generally, specifically or selectively cleaves the base portion of a particular methylated nucleotide), whereby about any of 98%, 95%, 90%, 85%, or 80% of the base portions cleaved are base portions of methylated nucleotides. However, extent of cleavage can be less. Thus, reference to specific cleavage is exemplary.

[0094] As noted herein, for convenience, cleavage of a base portion of a methylated nucleotide (whereby an abasic site is generated) has been described as a separate step. It is

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understood that this step may be performed simultaneously with cleavage of the backbone at an abasic site (fragmentation) and/or labeling at an abasic site.

[0095] It is understood that the frequency (or spacing) of abasic sites in the resulting polynucleotide comprising an abasic site (following cleavage of a base portion of a methylated nucleotide, and thus the average size of fragments generated using the methods of the invention (i.e., following cleavage of a phosphodiester backbone at an abasic site), is controlled by variables known in the art, including: frequency of methylated nucleotide(s) in the polynucleotide (or other measures of nucleotide content of a sequence, such as average G-C content), length of the polynucleotide comprising a methylated nucleotide, and the reaction conditions used during generation of abasic site, as is further discussed herein. In some embodiments, polynucleotide are additionally cleaved using other means (e.g., restriction digestion, mechanical cleavage) before or after cleavage and labeling at an abasic site using the methods of the invention.

2. Cleaving the backbone at the abasic site of the polynucleotide comprising an abasic site and labeling at the abasic site

[0096] The backbone of the polynucleotide is cleaved at the abasic site, and the abasic site is labeled, whereby labeled polynucleotide fragments are generated. It is understood that cleavage of the backbone and labeling can be performed in any order, or simultaneously. For convenience, however, these reactions are described as separate steps.

i. Cleaving the backbone at the abasic site of the polynucleotide comprising an abasic site

[0097] Following generation of an abasic site, the backbone of the polynucleotide is cleaved at the abasic site (i.e., the site generated following cleavage of the base portion of the methylated nucleotide) with an agent capable of effecting cleavage of the backbone at the abasic site. Cleavage at the backbone (also termed "fragmentation") results in at least two fragments (depending on the number of abasic sites present in the polynucleotide comprising an abasic site, and the extent of cleavage).

[0098] Suitable agents (for example, an enzyme, a chemical and/or reaction conditions such as heat) capable of cleavage of the backbone at an abasic site are well known in the art, and include: heat treatment and/or chemical treatment (including basic

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conditions, acidic conditions, alkylating conditions, or amine mediated cleavage of abasic sites, (see e.g., co-pending co-owned U.S. Patent Application Serial No. 10/441,663; McHugh and Knowland, Nucl. Acids Res. (1995) 23(10):1664-1670; Bioorgan. Med. Chem (1991) 7:2351; Sugiyama, Chem. Res. Toxicol. (1994) 7: 673-83; Horn, Nucl. Acids. Res., (1988) 16:11559-71), and use of enzymes that catalyze cleavage of polynucleotides at abasic sites, for example AP endonucleases (also called "apurinic, apyrimidinic endonucleases") (e.g., *E. coli* Endonuclease IV, available from Epicentre Tech., Inc, Madison WI), *E. coli* endonuclease III or endonuclease IV, *E. coli* exonuclease III in the presence of calcium ions. See, e.g. co-pending co-owned U.S. Patent Application Serial No. 10/441,663; Lindahl, PNAS (1974) 71(9):3649-3653; Jendrisak, U.S. Patent No. 6,190,865 B1; Shida, Nucleic Acids Res. (1996) 24(22):4572-76; Srivastava, J. Biol Chem. (1998) 273(13):21203-209; Carey, Biochem. (1999) 38:16553-60; Chem Res Toxicol (1994) 7:673-683. As used herein "agent" encompasses reaction conditions such as heat. In one embodiment, the AP endonuclease, *E. coli* endonuclease IV, is used to cleave the phosphodiester backbone at an abasic site. In another embodiment, cleavage is with an amine, such as N, N'-dimethylethylenediamine. See, e.g. McHugh and Knowland, *supra*.

[0099] Generally, cleavage is between the nucleotide immediately 5' to the abasic residue and the abasic residue, or between the nucleotide immediately 3' to the abasic residue and the abasic residue (though, as explained herein, 5' or 3' cleavage of the phosphodiester backbone may or may not result in retention of the phosphate group 5' or 3' to the abasic site, respectively, depending on the fragmentation agent used). As is well known in the art, cleavage can be 5' to the abasic site (such as endonuclease IV treatment which generally results in cleavage of the backbone at a location immediately 5' to the abasic site between the 5'-phosphate group of the abasic residue and the deoxyribose ring of the adjacent nucleotide, generating a free 3' hydroxyl group on the adjacent nucleotide), such that an abasic site is located at the 5' end of the resulting fragment. Cleavage can also be 3' to the abasic site (e.g., cleavage between the deoxyribose ring and 3' -phosphate group of the abasic residue and the deoxyribose ring of the adjacent nucleotide, generating a free 5' phosphate group on the deoxyribose ring of the adjacent nucleotide), such that an abasic site is located at the 3' end of the resulting fragment. Treatment under basic conditions or with amines (such as N, N'-dimethylethylenediamine) results in cleavage of the phosphodiester

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backbone immediately 3' to the abasic site. In addition, more complex forms of cleavage are also possible, for example, cleavage such that cleavage of the phosphodiester backbone and cleavage of (a portion of) the abasic site results. For example, under certain conditions, cleavage using chemical treatment and/or thermal treatment may comprise a β-elimination step which results in cleavage of a bond between the abasic site deoxyribose ring and its 3' phosphate, generating a reactive α,β-unsaturated aldehyde which can be labeled or can undergo further cleavage and cyclization reactions. See, e.g. Sugiyama, Chem. Res. Toxicol. (1994) 7: 673-83; Horn, Nucl. Acids. Res., (1988) 16:11559-71. It is understood that more than one method of cleavage can be used, including two or more different methods which result in multiple, different types of cleavage products (e.g., fragments comprising an abasic site at the 3' end, and fragments comprising an abasic site at the 5' end).

[00100] Generally, cleavage of the backbone at an abasic site is general, specific or selective cleavage (in the sense that the agent (such as an enzyme) capable of cleaving the backbone at an abasic site specifically or selectively cleaves the backbone at an abasic site), whereby greater than about 98%, about 95%, about 90%, about 85%, or about 80% of the cleavage is at an abasic site. However, extent of cleavage can be less. Thus, reference to specific cleavage is exemplary. In some embodiments, specific or selective cleavage is desirable for control of the fragment size in the methods of generating labeled polynucleotide fragments of the invention. In some embodiments, reaction conditions can be selected such that the cleavage reaction is performed in the presence of a large excess of reagents and allowed to run to completion. In other embodiments, extent of cleavage can be less, such that polynucleotide fragments are generated comprising an abasic site at an end and an abasic site(s) within or internal to the polynucleotide fragment (i.e., not at an end). As disclosed herein, polynucleotide fragments comprising internal abasic sites are useful e.g., in embodiments involving immobilization of a labeled polynucleotide (wherein one abasic site is used for immobilization and another abasic site(s) are labeled at an abasic site).

[00101] As noted herein, the approximate or average size of the fragments (following cleavage of an abasic site, and cleavage of the backbone at the abasic site as described herein) is controlled by variables known in the art, including: frequency of cleavable nucleotides in the polynucleotide (in some embodiments involving methylation analysis, frequency of methylated nucleotides), or other measures of nucleotide content of a sequence,

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such as average G-C content), length of the polynucleotide, and the reaction conditions used during generation of abasic site and cleavage of the backbone at the abasic site. In some embodiments, polynucleotide are additionally cleaved using other means (e.g., restriction digestion, mechanical cleavage) before or after cleavage and labeling at an abasic site using the methods of the invention. Generally, suitable fragment sizes are about 5, 10, 15, 20, 25, 30, 40, 50, 65, 75, 85, 100, 123, 150, 175, 200, 225, 250, 300, 350, 400, 450, 500, 550, 600, 650 or more, such as 1kb, 2kb, 3kb, 4kb, 5kb or more nucleotides in length. In some embodiments, the fragment is about 200 nucleotides, about 100 nucleotides, or about 50 nucleotides in length. In another embodiment, the size of a population of fragments is about 50 to 200 nucleotides. It is understood that the fragment size may be approximate, particularly when populations of fragments are generated, because the frequency and spacing of abasic sites (which relates to the fragment size following cleavage) will vary from template to template and also between copies of the same template, due to representation of the methylated nucleotide, reactions conditions selected for generation of abasic sites, and reaction conditions selected for fragmentation. Thus, in some embodiments, fragments generated from same starting material (such as a single polynucleotide template) may have different (and/or overlapping) sequence, while still having the same approximate size or size range.

[00102] Following cleavage of the polynucleotide backbone at the abasic site, every fragment will comprise one abasic site (if cleavage is completely efficient), except for either the 5'- or 3'-most fragment, which may lack an abasic site depending on the cleavage agent. If the cleavage is 5' to the abasic site, the 5' most fragment will not comprise an abasic site. If cleavage is 3' to the abasic site, the 3' most fragment will not comprise an abasic site.

[00103] It is understood that the preceding disclosure regarding cleavage of the backbone at the abasic site is applicable to embodiments involving cleavage of the backbone at the abasic site wherein the abasic site was generated by cleavage of a cleavable canonical nucleotide with an agent capable of cleaving a base portion of a canonical nucleotide, as described infra.

ii. Labeling the abasic site and detection

[00104] The abasic site is labeled, whereby a polynucleotide (or polynucleotide fragment) comprising a label is generated. In some embodiments, a polynucleotide fragment

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comprising an abasic site is contacted with an agent capable of labeling at the abasic site; whereby labeled fragments of the polynucleotide are generated. As used herein, a "label" (interchangeably called a "detectable moiety") is attached to or associated with a polynucleotide, such that the polynucleotide comprising an abasic site is attached to or associated with a label.

[00105] Thus, in some embodiments, the label attaches to or associates with a chemical structure remaining following treatment of a methylated nucleotide (present in a polynucleotide chain) with an agent (e.g., an enzyme, or acidic conditions, or a chemical reagent) capable of effecting cleavage of a base portion of the methylated nucleotide. In embodiments involving fragmentation, the label attaches to or associates with any chemical structure remaining following treatment of the methylated nucleotide (present in a polynucleotide chain) with an agent (e.g., an enzyme, or acidic conditions, or a chemical reagent) capable of effecting cleavage of a base portion of the methylated nucleotide (in embodiments involving cleavage of a methylated nucleotide), and following treatment with an agent capable of cleaving the backbone at the abasic site. In one embodiment, the label covalently bonds with a reactive aldehyde form of a hemiacetal ring in an abasic site. In some embodiments, labeling "at" an abasic site encompasses labels that bind to an abasic site, but do not bind to the intact (uncleaved) methylated nucleotide (in embodiments involving cleavage of a methylated nucleotide) whether incorporated or present as a single methylated nucleotide). In some embodiments, labeling "at" an abasic site specifically excludes labels that attach (e.g., covalently bind) to a phosphate group of a nucleotide (or polynucleotide) or a phosphate group of an abasic site. In some embodiments, labeling "at" an abasic site specifically excludes labels that attach or associate at the 3' position of the sugar. In still other embodiments, labeling "at" an abasic site specifically excludes a label comprised of a phosphine. As made clear from the disclosure herein, "label" refers to any component of a labeling system.

[00106] It is understood that cleavage of the phosphodiester backbone at the abasic site, and labeling at an abasic site can be performed in any order, or simultaneously.

[00107] The label can be detectable, or the label can be indirectly detected, for example as when the label (attached at an abasic residue) is covalently or non-covalently associated with another moiety which is itself detected. For example, biotin can be attached

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to the label capable of associating with the abasic site. In another example, an antibody (that can be detectably labeled) binds the label that is attached at the abasic site. In some embodiments, the label comprises an organic molecule, a hapten, or a particle (such as a polystyrene bead). In some embodiments, the label is detected using antibody binding, biotin binding, or via fluorescence or enzyme activity. In some embodiments, the detectable signal is amplified.

[0100] Generally, labeling at an abasic site is general, specific, or selective labeling (in the sense that the agent capable of labeling at an abasic site specifically or selectively labels the abasic site), whereby greater than about 98%, about 95%, about 93%, about 90%, about 85%, or about 80% of the labels bind abasic sites. However, extent of labeling can be less. Thus, reference to specific labeling is exemplary. In some embodiments, reaction conditions are selected such that the reaction in which the abasic site(s) are labeled can run to completion.

[0101] In some embodiments, labeled polynucleotide fragments are produced which each comprise a single label (to the extent that cleavage of the phosphodiester backbone is generally complete, in the sense that many or essentially all of the polynucleotide fragments comprise a single abasic site). This aspect is useful in quantitating level of hybridization, because signal is proportional to number of bound fragments, and does not relate to the length of the hybridizing fragment or the number of labels per fragment. Thus, hybridization intensity can generally be directly compared, regardless of fragment length. In another embodiment, labeled fragments are produced which comprise a labeled abasic site at an end (such as the 3' end and/or the 5' end) and a labeled internal abasic site.

[0102] Methods and reaction conditions for labeling abasic sites are known in the art. See, e.g., co-pending co-owned U.S. Patent Application Serial No. 10/441,663. For example, a common functional group exposed in an abasic site (and therefore suitable for use in labeling) is the highly reactive aldehyde form of the hemiacetal ring which can be covalently attached to or noncovalently associated with a label using reaction conditions that are known in the art. Many labels comprise substituted hydrazines or hydroxylamines which readily form imine bonds with aldehydes, for example, 5-(((2-(carbohydrazino)-methyl)thio)acetyl)aminofluorescein, aminooxyacetyl hydrazide (FARP). See Makrigiorgos, PCT Publication No. WO 00/39345. The stable oxime formed by this

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compound can be detected directly by fluorescence or the signal can be amplified using an antibody-enzyme conjugate. See, e.g., Srivastava, J. Biol. Chem. (1998) 273(33): 21203-209; Makrigiorgos, Int J. Radiat. Biol. (1998) 74(1):99-109; Makrigiorgos, U.S. Patent No. 6,174,680 B1; Makrigiorgos, WO 00/39345. Suitable sidechains (present on the substrate) to react with the aldehyde (of the abasic site) include at least the following: substituted hydrazines, hydrazides, or hydroxylamines (which readily form imine bonds with aldehydes), and the related semicarbazide and thiosemicarbazide groups, and other amines which can form stable carbon-nitrogen double bonds, that can catalyze simultaneous cleavage and binding (see Horn, Nucl. Acids. Res., (1988) 16:11559-71), or can be coupled to form stable conjugates, e.g. by reductive amination. Other methods for attaching a reactive group present in an abasic site to a reactive group present on a label are known in the art. In another example, the abasic site may be chemically modified, then the modified abasic site covalently attached to or non-covalently associated with a suitable reactive group on a substrate. For example, the aldehyde (in the abasic site) can be oxidized or reduced (using methods known in the art), then covalently immobilized to a substrate using, e.g., reductive amination or various oxidative processes.

[0103] Other suitable reagents are known in the art, e.g., fluorescein aldehyde reagents. See, e.g., Boturyn (1999) Chem. Res. Toxicol. 12:476-482. See, also, Adamczyk (1998) Bioorg.Med. Chem. Lett. 8(24):3599-3602; Adamczyk (1999) Org. Lett. 1(5):779-781; Kow (2000) Methods 22(2):164-169; Molecular Probes Handbook, Section 3.2 (www.probes.com). For example, detectable moieties comprising aminooxy groups can be used. See, Boturyn, *supra*. The aminoooxy group readily reacts with the highly reactive aldehyde form of the hemiacetal ring of an abasic site. In one embodiment, the label comprising an aminooxy reactive group is N-(aminooxyacetyl)-N'-(D-biotinoyl) hydrazine, trifluoroacetic acid salt (ARP) (available from Molecular Probes, Eugene OR, catalog No. A-10550). See, e.g., Kubo et al., Biochem 31:3703-3708 (1992); Ide et al., Biochem. 32:8276-8283 (1993).

[0104] In yet another example, labels comprising a hydrazide linker can be converted to an aminooxy (interchangeably termed "hydroxylamine") derivative, then used to label abasic sites as described herein. In one embodiment, the label comprises an aminooxy derivatized Alexa Fluor 555 reagent as disclosed in co-pending U.S. Patent

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Application Serial No. 10/441,663, Use of the aminooxy-derivatized Alexa Fluor 555 resulted in greater labeling efficiency, as well as increased fluorescence as compared to labeling with unmodified Alexa Fluor 555 hydrazide (Order No. A-20501, Molecule Probes, Eugene OR).

[0105] In another example, the abasic site may be chemically modified (before, during or after cleavage of the phosphodiester backbone as described herein), then the modified abasic site detected directly or indirectly. For example, fluorescent cadaverine can be incorporated into an abasic site as described in Horn (Nucl. Acids. Res., (1988) 16:11559-71). In another example, the abasic site may be chemically modified by reaction with NHBA (0-4-nitrobenzyl hydroxylamine), then the NBHA-modified abasic site is detected with an antibody that specifically binds to the NBHA-modified abasic sites. See Kow et al, PCT Publication No. WO 92/07951 (1992).

[0106] In another example, the abasic site may be labeled with an antibody (such as a monoclonal or polyclonal antibody or antigen binding fragment). Methods for detecting specific antibody binding are well known in the art.

[0107] In another example, the aldehyde and/or hemiacetal ring may itself be detected, as when, for example, detectable signal is generated using chemical or electrochemical reactions specific to those chemical structures, including for example, oxidation reactions, enzymes with dehydrogenase or oxidase activity, and the like. In another example, many aldehydes are substrates for enzymes, such that a detectable product is generated in the presence of the aldehyde. For example, dehydrogenases typically couple oxidation of an aldehyde with reduction of NAD+ which can be detected spectrophotometrically. In another example, glucose oxidases generate hydrogen peroxide in the presence of sugar aldehydes. Hydrogen peroxide is readily detectable by coupling to horseradish peroxidase with suitable substrates. Thus, the invention provides methods for detecting an abasic site.

[0108] Methods of signal detection are known in the art. Signal detection may be visual or utilize a suitable instrument appropriate to the particular label used, such as a spectrometer, fluorimeter, or microscope. For example, where the label is a radioisotope, detection can be achieved using, for example, a scintillation counter, or photographic film as in autoradiography. Where a fluorescent label is used, detection may be by exciting the

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fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence, such as by microscopy, visual inspection or photographic film, fluorometer, CCD cameras, scanner and the like. Where enzymatic labels are used, detection may be by providing appropriate substrates for the enzyme and detecting the resulting reaction product. For example, many substrates of horseradish peroxidase, such as o-phenylenediamine, give colored products. Simple colorimetric labels can usually be detected by visual observation of the color associated with the label; for example, conjugated colloidal gold is often pink to reddish, and beads appear the color of the bead. Instruments suitable for high sensitivity detection are known in the art.

[0109] It is understood that the polynucleotide or polynucleotide fragments can be additionally labeled using other methods known in the art.

[0110] Labeled polynucleotide fragments can be immobilized to a substrate, as described herein.

[0111] It is understood that the preceding disclosure regarding labeling at the abasic site is applicable to embodiments involving labeling at the abasic site wherein the abasic site was generated by cleavage of a cleavable canonical nucleotide with an agent capable of cleaving a base portion of a canonical nucleotide, as described infra.

B. Methods for labeling a polynucleotide comprising a methylated nucleotide

[0112] The invention provides methods for generating labeled nucleic acid(s).

[0113] Generally, the methods comprise use of an agent (such as an enzyme) that cleaves a base portion from a methylated nucleotide (such as 5-methylcytosine), whereby an abasic site is generated; and labeling at the abasic site, whereby labeled polynucleotides are generated. Generally, the polynucleotide comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated nucleotide) is labeled at the abasic site (which is generated by cleavage of a base portion of the methylated nucleotide). The methods of the invention generate labeled polynucleotides which are useful for, e.g., hybridization to a microarray and other uses described herein. The methods of the invention are suitable for multiplexing.

[0114] The methods involve the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with

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an agent capable of cleaving a base portion of the methylated nucleotide (i.e., cleaving a base portion of the methylated nucleotide), whereby an abasic site is generated; and (b) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotides are generated.

[0115] It is understood that labeled polynucleotides may be cleaved using other means (i.e. other means than cleavage at an abasic site as described herein, e.g., restriction digestion, mechanical cleavage) before or after labeling at an abasic site using the methods of the invention. In some embodiments, the methods involving labeling of a polynucleotide comprising a methylated nucleotide are suitable for multiplex analysis involving hybridization to an array.

1. Cleaving a base portion of a methylated nucleotide to create an abasic site

[0116] The polynucleotide comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated nucleotide) is treated with an agent, such as an enzyme, capable of generally, specifically, or selectively cleaving a base portion of the methylated deoxyribonucleoside to create an abasic site, as described herein.

2. Labeling the abasic site and detection

[0117] The abasic site is labeled, whereby a polynucleotide comprising a label is generated, as described herein.

C. Methods for fragmenting a polynucleotide comprising a methylated nucleotide

[0118] The methods involve the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an agent capable of cleaving a base portion of the methylated nucleotide (i.e., cleaving a base portion of the methylated nucleotide), whereby an abasic site is generated; and (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site, whereby polynucleotide fragments are generated.

1. Cleaving a base portion of a methylated nucleotide to create an abasic site

[0119] The polynucleotide comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated nucleotide) is treated with an agent, such as an enzyme, capable of generally, specifically, or selectively cleaving a base portion of the methylated deoxyribonucleoside to create an abasic site, as described herein.

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2. Cleaving the backbone at the abasic site of the polynucleotide comprising an abasic site and labeling at the abasic site

[0120] The backbone of the polynucleotide is cleaved at the abasic site, and the abasic site is labeled, whereby polynucleotide fragments are generated, as described herein.

D. Methods for labeling and fragmenting a polynucleotide comprising a methylated nucleotide using an enzyme that cleaves a base portion of a nonmethylated nucleotide

[0121] In another aspect, the invention comprises use of an enzyme (such as cytosine deaminase in conjunction with uracil N deglycosylase (UNG)) that cleaves a base portion from an unmethylated nucleotide, whereby an abasic site is generated, wherein the enzyme is not capable of cleaving a methylated nucleotide; cleavage of the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and labeling at the abasic site, whereby labeled polynucleotide fragments are generated. Generally, the polynucleotide comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated nucleotide) is fragmented and labeled at the abasic site (which is generated by cleavage of a base portion of the non-methylated nucleotide). Thus, the methods of the invention are useful for, e.g., detecting methylation (including presence and/or absence and/or quantity or level of methylation), identifying methylated polynucleotide sequences, isolating methylated polynucleotide sequences, identifying and/or isolating methylatable polynucleotide sequences, and other applications as described herein. The methods of the invention generate labeled polynucleotide fragments which are useful for, e.g., hybridization to a microarray and other uses described herein.

[0122] The methods comprise the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an enzyme capable of cleaving a base portion of an unmethylated nucleotide (i.e., cleaving a base portion of the unmethylated nucleotide), whereby an abasic site is generated, wherein the enzyme is not capable of cleaving a methylated nucleotide; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated. In

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some embodiments, the enzyme is cytosine deaminase in conjunction with uracil DNA glycosylase.

1. Cleaving a base portion of an unmethylated nucleotide

[0123] In another aspect, the invention comprises use of an enzyme (such as cytosine deaminase in conjunction with uracil DNA glycosylase) that cleaves a base portion of an unmethylated nucleotide, wherein the enzyme is not capable of cleaving a methylated nucleotide; cleavage of the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and labeling at the abasic site, whereby labeled polynucleotide fragments are generated.

[0124] Generally, the methods involve the following steps: (a) contacting a polynucleotide comprising (in some embodiments, suspected of comprising) a methylated nucleotide with an agent (such as an enzyme) capable of cleaving a base portion of an unmethylated nucleotide (i.e., cleaving a base portion of the unmethylated nucleotide), whereby an abasic site is generated, wherein the agent (such as an enzyme) is not capable of cleaving the methylated nucleotide; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated.

[0125] In some embodiments, the enzyme is cytosine deaminase. See Sohail et al, NAR 2003, 31: 2990-94. Cytosine deaminase catalyzes the deamination of cytosine, such that dUTP is generated. Cleavage of a base portion of dUTP is necessary to generate the abasic site. Thus, the invention encompasses use of (a) an agent (such as cytosine deaminase) that modifies a nucleotide (such as dCTP), whereby dUTP is generated, in conjunction with (b) an agent (such as an enzyme, such as UNG) that cleaves a base portion of dUTP, whereby an abasic site is generated. Methods for cleaving a base portion of dUTP are known in the art. See, e.g., Lindahl, PNAS (1974) 71(9):3649-3653; Jendrisak, U.S. Patent No. 6,190,865 B1; U.S. Patent No. 5,035,996; U.S. Patent No. 5,418,149; Sartori et al (2002) EMBO J 21:3182-3191. As used herein, "in conjunction" encompasses simultaneous treatment (e.g., when cytosine deaminase and UNG cleavage occurs in the

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same reaction mixture) and/or treatment at different times (e.g., when cytosine deaminase and UNG treatment is conducted sequentially).

[0126] In some embodiments, the agent that cleaves the base portion of the unmethylated nucleotide is the same agent that cleaves a phosphodiester backbone at the abasic site.

[0127] Generally, cleavage of the base portion of the unmethylated nucleotides is general, specific or selective cleavage (in the sense that the agent (such as an enzyme) capable of cleaving a base portion of an unmethylated nucleotide generally, specifically or selectively cleaves the base portion of a particular unmethylated nucleotide), and generally, specifically and selectively does not cleave the base portion of the methylated nucleotide, whereby about any of 98%, 95%, 90%, 85%, or 80% of the base portions cleaved are base portions of unmethylated nucleotides. However, extent of cleavage can be less. Thus, reference to specific cleavage is exemplary. In some embodiments, the methylated nucleotide is 5-methylcytosine and the unmethylated nucleotide is cytosine.

[0128] As noted herein, for convenience, cleavage of a base portion of a unmethylated nucleotide (whereby an abasic site is generated) has been described as a separate step. It is understood that this step may be performed simultaneously with cleavage of the backbone at an abasic site (fragmentation) and/or labeling at an abasic site.

[0129] It is understood that the frequency (or spacing) of abasic sites in the resulting polynucleotide comprising an abasic site (following cleavage of a base portion of a unmethylated nucleotide, and thus the average size of fragments generated using the methods of the invention (i.e., following cleavage of a phosphodiester backbone at an abasic site), is controlled by variables known in the art, including: frequency of unmethylated nucleotide(s) in the polynucleotide (or other measures of nucleotide content of a sequence, such as average G-C content), length of the polynucleotide comprising a unmethylated nucleotide, and the reaction conditions used during generation of abasic site, as is further discussed herein (such as presence of a methylation binding protein, and/or presence of a DNA binding protein). In some embodiments, polynucleotide are additionally cleaved using other means (e.g., restriction digestion, mechanical cleavage) before or after cleavage and labeling at an abasic site using the methods of the invention.

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[0130] It is understood that the methods involving cleavage of a base portion of an unmethylated nucleotide relate to and comprise use of a polynucleotide with native methylation status, i.e., a polynucleotide in which methylation status or pattern exists in vivo, as described herein. Specifically excluded are labeling and/or fragmentation methods comprising synthesis of a polynucleotide comprising a non-canonical nucleotide as disclosed in co-pending co-owned U.S. Patent Application Serial No. 10/441,663 (publication no. 2004/0005614).

[0131] Generally, the methods involving cleavage of a nonmethylated nucleotide (but not cleavage of the corresponding methylated nucleotide) are suitable for use with polynucleotide templates that are heavily methylated (hypermethylated), though other polynucleotide templates are suitable for use in the methods of the invention. Methods involving cleavage in the presence of methyl binding proteins and/or DNA binding proteins are further described herein.

[0132] In some embodiments, the polynucleotide comprising methylated nucleotide is contacted with a methyl binding agent, such as a methyl binding antibody, and/or a methyl binding protein, prior to and/or during cleavage of the base portion of the unmethylated nucleotide. Binding with a methyl binding agent protects the portion of the polynucleotide bound from labeling and fragmentation (including cleavage of a nonmethylated nucleotide). For example, when using an enzyme for cleavage of the base, binding of the enzyme to a methylated nucleotide base or activity of the enzyme towards a methylated nucleotide, may be prevented by binding to the methyl binding agent. Thus, the methods generally produce polynucleotide fragments comprising a methylated nucleotide that are suitable for further analysis, such as by hybridization to a microarray. The methods are generally suitable, e.g., for the analysis of hypermethylated samples, where methods involving cleavage at a methylated nucleotide may result in excessive fragmentation. In some embodiments, the methods are useful for selectively fragmenting a non-hypermethylated sequence in the polynucleotide of interest, such that hypermethylated sequences may be isolated from the reaction mixture and further analyzed (including sequencing and/or cloning and/or hybridization to a microarray).

[0133] Antibodies that bind methylated nucleotides are known in the art. See, e.g., U.S. Patent Publication No. 2002/0197639; Erlanger and Beiser (PNAS, 52:68, 1964); Sano

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et al., (Biochemica et Biophysica Acta, 951:157, 1988); PCT Publication No. WO 99/10540 published on Mar. 4, 1999; Mizugaki et al (1996) Biol Pharm. Bull. 19:1537-40 (describing antibodies that recognize 5-methylcytosine) and Tohuku J. Exp Med. 1986, 149(2):151-161. Methyl binding proteins are also known in the art. See, e.g, Ballestar et al (2001) Eur J Biochem. 268: 1-6. As is well known in the art, a methyl binding protein (also termed methylation binding protein or MBP) encompasses proteins that bind methylated nucleotides as well as proteins that bind a region including a methylated nucleotide.

[0134] In other embodiments, the polynucleotide comprising a methylated nucleotide is contacted with a polynucleotide (e.g. DNA) binding protein, such as a transcription factor, regulatory protein, and other DNA binding proteins, prior to cleavage of the base portion of the unmethylated nucleotide. The polynucleotide binding protein, e.g. a transcription factor, protects the bound portion of the polynucleotide from cleavage, thus generating a labeled and fragmented polynucleotide comprising the binding site of the particular DNA binding protein.

[0135] In some embodiments, methylation status may be compared in the presence or absence or methylation binding agents and/or other polynucleotide binding proteins. As further described herein, comparison of methylation status is useful to compare samples from, e.g., different stages of development, and/or different disease states (such as a control (normal) sample and a sample from diseased tissue).

2. Cleaving the backbone at the abasic site of the polynucleotide comprising an abasic site

[0136] Following generation of an abasic site, the backbone of the polynucleotide is cleaved at the abasic site (i.e., the site generated following cleavage of the base portion of the canonical nucleotide) with an agent capable of effecting cleavage of the backbone at the abasic site, as described herein.

3. Labeling the abasic site and detection

[0137] The abasic site is labeled, whereby a polynucleotide (or polynucleotide fragment) comprising a label is generated as described herein. In some embodiments, a polynucleotide fragment comprising an abasic site is contacted with an agent capable of

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labeling at the abasic site; whereby labeled fragments of the polynucleotide are generated as described herein.

E. Applications using the labeling and/or fragmentation methods for analysis of DNA methylation

[0138] The methods and compositions of the invention can be used for a variety of purposes. For purposes of illustration, methods of producing a hybridization probe or target characterizing and/or quantitating nucleic acid, preparing a subtractive hybridization probe, detection (using the hybridization probe), and determining methylation status, using the labeled and/or fragmented nucleic acids generated by the methods of the invention, are described.

1. Methods for characterizing methylated polynucleotides

[0139] The labeled and/or fragmented nucleic acids obtained by the methods of the invention are amenable to further characterization.

[0140] The labeled and/or fragmented nucleic acids (i.e., products of any of the methods for labeling and/or fragmenting a polynucleotide comprising a methylated nucleotide described herein), can be analyzed using, for example, probe hybridization techniques known in the art, such as Southern and Northern blotting, and hybridizing to probe arrays. They can also be analyzed by electrophoresis-based methods, such as differential display and size characterization, which are known in the art, e.g., capillary electrophoresis and gel electrophoresis using a sequencing gel.

[0141] In one embodiment, the methods of the invention are utilized to generate labeled and/or fragmented nucleic acids, and analyze the labeled and/or fragmented nucleic acids by contact with a probe.

[0142] In one embodiment, the methods of the invention are utilized to generate labeled and/or fragmented nucleic acids which are analyzed (for example, detection and/or quantification) by contacting them with, for example, microarrays (of any suitable substrate, which includes glass, chips, plastic), beads, or particles, that comprise suitable probes such as cDNA and/or oligonucleotide probes. Thus, the invention provides methods to characterize (for example, detect and/or quantify and/or identify) a labeled and/or

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fragmented nucleic acid by analyzing the labeled products, for example, by hybridization of the labeled products to, for example, probes immobilized at, for example, specific locations on a solid or semi-solid substrate, probes immobilized on defined particles (including beads, such as Bead Array, Illumina), or probes immobilized on blots (such as a membrane), for example arrays, or arrays of arrays. Immobilized probes include immobilized probes generated by the methods described herein, and also include at least the following microarrays: cDNA and synthetic oligonucleotides, which can be synthesized directly on the substrate. In some embodiments, the microarray is an Affymetrix Gene Chip array, an Agilent oligonucleotide array, or Amersham CodeLink array, or other high density or low density oligonucleotide or cDNA array, including genome or focused arrays. The identity of the probes provides characterization of the sequence identity of the products, and thus by extrapolation can also provide characterization of the identity of the polynucleotide comprising a methylated nucleotide (termed "template") from which the products were prepared. Thus, it is evident that polynucleotides comprising a methylated nucleotide may be identified using the methods of the invention.

[0143] Other methods of analyzing labeled products are known in the art, such as, for example, by contacting them with a solution comprising probes, followed by extraction of complexes comprising the labeled products and probes from solution. The identity of the probes provides characterization of the sequence identity of the products, and thus by extrapolation can also provide characterization of the identity of a template from which the products were prepared. In addition, hybridization of the labeled products is detectable, and the amount of labels that are detected is proportional to the amount of the labeled products prepared from a specific polynucleotide comprising a methylated nucleotide. This measurement is useful for, for example, measuring the relative amount (quantity, extent) of methylated species in a sample. The amount of labeled products (as indicated by, for example, detectable signal associated with the label) hybridized at defined locations on an array is indicative of the detection and/or quantification of the corresponding methylated polynucleotide species in the sample.

[0144] In another aspect, the invention provides methods for detecting methylated polynucleotides, including detecting presence or absence or quantity (level and/or extent) of methylation in a polynucleotide sample (which may comprise one or a multiplicity, a large

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multiplicity or a very large multiplicity of polynucleotides comprising a methylated nucleotide (in some embodiments, suspected of comprising a methylated polynucleotide), including essentially all genomic DNA. In some embodiments, presence or absence or quantity of methylation is determined by hybridizing the labeled and/or fragmented polynucleotides (generated using any of the methods for labeling and/or fragmenting methylated polynucleotides described herein) to a polynucleotide (probe) of defined sequence (which may be immobilized, for example, on a microarray), as described further herein. In some embodiments, the microarray is a cDNA microarray. In some embodiments, the microarray is an Affymetrix Gene Chip array, an Agilent oligonucleotide array, or Amersham CodeLink array, or other high density or low density oligonucleotide or cDNA array, including genome or focused arrays. It is understood that amount of labeled and/or fragmented nucleic acids produced (and thus the amount of product) may be determined using quantitative and/or qualitative methods. Determining amount of labeled and/or fragmented nucleic acids includes determining whether labeled and/or fragmented nucleic acids are present or absent. Thus, amount or extent of methylation includes information relating to absence of methylation. "Absent" or "absence" of product, and "lack of detection of product" as used herein includes insignificant, or de minimus levels.

[0145] As described above, labeled and/or fragmented nucleic acids can be detected and quantified by various methods, as described herein and/or known in the art. Determination of the amount of methylated polynucleotide of interest present in a sample, as determined by quantifying products (for example labeled and/or fragmented products) of the methods described herein, provides for determination of the methylation status of the sample source. It is understood that presence or absence or quantity of methylation may be determined for one polynucleotide of interest, a multiplicity of polynucleotides, a large multiplicity, or a very large multiplicity of polynucleotides (including all or essentially all genomic DNA). In some embodiments, presence or absence or quantity (extent or pattern) of methylation is determined for a specific polynucleotide sequence. In other embodiments, presence or absence or quantity is determined for a multiplicity, a large multiplicity or a very large multiplicity of polynucleotide sequences. Polynucleotide sequences (for which methylation analysis is desired) may be known (e.g., a particular locus, genomic region,

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CpG island, CpG dinucleotide, etc.) or may be unknown or unidentified polynucleotide sequences.

[0146] In another aspect, the invention provides methods for isolating, enriching and/or identifying methylated polynucleotides. The methods of the invention are utilized to generate labeled and/or fragmented nucleic acids which are isolated, enriched and or identified. In some embodiments, labeled and/or fragmented polynucleotides are physically captured (for example, by binding to probes in solution or microarray), and captured polynucleotide products are isolated and or enriched. In other embodiments, labeled and/or fragmented polynucleotide products are identified via hybridization to a known probe or other means for characterizing the sequence thereof.

2. Comparison of methylation status

[0147] The labeled and/or fragmented nucleic acids generated according to the methods of the invention are also suitable for analysis for the detection of any alteration in methylation status (e.g., presence or absence or quantity, extent and/or pattern) in the template polynucleotide sequence (from which the labeled and/or fragmented nucleic acids are synthesized), as compared to a reference nucleic acid sequence which is identical to the template nucleic acid sequence other than the alteration in methylation status, if any.

[0148] Accordingly, the invention provides methods of comparing methylation status in a sample, said method comprising: (a) generating labeled and/or fragmented from at least one polynucleotide template in the sample using any of the methods described herein; and (b) determining amount of labeled polynucleotide or fragments thereof of each polynucleotide template, wherein each said amount is indicative of amount of each polynucleotide template in the sample.

[0149] The methods are useful in a wide variety of molecular diagnostics, and especially in the study of essentially any cell (including a single cell) or cell population. A cell or cell population (e.g. a tissue) may be from, for example, blood, brain, spleen, bone, heart, vascular, lung, kidney, pituitary, endocrine gland, embryonic cells, tumors, or the like. Comparison of methylation status is also useful for comparing a control (normal) sample to a test sample, including test samples collected at different times, including before, after, and/or during development, a treatment, and the like.

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3. Comparative hybridization

[0150] In another aspect, the invention provides methods for comparative hybridization (such as comparative genomic hybridization), said method comprising: (a) preparing a first population of labeled polynucleotides or fragments thereof from a first template polynucleotide sample using any of the methods described herein; (b) comparing hybridization of the first population to at least one probe with hybridization of a second population of labeled polynucleotides or fragments thereof. In still other embodiments, the at least one probe is provided as a microarray. In some embodiments, the first and second population comprises detectably different labels. In other embodiments, the second population of labeled polynucleotides, or fragments thereof, are prepared from a second polynucleotide sample using any of the methods described herein. In some embodiments, step (b) of comparing comprises determining amount of said products, whereby the amount of the first and second polynucleotide templates is quantified.

[0151] In some embodiments, comparative hybridization comprises preparing a first population of labeled polynucleotides (which can be polynucleotide fragments) according to any of the methods described herein. A second population of labeled polynucleotides (to which the first population is desired to be compared) is prepared from a second genomic DNA template. The first and second population may be hybridized to a single array (in which case, detectably different labels are generally used) or hybridized to different arrays (in which case the labels may be the same or different). Hybridization of the first and second populations is detected and compared.

Methods for labeling and fragmenting polynucleotides comprising a cleavable canonical nucleotide, and methods for labeling a polynucleotide comprising a cleavable canonical nucleotide

A. Methods for labeling and fragmenting polynucleotides comprising a cleavable canonical nucleotide

[0152] The invention provides methods for generating labeled fragments of a polynucleotide comprising a cleavable canonical nucleotide (interchangeably termed

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"canonical nucleotide"). In some aspects, namely the RNA context, a canonical nucleotide includes a nucleotide comprising the base uracil (U) (as well as respective forms such as ribonucleoside, etc.).

[0153] The methods generally comprise cleavage of a base portion of a canonical nucleotide present in a polynucleotide comprising the canonical nucleotide with an agent capable of cleaving a base portion of the canonical nucleotide; and cleavage of the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and labeling at the abasic site, whereby labeled nucleic acid fragments are generated. Generally, the polynucleotide comprising a canonical nucleotide is fragmented and labeled at the abasic site (generated by cleavage of a base portion of a canonical nucleotide). Thus, the frequency of abasic sites generated by cleavage of the base portion of the canonical nucleotide generally relates to and determines the size range of the labeled fragments produced from the polynucleotide. The methods of the invention generate labeled nucleic acid fragments, which are useful for, for example, hybridization to a microarray and other uses described herein.

[0154] The methods involve the following steps: (a) contacting a polynucleotide comprising a canonical nucleotide with an agent capable of cleaving a base portion of the canonical nucleotide (i.e., cleaving a base portion of the canonical nucleotide), whereby an abasic site is created; (b) cleaving the backbone of the polynucleotide comprising the abasic site at the abasic site; and (c) contacting the polynucleotide comprising the abasic site with an agent capable of labeling the abasic site (i.e., labeling the abasic site), whereby labeled polynucleotide fragments are generated.

[0155] For simplicity, individual steps of the labeling and fragmentation method are discussed below. It is understood, however, that the steps may be performed simultaneously and/or in varied order, as discussed herein.

[0156] In some embodiments, an abasic site is generated at about every 5, 10, 15, 20, 25, 30, 40, 50, 65, 75, 85, 100, 123, 150, 175, 200, 225, 250, 300, 350, 400, 450, 500, 550, 600, 650 or more nucleotides apart. In one embodiment, the abasic site is generated about every 200 nucleotides, about every 100 nucleotide, or about every 50 nucleotide. In another embodiment, the abasic site is generated about every 50 to about 200 nucleotides.

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[0157] The frequency (or spacing) of abasic sites in the resulting polynucleotide comprising an abasic site (following cleavage of a base portion of a canonical nucleotide, and thus the average size of fragments generated using the methods of the invention (i.e., following cleavage of a phosphodiester backbone at an abasic site), is controlled by variables known in the art, including: frequency of canonical nucleotide(s) in the polynucleotide (or other measures of nucleotide content of a sequence, such as average G-C content),and the reaction conditions used during generation of abasic site, as is further discussed herein. The reaction conditions can be empirically determined, for example, by assessing average fragment size generated using the methods of the invention taught herein. In some embodiments, about any of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9 %, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or more canonical bases are cleaved.

[0158] The polynucleotide comprising a canonical nucleotide may be any template from which labeled polynucleotide fragments are desired to be produced, including double-stranded, partially double-stranded, and single-stranded nucleic acids from any source in purified or unpurified form, which can be DNA (dsDNA and ssDNA) or RNA, including tRNA, mRNA, rRNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, humans, and fragments thereof. Obtaining and purifying nucleic acids use standard techniques in the art. RNAs can be obtained and purified using standard techniques in the art. A DNA template (including genomic DNA template) can be transcribed into RNA form, which can be achieved using methods disclosed in Kurn, U.S. Patent No. 6,251,639 B1, and by other techniques (such as expression systems) known in the art. RNA copies of genomic DNA would generally include untranscribed sequences generally not found in mRNA, such as introns, regulatory and control elements, etc. DNA copies of an RNA template can be synthesized using methods described in Kurn, U.S. Patent Publication No. 2003/0087251 A1 or other techniques known in the art). Synthesis of polynucleotide from a DNA-RNA hybrid can be accomplished by denaturation of the hybrid to obtain a ssDNA and/or RNA, cleavage with an agent capable of cleaving RNA from an RNA/DNA hybrid, and other methods known in the art. The template can be only a minor

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fraction of a complex mixture such as a biological sample and can be obtained from various biological materials by procedures well known in the art. The template can be known or unknown and may contain more than one desired specific nucleic acid sequence of interest, each of which may be the same or different from each other. The template DNA can be a sub-population of nucleic acids, for example, a subtractive hybridization probe, total genomic DNA, restriction fragments, a cDNA library, cDNA prepared from total mRNA, a cloned library, or amplification products of any of the templates described herein. In some cases, the initial step of the synthesis of the complement of a portion of a template nucleic acid sequence is template denaturation. The denaturation step may be thermal denaturation or any other method known in the art, such as alkali treatment.

[0159] For simplicity, the polynucleotide comprising a canonical nucleotide is described as a single nucleic acid. It is understood that the polynucleotide can be a single polynucleotide, or a population of polynucleotides (from a few to a multiplicity to a very large multiplicity of polynucleotides). It is further understood that a polynucleotide comprising a canonical nucleotide can be a multiplicity (from small to very large) of different polynucleotide molecules. Such populations can be related in sequence (e.g., member of a gene family or superfamily) or extremely diverse in sequence (e.g., generated from all mRNA, generated from all genomic DNA, etc.). Polynucleotides can also correspond to single sequence (which can be part or all of a known gene, for example a coding region, genomic portion, etc.). Methods, reagents, and reaction conditions for generating specific polynucleotide sequences and multiplicities of polynucleotide sequences are known in the art.

[0160] Generally, the polynucleotide is DNA, though, as noted herein, the polynucleotide can comprise altered and/or modified nucleotides, internucleotide linkages, ribonucleotides, etc. As generally used herein, it is understood that "DNA" applies to polynucleotide embodiments. In some aspects, namely the RNA context, a canonical nucleotide includes a nucleotide comprising the base uracil (U) (as well as respective forms such as ribonucleoside, etc.).

[0161] Polynucleotides may be generated or isolated, e.g., from a sample. Methods for preparing polynucleotide are well known in the art. Methods for synthesizing polynucleotides, e.g., single and double stranded DNA, from a template are well known in

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the art, and include, for example, single primer isothermal amplification (SPIA™), Ribo-SPIA™, PCR, reverse transcription, primer extension, limited primer extension, replication (including rolling circle replication), strand displacement amplification (SDA), nick translation, multiple displacement amplification (MDA). See, e.g., Kurn, U.S. Patent No. 6,251,639 B1; Kurn, PCT Publication No. WO 02/00938; Kurn, U.S. Patent Publication No. 2003/0087251 A1; Mullis, U.S. Patent No. 4,582,877; Wallace, U.S. patent No. 6,027,923; U.S. Patent Nos. 5,508,178; 5,888,819; 6,004,744; 5,882,867; 5,710,028; 6,027,889; 6,004,745; 5,763,178; 5,011,769; see also Sambrook (1989) "Molecular Cloning: A Laboratory Manual", second edition; Ausebel (1987, and updates) "Current Protocols in Molecular Biology"; Mullis, (1994) "PCR: The Polymerase Chain Reaction". One or more methods known in the art can be used to synthesize polynucleotides. Suitable methods include methods that result in one single- or double-stranded polynucleotide comprising a canonical nucleotide (for example, reverse transcription, production of double stranded cDNA, a single round of DNA replication), as well as methods that result in multiple single stranded or double stranded copies or copies of the complement of a template (for example, single primer isothermal amplification or Ribo-SPIA™ or PCR). In one embodiment, a single-stranded polynucleotide comprising a canonical nucleotide is synthesized using single primer isothermal amplification. See Kurn, U.S. Patent No. 6,251,639 B1.

1. Cleaving a base portion of a canonical nucleotide to create an abasic site

[0162] The polynucleotide comprising a canonical nucleotide is treated with an agent, such as an enzyme, capable of generally, specifically, or selectively cleaving a base portion of the canonical deoxyribonucleoside to create an abasic site. As used herein, "abasic site" encompasses any chemical structure remaining following removal of a base portion (including the entire base) of a canonical nucleotide with an agent capable of cleaving a base portion of a nucleotide. In some embodiments, the agent (such as an enzyme) catalyzes hydrolysis of the bond between the base portion of the canonical nucleotide and a sugar in the canonical nucleotide to generate an abasic site comprising a hemiacetal ring (in some embodiments, comprising an aldehyde moiety) and lacking the base (interchangeably called "AP" site), though other cleavage products are contemplated for use in the methods of the invention. Suitable agents and reaction conditions for cleavage of

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base portions of cleavable canonical nucleotides are known in the art, and include the agents shown in **Table 1**.

Table 1:

Canonical nucleotide	Agent	Mechanism	Reference
dC	bisulfite	dC + HSO_3^- → dU → AP site**	U.S. Patent No. 6,017,704
dC	Cytosine deaminase	dC → dU → AP site**	Sohail et al, NAR 2003,31: 2990-94.
dG	Acidic conditions; alkylation (e.g., dimethyl sulfate treatment)	dG → AP site	

** UNG catalyzes dU →AP (abasic site).

[0163] One or more agents may be used. In some embodiments, the one or more agents cleave a base portion of the same canonical nucleotide. In other embodiments, the one or more agents cleave a base portion of different canonical nucleotides. In some embodiments, the agent is an enzyme. In other embodiments, the agent is a chemical agent. In still other embodiments, the agent is a reaction condition (such as presence of acidic conditions).

[0164] As is evident, in some embodiments dUTP is generated as an intermediate and cleavage of a base portion of dUTP is necessary to generate the abasic site. Methods for cleaving a base portion of dUTP are known in the art. See, e.g., Lindahl, PNAS (1974) 71(9):3649-3653; Jendrisak, U.S. Patent No. 6,190,865 B1; U.S. Patent No. 5,035,996; U.S. Patent No. 5,418,149. For example, in some embodiments, the enzyme cytosine deaminase is used to deaminate dCTP, whereby dUTP is generated. UNG is then used to cleave the base portion of dUTP, whereby an abasic site is generated. Thus, in some embodiments, cytosine deaminase is used in conjunction with UNG to generate an abasic site from the canonical nucleotide dCTP. As used herein, "in conjunction" encompasses simultaneous

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treatment (e.g., when cytosine deaminase and UNG cleavage occurs in the same reaction mixture) and/or treatment at different times (e.g., when cytosine deaminase and UNG treatment is conducted sequentially).

[0165] As is evident, in some embodiments, different agents cleave the base portion of the canonical nucleotide and cleave the phosphodiester backbone at the abasic site. In other embodiments, the agent that cleaves the base portion of the canonical nucleotide is the same agent that cleaves a phosphodiester backbone at the abasic site. In some embodiments, fragmentation and labeling is performed under acidic conditions (in some embodiments, at pH 2-5; in some embodiments, pH 3-3.5). Example 1 exemplifies reaction conditions suitable for efficient labeling and fragmentation under acidic conditions. In still other embodiments, ARP labeling and fragmentation are performed under substantially similar reaction conditions.

[0166] In some embodiments, cleavage of base portions of canonical nucleotides is general, specific or selective cleavage (in the sense that the agent (such as an enzyme) capable of cleaving a base portion of a canonical nucleotide generally, specifically or selectively cleaves the base portion of a particular canonical nucleotide), whereby about 90%, about 85%, or about 80% of the base portions cleaved are base portions of canonical nucleotides. However, extent of cleavage can be less. Thus, reference to specific cleavage is exemplary.

[0167] As noted herein, for convenience, cleavage of a base portion of a canonical nucleotide (whereby an abasic site is generated) has been described as a separate step. It is understood that this step may be performed simultaneously with synthesis of the polynucleotide comprising a canonical nucleotide (in embodiments involving synthesis of the polynucleotide comprising the cleavable canonical nucleotide), cleavage of the backbone at an abasic site (fragmentation) and/or labeling at an abasic site. Example 1 exemplifies the use of acidic conditions for cleavage of a canonical nucleotide (generally, dGTP) and cleavage of the phosphodiester backbone at the abasic site.

[0168] In some embodiments involving synthesis of the polynucleotide comprising the canonical nucleotide, the polynucleotide comprising a canonical nucleotide is purified following synthesis of the canonical polynucleotide (to eliminate, for example, residual free canonical nucleotides that are present in the reaction mixture). In other embodiments, there

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is no intermediate purification between the synthesis of the polynucleotide comprising the canonical nucleotide and subsequent steps (such as cleavage of a base portion of the canonical nucleotide and cleavage of a phosphodiester backbone at the abasic site).

2. Cleaving the backbone at the abasic site of the polynucleotide comprising an abasic site

[0169] Following generation of an abasic site, the backbone of the polynucleotide is cleaved at the abasic site (i.e., the site generated following cleavage of the base portion of the canonical nucleotide) with an agent capable of effecting cleavage of the backbone at the abasic site, as described herein.

3. Labeling the abasic site and detection

[0170] The abasic site is labeled, whereby a polynucleotide (or polynucleotide fragment) comprising a label is generated as described herein. In some embodiments, a polynucleotide fragment comprising an abasic site is contacted with an agent capable of labeling at the abasic site; whereby labeled fragments of the polynucleotide are generated as described herein.

B. Methods for labeling nucleic acids comprising a cleavable canonical nucleotide

[0171] The invention provides methods for generating labeled nucleic acid(s). The methods generally comprise cleavage of a base portion of a canonical nucleotide present in a polynucleotide with an agent capable of cleaving the base portion of the canonical nucleotide; and labeling the abasic site, whereby labeled polynucleotide(s) is generated. Generally, the polynucleotide comprising a canonical nucleotide is labeled at the site of the canonical nucleotide in the polynucleotide (following generation of an abasic site by cleavage of a base portion of the canonical nucleotide). The methods of the invention generate labeled polynucleotide(s), which are useful for, for example, hybridization to a microarray and other uses described herein.

[0172] The methods involve the following steps: (a) contacting the polynucleotide comprising a canonical nucleotide with an agent capable of cleaving a base portion of the

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canonical nucleotide, whereby an abasic site is created; and (b) labeling the abasic site in the polynucleotide comprising the abasic site, whereby labeled polynucleotide(s) is generated.

[0173] For simplicity, individual steps of the labeling methods are discussed below. It is understood, however, that the steps may be performed simultaneously and in varied order, as discussed herein.

[0174] In some embodiments, an basic site is generated at about every 5, 10, 15, 20, 25, 30, 40, 50, 65, 75, 85, 100, 123, 150, 175, 200, 225, 250, 300, 350, 400, 450, 500, 550, 600, 650 or more nucleotides apart. In one embodiment, the abasic site is generated about every 200 nucleotides, about every 100 nucleotide, or about every 50 nucleotide. In another embodiment, the abasic site is generated about every 50 to about 200 nucleotides.

[0175] The frequency (or spacing) of abasic sites in the resulting polynucleotide comprising an abasic site (following cleavage of a base portion of a canonical nucleotide, and thus the average size of fragments generated using the methods of the invention (i.e., following cleavage of a phosphodiester backbone at a canonical nucleotide), is controlled by variables known in the art, including: frequency of canonical nucleotide(s) in the polynucleotide (or other measures of nucleotide content of a sequence, such as average G-C content),and the reaction conditions used during generation of abasic site, as is further discussed herein. The reaction conditions can be empirically determined, for example, by assessing average fragment size generated using the methods of the invention taught herein. In some embodiments, about any of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9 %, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more canonical bases are cleaved.

1. Cleaving a base portion of a canonical nucleotide to create an abasic site

[0176] The polynucleotide comprising a canonical nucleotide is treated with an agent, such as an enzyme, capable of generally, specifically, or selectively cleaving a base portion of the canonical deoxyribonucleoside to create an abasic site, as described herein.

2. Labeling the abasic site and detection

[0177] The abasic site is labeled, whereby a polynucleotide comprising a label is generated, as described herein.

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Methods for preparing polynucleotides (or fragments thereof) and immobilizationd on a substrate

[0177] The invention provides methods for generating polynucleotides or polynucleotide fragments and immobilization of the fragments on a substrate (interchangeably termed a "surface", herein). The methods generally comprise immobilizing the polynucleotide, or fragments thereof, on a substrate, wherein the polynucleotide or fragment thereof is immobilized at the abasic site, wherein the polynucleotide, or fragment thereof, is generated using any of the methods described herein.. Optionally, the polynucleotide comprising an abasic site can be labeled at an abasic site according to the labeling methods described herein. The methods of the invention generate polynucleotides, and fragments thereof, immobilized on a substrate, for example, a microarray. In some embodiments, one or more abasic site(s) are labeled (as described herein) and one or more abasic site(s) are immobilized to a substrate.

[0178] The methods involve the following steps: immobilizing a polynucleotide (or polynucleotide fragments) on a substrate, wherein the polynucleotide is immobilized to the substrate at the abasic site; wherein the polynucleotide (or polynucleotide fragment) comprising an abasic site is generated by any of the methods described herein.

[0179] After generation of the polynucleotide comprising an abasic site, the polynucleotide (or polynucleotide fragment, if the backbone is cleaved) is immobilized to a substrate at the abasic site. In embodiments involving cleavage of the backbone at an abasic site (whereby fragments of the synthesized nucleic acid are generated), the cleaved fragments are immobilized to a substrate at the cleaved abasic site. Immobilizing a polynucleotide(s) is useful, for example, to tag an analyte, or to create a microarray. Single stranded polynucleotides (including polynucleotide fragments) are particularly suitable for preparing microarrays comprising the single stranded polynucleotides. Single stranded polynucleotide fragments (in embodiments involving cleavage of the phosphodiester backbone at an abasic site) are advantageous, because the orientation of the fragment with respect to the substrate (upon which the fragment is immobilized) can be controlled by selection of the method used to cleave the phosphodiester backbone, such that an abasic site is positioned at the 3' end of a fragment or at the 5' end of a fragment. Immobilizing polynucleotides in a defined orientation (e.g., at the 3' end, at the 5' end) enhances

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hybridization of complementary oligonucleotides, and permits a higher density of immobilization.

[0180] The polynucleotide comprising the abasic site is immobilized to a substrate as follows: generally, reagents are used that are capable of covalently or non-covalently attaching a reactive group present in the abasic site to a reactive group present on a substrate. For example, a common functional group exposed in an abasic site (and therefore suitable for use in labeling) is the aldehyde of the hemiacetal ring which can be covalently or noncovalently attached to a reactive group on a suitable substrate using reaction conditions that are known in the art. Suitable sidechains (present on the substrate) to react with the aldehyde (of the abasic site) include at least the following: substituted hydrazines, hydrazides, or hydroxylamines (which readily form imine bonds with aldehydes), and the related semicarbazide and thiosemicarbazide groups, and other amines which can form stable carbon-nitrogen double bonds, that can catalyze simultaneous cleavage and binding (see Horn, Nucl. Acids. Res., (1988) 16:11559-71), or can be coupled to form stable conjugates, e.g. by reductive amination.

[0181] The substrate to which the polynucleotide is to be immobilized can be functionalized with suitable reactive groups using methods known in the art. For example, a solid or semi-solid substrate (e.g., silicon or glass slide) can be coated with polymers (e.g., polyacrylamide, dextran, acrylamide, or latex) comprising hydrazine, hydrazide, or amine derivatized substrates (e.g. semicarbazides). Methods for functionalizing substrates with suitable reactive groups are known in the art, and disclosed in, for example, Luktanov, U.S. Patent No. 6,339,147; Van Ness, U.S. Patent No. 5,667,976; Bangs Laboratories, Inc. TechNote 205 (available at bangslabs.com); Ghosh, Anal. Biochem (1989) 178:43-51; O'Shannessy, Anal. Biochem. (1990) 191:1-8; Wilchek, Methods Enzymol. (1987) 138:429-442; Baumgartner, Anal. Biochem. (1989) 181:182-189; Zalipsky, Bioconjugate Chem. (1995) 6: 150-165, and references cited therein.

[0182] Methods and reaction conditions for performing these reactions are known in the art. See, e.g. Luktanov, U.S. Patent No. 6,339,147; Van Ness, U.S. Patent No. 5,667,976; Bangs Laboratories, Inc. TechNote 205 (available at bangslabs.com); Ghosh, Anal. Biochem (1989) 178:43-51; O'Shannessy, Anal. Biochem. (1990) 191:1-8; Wilchek, Methods Enzymol. (1987) 138:429-442; Baumgartner, Anal. Biochem. (1989) 181:182-189;

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Zalipsky, Bioconjugate Chem. (1995) 6: 150-165, and references cited therein. It is appreciated that similar chemistry is described herein with respect to the methods of labeling an abasic site (i.e., embodiments in which a reactive group in the abasic site is covalently or non-covalently attached to a suitable reactive group on a label). See, e.g., Srivastava, J. Biol. Chem. (1998) 273(33): 21203-209; Makrigiorgos, Int J. Radiat. Biol. (1998) 74(1):99-109; Makrigiorgos, U.S. Patent No. 6,174,680 B1; Makrogiorgos, PCT Publication No. WO 00/39345.

[0183] In another example, the abasic site may be chemically modified, then the modified abasic site covalently or non-covalently attached to a suitable reactive group on a substrate. For example, the aldehyde (in the abasic site) can be oxidized or reduced (using methods known in the art), then covalently immobilized to a substrate using, e.g., reductive amination or various oxidative processes.

[0184] The substrate may consist of many materials, limited primarily by capacity to immobilize (or, in some embodiments, capacity for derivatization to immobilize) any of a number of chemically reactive groups and compatibility with the synthetic chemistry used to immobilize the polynucleotide comprising an abasic site. The substrate can be a solid or semi-solid support, which may be made, e.g., from glass, plastic (e.g., polystyrene, polypropylene, nylon), polyacrylamide, nitrocellulose, or other materials such as metals. As described herein, the substrate can be functionalized, if necessary to add a suitable reactive group (to which the abasic site is covalently or non-covalently immobilized). The polynucleotides may also be spotted as a matrix on substrates comprising paper, glass, plastic, polystyrene, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, optical fiber or any other suitable solid or semi-solid (e.g., thin layer of polyacrylamide gel, assuming that the substrate is suitably functionalized, as described herein (Khrapko, et al., DNA Sequence (1991), 1:375-388)).

[0185] An array may be assembled as a two-dimensional matrix on a planar substrate or may have a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, cylinders and any other arrangement suitable for hybridization and detection of template molecules. In one embodiment the substrate to which the polynucleotide (or fragments thereof) is immobilized is magnetic beads or particles. In another embodiment, the solid substrate comprises an optical fiber. In yet

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another embodiment, the polynucleotides are dispersed in fluid phase within a capillary which, in turn, is immobilized with respect to a solid phase.

[0186] In another embodiment, the substrate comprises a polypeptide, a protein, a peptide, carbohydrates, cells, microorganisms and fragments and products thereof, an organic molecule, an inorganic molecule, carrier molecules, PEG, amino-dextran, carbohydrates, supramolecular assemblies, organelles, cells, microorganisms, organic molecules, inorganic molecules, or any substance for which immobilization sites for polynucleotides comprising abasic sites naturally exist, can be created (e.g. by functionalizing or otherwise modifying the substrate) or can be developed. In one embodiment, the substrate is a polynucleotide.

[0187] The substrate may be an analyte. Typical analytes may include, but are not limited to antibodies, proteins (including enzymes), peptides, nucleic acid molecules or segments thereof, carrier molecules, PEG, amino-dextran, carbohydrates, supramolecular assemblies, organelles, cells, microorganisms, organic molecules, inorganic molecules, or any substance for which immobilization sites for polynucleotides comprising abasic sites naturally exist, can be created (e.g. by functionalizing the analyte) or can be developed.

[0188] It is understood that a substrate may be a member(s) of a binding pair. Non-limiting examples of a binding pair include a protein:protein binding pair, and a protein: antibody binding pair. In another embodiment, polynucleotides (or fragments thereof) are immobilized to (tag) a molecular library of substrates, e.g., a molecular library of chemical compounds, a phage peptide display library, or a library of antibodies.

[0189] In some embodiments, the substrate (to which the polynucleotide is immobilized) is an enzyme, such that enhanced detection of hybridization of the polynucleotide is provided. For example, a polynucleotide immobilized to an enzyme can be hybridized to a microarray, and hybridized polynucleotide detected by contacting the microarray with a defined substrate.

[0190] In embodiments of the invention involving cleavage of the phosphodiester backbone at an abasic site (whereby fragments of the synthesized nucleic acid are generated), the cleaved fragments can also be immobilized to a substrate using any method known in the art for immobilization of a nucleic acid to a substrate. As used herein, "immobilization" includes both covalent attachment and non-covalent association. In one

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embodiment, a polynucleotide is immobilized to a substrate directly via the abasic site. In another embodiment, a polynucleotide is immobilized to a substrate directly or indirectly via an attached or associated label.

[0191] For example, single or double stranded polynucleotide fragments (generally single stranded) can be immobilized to a solid or semi-solid support or substrate, which may be made, e.g., from plastics, ceramics, metals, acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, polysilicates, polycarbonates, Teflon®, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids, and other materials. Substrates may be two-dimensional or three-dimensional in form, such as gels, membranes, thin films, glasses, plates, cylinders, beads, magnetic beads, optical fibers, woven fibers, microtiter well, capillaries, etc.. For example, the fragments can be contacted with a solid or semi-solid substrate, such as a glass slide, which is coated with a reactive group which will form a covalent link with the reactive group that is on the polynucleotide fragment and become covalently immobilized to the substrate.

[0192] Microarrays comprising the nucleotide fragments can be fabricated using a Biodot (BioDot, Inc. Irvine, CA) spotting apparatus and aldehyde-coated glass slides (CEL Associates, Houston, TX). Polynucleotide fragments can be spotted onto the aldehyde-coated slides following suitable functionalization, and processed according to published procedures (Schena et al., Proc. Natl. Acad. Sci. U.S.A. (1995) 93:10614-10619), provided suitable care is taken to avoid interfering with other desired reactions at the abasic sites. Arrays can also be printed by robotics onto glass, nylon (Ramsay, G., Nature Biotechnol. (1998), 16:40-44), polypropylene (Matson, et al., Anal Biochem. (1995), 224(1):110-6), and silicone slides (Marshall, A. and Hodgson, J., Nature Biotechnol. (1998), 16:27-31). Other approaches to array assembly include fine micropipetting within electric fields (Marshall and Hodgson, *supra*), and spotting the polynucleotides directly onto positively coated plates. Methods such as those using amino propyl silane surface chemistry are also known in the art, as disclosed at http://www.cmt.corning.com and http://cmgm.stanford.edu/pbrown/.

[0193] One method for making microarrays is by making high-density polynucleotide arrays. Techniques are known for rapid deposition of polynucleotides

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(Blanchard et al., Biosensors & Bioelectronics, 11:687-690). In principle, and as noted above, any type of array, for example, dot blots on a nylon hybridization membrane, could be used. However, as will be recognized by those skilled in the art, very small arrays will frequently be preferred because hybridization volumes will be smaller. Very small arrays may include a high or low density of probes.

[0194] Methods for immobilizing polynucleotide fragments to analytes (as described herein) are known in the art. See, e.g., U.S. Patent Nos. 6,309,843; 6,306,365; 6,280,935; and 6,087,103 (and methods discussed therein).

Applications using the labeling and/or fragmentation and/or immobilization methods of the invention

[0195] The methods and compositions of the invention can be used for a variety of purposes. For purposes of illustration, methods of producing a hybridization probe or target, characterizing and/or quantitating nucleic acid, detecting a mutation, preparing a subtractive hybridization probe, detection (using the hybridization probe), and determining a gene expression profile, using the labeled and/or fragmented nucleic acids generated by the methods of the invention, are described.

[0196] Immobilized polynucleotides, for example on a microarray, prepared according to any of the methods of the invention, are also useful for methods of analyzing and characterizing nucleic acids, including methods of hybridizing nucleic acids, methods of characterizing and/or quantitating nucleic acids, methods of detecting a mutation in a nucleic acids, and methods of determining a gene expression profile, as described below, and these applications likewise apply to immobilized polynucleotides.

A. Method of producing a hybridization probe or target

[0197] The polynucleotides obtained by the methods of the invention are useful as hybridization probes or targets. As used herein, "probe" refers to a reference polynucleotide or oligonucleotide, for example immobilized to a substrate or in solution, and "target" refers to a polynucleotide or oligonucleotide from a sample to be analyzed by hybridization to the probe. In some embodiments, the target is detectably labeled. In other embodiments, a hybridization target generated by a method of the invention is unlabeled and its interaction with a probe is detected indirectly. Accordingly, in one aspect, the invention provides

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methods for generating hybridization targets, comprising generating labeled polynucleotides using any of the methods described herein, and using the labeled polynucleotides as a hybridization target. In another embodiment, the invention provides methods for generating a hybridization target, comprising generating labeled polynucleotide fragments using any of the methods described herein, and using the labeled polynucleotide fragments as a hybridization target. The labeled polynucleotide (or polynucleotide fragments) can be produced from any template, including RNA, DNA, genomic DNA (including global genomic DNA amplification), and libraries (including cDNA, genomic or subtractive hybridization library). The invention also provides methods of hybridizing using the hybridization targets to a probe as described herein. In another aspect, the invention provides methods for generating hybridization probes, comprising generating polynucleotides or polynucleotide fragments using any of the methods described herein. In one embodiment, a probe comprising a polynucleotide or polynucleotide fragment generated by a method of the invention is immobilized to a solid support. In one embodiment, the probe is labeled and is immobilized to a support via the label (for example, indirect immobilization via a member of a specific binding pair). In another embodiment, the probe is unlabeled.

B. Characterization of nucleic acids

[0198] The labeled and/or fragmented nucleic acids obtained by the methods of the invention are amenable to further characterization.

[0199] The labeled and/or fragmented nucleic acids (i.e., products of any of the methods described herein), can be analyzed using, for example, probe hybridization techniques known in the art, such as Southern and Northern blotting, and hybridizing to probe arrays. They can also be analyzed by electrophoresis-based methods, such as differential display and size characterization, which are known in the art.

[0200] In one embodiment, the methods of the invention are utilized to generate labeled and/or fragmented nucleic acids, and analyze the labeled and/or fragmented nucleic acids by contact with a probe. The labeled and/or fragmented nucleic acid can be produced from any template known in the art, including RNA, DNA, genomic DNA (including global genomic DNA amplification), and libraries (including cDNA, genomic or subtractive hybridization library).

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[0201] In one embodiment, the methods of the invention are utilized to generate labeled and/or fragmented nucleic acids which are analyzed (for example, detection and/or quantification) by contacting them with, for example, microarrays (of any suitable substrate, which includes glass, chips, plastic), beads, or particles, that comprise suitable probes such as cDNA and/or oligonucleotide probes. Thus, the invention provides methods to characterize (for example, detect and/or quantify and/or identify) a labeled and/or fragmented nucleic acid by analyzing the labeled products, for example, by hybridization of the labeled products to, for example, probes immobilized at, for example, specific locations on a solid or semi-solid substrate, probes immobilized on defined particles (including beads, such as Bead Array, Illumina), or probes immobilized on blots (such as a membrane), for example arrays, or arrays of arrays. Immobilized probes include immobilized probes generated by the methods described herein, and also include at least the following: cDNA and synthetic oligonucleotides, which can be synthesized directly on the substrate.

[0202] Other methods of analyzing labeled products are known in the art, such as, for example, by contacting them with a solution comprising probes, followed by extraction of complexes comprising the labeled products and probes from solution. The identity of the probes provides characterization of the sequence identity of the products, and thus by extrapolation can also provide characterization of the identity of a template from which the products were prepared (for example, the identity of an RNA in a solution). For example, hybridization of the labeled products is detectable, and the amount of specific labels that are detected is proportional to the amount of the labeled products prepared from a specific RNA sequence of interest. This measurement is useful for, for example, measuring the relative amounts of the various RNA species in a sample, which are related to the relative levels of gene expression, as described herein. The amount of labeled products (as indicated by, for example, detectable signal associated with the label) hybridized at defined locations on an array can be indicative of the detection and/or quantification of the corresponding template RNA species in the sample.

[0203] Methods of characterization include sequencing by hybridization (see, e.g., Dramanac, U.S. Patent No. 6,270,961) and global genomic hybridization (also termed comparative genome hybridization) (see, e.g., Pinkel, U.S. Patent No. 6,159,685).

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[0204] In another aspect, the invention provides a method of quantitating labeled and/or fragmented nucleic acids comprising use of an oligonucleotide (probe) of defined sequence (which may be immobilized, for example, on a microarray).

C. Mutation detection utilizing the methods of the invention

[0205] The labeled and/or fragmented nucleic acids generated according to the methods of the invention are also suitable for analysis for the detection of any alteration in the template nucleic acid sequence (from which the labeled and/or fragmented nucleic acids are synthesized), as compared to a reference nucleic acid sequence which is identical to the template nucleic acid sequence other than the sequence alteration. The sequence alterations may be sequence alterations present in the genomic sequence or may be sequence alterations which are not reflected in the genomic DNA sequences, for example, alterations due to post transcriptional alterations, and/or mRNA processing, including splice variants. Sequence alterations (interchangeably called "mutations") include deletion, substitution, insertion and/or transversion of one or more nucleotide.

[0206] Accordingly, the invention provides methods of detecting presence or absence of a mutation in a template, comprising: (a) generating a labeled polynucleotide, or fragments thereof, by any of the methods described herein; and (b) analyzing the labeled polynucleotide, or fragments thereof, whereby presence or absence of a mutation is detected. In some embodiments, the labeled polynucleotide, or fragments thereof, is compared to a labeled reference template, or fragments thereof. Step (b) of analyzing the labeled polynucleotide, or fragments thereof, whereby presence or absence of a mutation is detected, can be performed by any method known in the art. In some embodiments, probes for detecting mutations are provided as a microarray.

[0207] Any alteration in the test nucleic acid sequence, such as base substitution, insertions or deletion, could be detected using this method. The method is expected to be useful for detection of specific single base polymorphism, SNP, and the discovery of new SNPs.

[0208] Other art recognized methods of analysis for the detection of any alteration in the template nucleic acid sequence, as compared to a reference nucleic acid sequence, are suitable for use in the methods of the present invention. For example, essentially any

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hybridization-based method of detection of mutations is suitable for use with the labeled and/or fragmented nucleic acids produced by the methods of the invention.

D. Methods of preparing a subtractive hybridization probe

[0209] The labeled and/or fragmented nucleic acids methods of the invention are particularly suitable for use in preparation of labeled and/or fragmented subtractive hybridization probes. For example, two nucleic acid populations, one sense and one antisense, can be allowed to mix together with one population present in molar excess ("driver"). Sequence present in both populations will form hybrids, while sequences present in only one population remain single-stranded. Thereafter, various well-known techniques are used to separate the unhybridized molecules representing differentially expressed sequences. See, e.g., Hamson et al., U.S. Patent No. 5,589,339; Van Gelder, U.S. Patent No. 6,291,170. Labeled and/or fragmented subtractive hybridization probe is then labeled and/or fragmented according to the methods of the invention described herein.

E. Comparative hybridization

[0210] In another aspect, the invention provides methods for comparative hybridization (such as comparative genomic hybridization), said method comprising: (a) preparing a first population of labeled polynucleotides or fragments thereof from a first template polynucleotide sample using any of the methods described herein; (b) comparing hybridization of the first population to at least one probe with hybridization of a second population of labeled polynucleotides or fragments thereof. In some embodiments, the at least one probe is a chromosomal spread. In still other embodiments, the at least one probe is provided as a microarray. In other embodiments, the second population of labeled polynucleotides, or fragments thereof, are prepared from a second polynucleotide sample using any of the methods described herein. In some embodiments, the first population and second population comprise detectably different labels. In some embodiments, step (b) of comparing comprises determining amount of said products, whereby the amount of the first and second polynucleotide templates is quantified.

[0211] In some embodiments, comparative hybridization comprises preparing a first population of labeled polynucleotides (which can be polynucleotide fragments) according to

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any of the methods described herein, wherein the template from which the first population is synthesized is genomic DNA. A second population of labeled polynucleotides (to which the first population is desired to be compared) is prepared from a second genomic DNA template. The first and second populations are labeled with different labels. The hybridized first and second populations are mixed, and hybridized to an array or chromosomal spread. The different labels are detected and compared.

Reaction conditions and detection

[0212] Appropriate reaction media and conditions for carrying out the methods of the invention are those that permit nucleic acid synthesis according to the methods of the invention. Such media and conditions are known to persons of skill in the art, and are described in various publications, such as U.S. Pat. Nos. 6,190,865; 5,554,516; 5,716,785; 5,130,238; 5,194,370; 6,090,591; 5,409,818; 5,554,517; 5,169,766; 5,480,784; 5,399,491; 5,679,512; PCT Publication No. WO 99/42618; *Mol. Cell Probes* (1992) 251-6; and Anal. Biochem. (1993) 211:164-9. For example, a buffer may be Tris buffer, although other buffers can also be used as long as the buffer components are non-inhibitory to enzyme components of the methods of the invention. The pH is from about 5 to about 11, more preferably from about 6 to about 10, from about 7 to about 9, and from about 7.5 to about 8.5. The reaction medium can also include bivalent metal ions such as Mg^{2+} or Mn^{2+}, at a final concentration of free ions that is within the range of from about 0.01 to about 15 mM, and most preferably from about 1 to 10 mM. The reaction medium can also include other salts, such as KCl or NaCl, that contribute to the total ionic strength of the medium. For example, the range of a salt such as KCl is preferably from about 0 to about 125 mM, more preferably from about 0 to about 100 mM, and most preferably from about 0 to about 75 mM. The reaction medium can further include additives that could affect performance of the amplification reactions, but that are not integral to the activity of the enzyme components of the methods. Such additives include proteins such as BSA, single strand binding proteins (e.g., T4 gene 32 protein), and non-ionic detergents such as NP40 or Triton. Reagents, such as DTT, that are capable of maintaining enzyme activities can also be included. Such reagents are known in the art. Where appropriate, an RNase inhibitor (such as Rnasin) that does not inhibit the activity of the RNase employed in the method (if any) can also be

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included. Any aspect of the methods of the invention can occur at the same or varying temperatures. The synthesis reactions (particularly, primer extension other than the first and second strand cDNA synthesis steps, and strand displacement) can be performed isothermally, which avoids the cumbersome thermocycling process. The synthesis reaction is carried out at a temperature that permits hybridization of oligonucleotides (primer) to the template polynucleotide and primer extension products, and that does not substantially inhibit the activity of the enzymes employed. The temperature can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 80°C, and most preferably about 37°C to about 75°C. In some embodiments that include RNA transcription, the temperature for the transcription steps is lower than the temperature(s) for the preceding steps. In these embodiments, the temperature of the transcription steps can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 75°C, and most preferably about 37°C to about 70°C.

[0213] Nucleotides that can be employed for synthesis of the nucleic acids in the methods of the invention are provided in the amount of from about 50 to about 2500 μM, about 100 to about 2000 μM, about 200 to about 1700 μM, and about 250 to about 1500 μM. The oligonucleotide components of the synthesis reactions of the invention are generally in excess of the number of template nucleic acid sequence to be replicated. They can be provided at about or at least about any of the following: 10, 10^2, 10^4, 10^6, 10^8, 10^{10}, 10^{12} times the amount of target nucleic acid. Composite primers can be provided at about or at least about any of the following concentrations: 50 nM, 100 nM, 500 nM, 1000 nM, 2500 nM, 5000 nM.

[0214] Optionally, the polynucleotide template (i.e., polynucleotide comprising a canonical nucleotide, or polynucleotide comprising a methylated nucleotide) can be treated with hydroxylamine (or any other suitable agent) to remove any aldehydes that may have formed spontaneously in the nucleic acid. *See, e.g.*, Makrogiorgos, PCT Publication No. WO 00/39345.

[0215] For convenience, the cleavage of a base portion of that polynucleotide by an enzyme capable of cleaving a base portion of the canonical nucleotide (or capable of cleaving a base portion of the methylated nucleotide, in embodiments involving methylation), and the cleavage of the phosphodiester backbone at the abasic site, are

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described as separate steps. It is understood that these steps may be performed simultaneously.

[0216] Appropriate reaction media and conditions for carrying out the cleavage of a base portion of a canonical nucleotide according to the methods of the invention are those that permit cleavage of a base portion of a canonical nucleotide. Such media and conditions are known to persons of skill in the art, and are described in various publications, such as Sohail et al, NAR 2003,31: 2990-94; A. Sartori et al, JBC 2001, 276: 29979-29986; US Patent No. 6,017,704. In some embodiments involving cleavage of a canonical nucleotide, dUTP is generated as an intermediate and cleavage of a base portion of dUTP is necessary to generate the abasic site. Methods for cleaving a base portion of dUTP are known in the art. See, e.g., Lindahl, *PNAS* (1974) 71(9):3649-3653; Jendrisak, U.S. Patent No. 6,190,865 B1; U.S. Patent No. 5,035,996; U.S. Patent No. 5,418,149.

[0217] Appropriate reaction media and conditions for carrying out the cleavage of a base portion of a methylated nucleotide according to the methods of the invention are those that permit cleavage of a base portion of a methylated nucleotide. Such media and conditions are known to persons of skill in the art, and are described in various publications, such as Wolffe et al., Proc. Nat. Acad. Sci. USA 96:5894-5896, 1999); Zhu et al., Proc. Natl. Acad. Sci. USA 98:5031-6, 2001; Zhu et al., Nuc. Acid Res. 28:4157-4165, 2000; Neddermann et al., J.B.C. 271:12767-74, 1996; Vairapandi & Duker, Oncogene 13:933-938, 1996; Vairapandi et al., J. Cell. Biochem. 79:249-260, 2000.

[0218] In embodiments involving cleavage of the phosphodiester backbone, appropriate reaction media and conditions for carrying out the cleavage of the phosphodiester backbone at an abasic site according to the methods of the invention are those that permit cleavage of the phosphodiester backbone at an abasic site. Such media and conditions are known to persons of skill in the art, and are described in various publications, such as Bioorgan. Med. Chem (1991) 7:2351; Sugiyama, Chem. Res. Toxicol. (1994) 7: 673-83; Horn, Nucl. Acids. Res., (1988) 16:11559-71); Lindahl, PNAS (1974) 71(9):3649-3653; Jendrisak, U.S. Patent No. 6,190,865 B1; Shida, Nucleic Acids Res. (1996) 24(22):4572-76; Srivastava, J. Biol Chem. (1998) 273(13):21203-209; Carey, Biochem. (1999) 38:16553-60; Chem Res Toxicol (1994) 7:673-683. For example, E. *coli* AP endonuclease IV is added to reaction conditions as described above. AP Endonuclease IV

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can be added at the same or different time as the agent (such as an enzyme) capable of cleaving the base portion of a canonical nucleotide.

[0219] In another example, nucleic acids containing abasic sites are heated in a buffer solution containing an amine, for example, 25mM Tris-HCl and 1-5 mM magnesium ions, for 10-30 minutes at 70°C to 95°C. Alternatively, 1.0 M piperidine (a base) is added to polynucleotide comprising an abasic site which has been precipitated with ethanol and vacuum dried. The solution is then heated for 30 minutes at 90°C and lyophilized to remove the piperidine. In another example, cleavage is affected by treatment with basic solution, e.g., 0.2 M sodium hydroxide at 37° for 15 minutes. See Nakamura (1998) Cancer Res. 58:222-225. In yet another example, incubation at 37C with 100 mM N, N'-dimethylethylenediamine acetate, pH 7.4 is used to cleave. See McHugh and Knowland, (1995) Nucl. Acids Res. 23(10) 1664-1670; see also co-pending co-owned U.S. Patent Application Serial No. 10/441,663.

[0220] In one embodiment, the reaction conditions are the same for the cleavage of a base portion of the canonical nucleotide (or in embodiments, involving methylation analysis, cleavage of a base portion of the methylated nucleotide) and for the cleavage of the phosphodiester backbone at abasic sites. In another embodiment, different reaction conditions are used for these reactions.

[0221] In embodiments involving labeling at an abasic site, appropriate reaction media and conditions for carrying out the labeling at an abasic site according to the methods of the invention are those that permit labeling at an abasic site. Such reaction mixtures and conditions are known to persons of skill in the art, and are described in various publications, such as co-pending co-owned U.S. Patent Application Serial No. 10/441,663; Makrigiorgos, PCT Publication No. WO 00/39345; Srivastava, *J. Biol. Chem.* (1998) 273(33): 21203-209; Makrigiorgos, Int J. Radiat. Biol. (1998) 74(1):99-109; Makrigiorgos, U.S. Patent No. 6,174,680 B1; Makrigiorgos, PCT Publication No. WO 00/39345; Boturyn (1999) Chem. Res. Toxicol. 12:476-482. See, also, Adamczyk (1998) Bioorg. Med. Chem. Lett. 8(24):3599-3602; Adamczyk (1999) Org. Lett. 1(5):779-781; Kow (2000) Methods 22(2):164-169; Molecular Probes Handbook, Section 3.2 (www.probes.com); Horn (Nucl. Acids. Res., (1988) 16:11559-71). For example, 5-(((2-(carbohydrazino)-methyl)thio)acetyl)aminofluorescein, aminooxyacetyl hydrazide (FARP); N-

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(aminooxyacetyl)-N'-(D-biotinoyl) hydrazine, trifluorecetic acid salt (ARP); Alexa Fluor 555 (Molecular Probes); aminooxy-derivatized Alexa Fluor 555; and other aldehyde-reactive reagents can be reacted with a polynucleotide comprising abasic sites. The buffer can be sodium citrate or sodium phosphate buffer, though other buffers are acceptable as long as the buffer components are non-inhibitory to enzyme components and/or desired chemical reactions used in the methods of the invention. The pH is preferably from about 3 to about 11, more preferably from about 4 to about 10, even more preferably from about 4 to about 8, and most preferably from about 4 to about 7. The reaction can be conducted at room temperature to 85°C (in some embodiments, at about 55°C), though other temperatures are suitable as long as the temperature is non-inhibitory to enzyme components and/or desired chemical reactions used in the methods of the invention. Generally, the label (e.g. ARP or FARP) is added at about 1-10 mM, in some embodiments at 2-5 mM, though other concentrations are suitable. If an antibody label is used, conditions for antibody binding are well-known in the art, and can be as described herein. Optionally, a stop buffer can be used that neutralizes the pH of the labeling reaction, thereby stopping the labeling reaction and optionally, facilitating subsequent purification of labeled product.

[0222] In embodiments involving immobilization of a polynucleotide at an abasic site, appropriate reaction media and conditions for carrying out the immobilization at an abasic site according to the methods of the invention are those that permit immobilization at an abasic site. Such reaction mixtures and conditions are known to persons of skill in the art, and are described in various publications, such as Luktanov, U.S. Patent No. 6,339,147; Van Ness, U.S. Patent No. 5,667,976; Bangs Laboratories, Inc. TechNote 205 (available at bangslabs.com); Ghosh, Anal. Biochem (1989) 178:43-51; O'Shannessy, Anal. Biochem. (1990) 191:1-8; Wilchek, Methods Enzymol. (1987) 138:429-442; Baumgartner, Anal. Biochem. (1989) 181:182-189; Zalipsky, Bioconjugate Chem. (1995) 6: 150-165, and references cited therein. In some cases, the initial product can be stabilized by reduction with sodium cyanoborohydride or similar agents known in the art. See, e.g., O'Shannessy, supra.

[0223] In one embodiment, the foregoing components are added simultaneously at the fragmentation and/or labeling and/or immobilization processes. In another embodiment, components are added in any order prior to or after appropriate timepoints during the

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synthesis step. Such timepoints, some of which are noted below, can be readily identified by a person of skill in the art. In these embodiments, the reaction conditions and components may be varied between the different reactions.

[0224] The fragmenting and/or labeling and/or immobilization process can be stopped at various timepoints, and resumed at a later time. Said timepoints can be readily identified by a person of skill in the art. Methods for stopping the reactions are known in the art, including, for example, cooling the reaction mixture to a temperature that inhibits enzyme activity or heating the reaction mixture to a temperature that destroys an enzyme. Methods for resuming the reactions are also known in the art, including, for example, raising the temperature of the reaction mixture to a temperature that permits enzyme activity or replenishing a destroyed (depleted) enzyme or other reagent. In some embodiments, one or more of the components of the reactions is replenished prior to, at, or following the resumption of the reactions. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

[0225] The reaction can be allowed to proceed without purification of intermediate complexes, for example, to remove primer. Products can be purified at various timepoints, which can be readily identified by a person of skill in the art.

Compositions and kits of the invention

[0226] The invention also provides compositions and kits used in the methods described herein. The compositions may be any component(s), reaction mixture and/or intermediate described herein, as well as any combination. For example, the invention provides a composition comprising an agent (such as an enzyme) capable of cleaving a base portion of a canonical nucleotide, optionally an agent (such as an enzyme) capable of effecting cleavage of a phosphodiester backbone at an abasic site, and an agent capable of labeling an abasic site. In some embodiments, the invention provides compositions further comprising a composite primer, said composite comprising a DNA portion and a 5' RNA portion. In still other embodiments, the 5' RNA portion of the composite primer is adjacent to the 3' DNA portion, the RNA portion of the composite primer consists of about 10 to about 20 nucleotides and the DNA portion of the composite primer consists of about 7 to about 20 nucleotides. In still other embodiments, the composition comprises a second,

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different composite primer. In some embodiments, the RNA portion of the composite primer comprises the following ribonucleotide sequence: 5'- GACGGAUGCGGUCU-3'. In still other embodiments, the compositions further comprise one or more of (a) a mixture of dATP, dTTP, dCTP, and dGTP; (b) a DNA polymerase; and (c) RNAse H. In still other embodiments, the compositions further comprise one or more of (a) MgCl2 solution; (b) acetic acid solution; and optionally, (c) a stop buffer comprising 1.5M Tris, pH 8.5. In some embodiments, the agent capable of cleaving the phosphodiester backbone at an abasic site is an amine (such as N, N'-dimethylethylenediamine); and/or E. coli Endonuclease IV. In some embodiments, the agent capable of labeling an abasic site is ARP, FARP, Alexa Fluor 555 hydrazide (Order No. A-20501, Molecule Probes, Eugene OR), and/or an aminooxy-modified Alexa Fluor 555 (*see* copending co-owned U.S. Patent Application Serial No. 10/441,663).

[0227] In another example, the invention provides a composition comprising an agent (such as an enzyme) capable of cleaving a base portion of a methylated nucleotide, optionally, an agent (such as an enzyme) capable of effecting cleavage of a phosphodiester backbone at an abasic site, and an agent capable of labeling an abasic site. In some embodiments, the agent capable of cleaving a base portion of a methylated nucleotide is 5-methylcytosine DNA glycosylase (5-MCDG), or 3-methyladenosine-DNA glycosylase. In some embodiments, the agent capable of cleaving the phosphodiester backbone at an abasic site is an amine (such as N, N'-dimethylethylenediamine); and/or E. coli Endonuclease IV. In some embodiments, the agent capable of labeling an abasic site is ARP, FARP, Alexa Fluor 555 hydrazide (Order No. A-20501, Molecule Probes, Eugene OR), and/or an aminooxy-modified Alexa Fluor 555 (see copending co-owned U.S. Patent Application Serial No. 10/441,663).

[0228] The compositions are generally in lyophilized or aqueous form (if appropriate), preferably in a suitable buffer.

[0229] The invention also provides compositions comprising the labeled and/or fragmented products described herein. Accordingly, the invention provides a population of labeled and/or fragmented polynucleotides, which are produced by any of the methods described herein (or compositions comprising the products).

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[0230] The invention also provides compositions comprising the immobilized polynucleotides or immobilized polynucleotide fragments described herein. In some embodiments, the immobilized polynucleotide (or immobilized fragment, in embodiments involving fragmentation) is labeled, as described herein. Accordingly, the invention provides a population of immobilized polynucleotides or immobilized polynucleotide fragments which are produced by any of the methods described herein (or compositions comprising the products).

[0231] The invention also provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. Examples of reaction mixtures have been described.

[0232] The invention provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein

[0233] The kits of the invention comprise one or more containers comprising any combination of the components described herein, and the following are examples of such kits.

[0234] In some embodiments, the invention provides kits comprising an agent (such as an enzyme) capable of cleaving a base portion of a canonical nucleotide, optionally an agent (such as an enzyme) capable of effecting cleavage of a phosphodiester backbone at an abasic site, and an agent capable of labeling an abasic site. In some embodiments, the invention provides kits further comprising a composite primer, said composite comprising a DNA portion and a 5' RNA portion. In still other embodiments, the 5' RNA portion of the composite primer is adjacent to the 3' DNA portion, the RNA portion of the composite primer consists of about 10 to about 20 nucleotides and the DNA portion of the composite primer consists of about 7 to about 20 nucleotides. In still other embodiments, the kits comprise a second, different composite primer. In some embodiments, the RNA portion of the composite primer comprises the following ribonucleotide sequence: 5'-GACGGAUGCGGUCU-3'. In still other embodiments, the kits further comprise one or more of (a) a mixture of dATP, dTTP, dCTP, and dGTP; (b) a DNA polymerase; and (c) RNAse H. In still other embodiments, the kits further comprise one or more of (a) MgCl2 solution; (b) acetic acid solution; and optionally, (c) a stop buffer comprising 1.5M Tris, pH

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8.5. In some embodiments, the invention provides a kit comprising RNAseH, and an agent (such as an enzyme) capable of cleaving a base portion of a canonical nucleotide. RNase H. In some embodiments, the invention provides a kit comprising a composite primer comprising an RNA portion and a 3' DNA portion and instructions for any of the methods for labeling and/or fragmenting a polynucleotide comprising a canonical nucleotide described herein. In some embodiments, the agent capable of cleaving the phosphodiester backbone at an abasic site is an amine (such as N, N'-dimethylethylenediamine); and/or E. coli Endonuclease IV. In some embodiments, the agent capable of labeling an abasic site is ARP, FARP, Alexa Fluor 555 hydrazide (Order No. A-20501, Molecule Probes, Eugene OR), and/or an aminooxy-modified Alexa Fluor 555 (see copending co-owned U.S. Patent Application Serial No. 10/441,663).

[0235] In another example, the invention provides kits comprising an agent (such as an enzyme) capable of cleaving a base portion of a methylated nucleotide, optionally, an agent (such as an enzyme) capable of effecting cleavage of a phosphodiester backbone at an abasic site, and an agent capable of labeling an abasic site. In some embodiments, the agent capable of cleaving a base portion of a methylated nucleotide is 5-methylcytosine DNA glycosylase (5-MCDG), or 3-methyladenosine-DNA glycosylase. In some embodiments, the agent capable of cleaving the phosphodiester backbone at an abasic site is an amine (such as N, N'-dimethylethylenediamine); and/or E. coli Endonuclease IV. In some embodiments, the agent capable of labeling an abasic site is ARP, FARP, Alexa Fluor 555 hydrazide (Order No. A-20501, Molecule Probes, Eugene OR), and/or an aminooxy-modified Alexa Fluor 555 (see copending co-owned U.S. Patent Application Serial No. 10/441,663).

[0236] Kits may also include one or more suitable buffers (as described herein). One or more reagents in the kit can be provided as a dry powder, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentrations for performing any of the methods described herein. Each component can be packaged in separate containers or some components can be combined in one container where cross-reactivity and shelf life permit.

[0237] The kits of the invention may optionally include a set of instructions, generally written instructions, although electronic storage media (e.g., magnetic diskette or

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optical disk) containing instructions are also acceptable, relating to the use of components of the methods of the invention for the intended methods of the invention, and/or, as appropriate, for using the products for purposes such as, for example preparing a hybridization probe or target, expression profiling, preparing a microarray, or characterizing a nucleic acid. The instructions included with the kit generally include information as to reagents (whether included or not in the kit) necessary for practicing the methods of the invention, instructions on how to use the kit, and/or appropriate reaction conditions.

[0238] The component(s) of the kit may be packaged in any convenient, appropriate packaging. The components may be packaged separately, or in one or multiple combinations.

[0239] The relative amounts of the various components in the kits can be varied widely to provide for concentrations of the reagents that substantially optimize the reactions that need to occur to practice the methods disclosed herein and/or to further optimize the sensitivity of any assay.

[0240] The following examples are provided to illustrate, but not to limit, the invention.

EXAMPLES

Example 1: Acid-catalyzed Fragmentation and Labeling of cDNA.

[0241] Single stranded cDNA was prepared from universal human total RNA (Stratagene, La Jolla CA.). Pooled purified cDNA product at a concentration of 145 μg/mL in water was aliquoted into each of five 200 μL PCR tubes, dispensing 13 μL or 1.89 μg into each tube. 12 μL water was then added to each tube, followed by 2.5 μL of 0.5 M glycolic acid buffer (prepared by dissolving glycolic acid in water at a concentration of 1.0 M, adjusting pH with 1 M NaOH, then diluting to 0.5 M with water). Three tubes received buffer at a pH of 3.0, and two received buffer of pH 3.5. The pH 3 tubes were heated at 95°C for 5 minutes and 65° for 5 minutes or 30 minutes. The pH 3.5 tubes were heated at 65°C for 5 minutes or 30 minutes, all in a MJ Research Peltier Effect thermal cycler. Tubes were then held briefly on ice for the next step.

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[0242] To each tube was added 5 μL of 0.5 M acetate buffer pH 4.33 (prepared by pH adjustment of acetic acid with NaOH), followed by 1 μL of 1 M NaOH in each pH 3.0 tube and 0.7 μL of 1 M NaOH in pH 3.5 tubes. All tubes then received 2 μL of 0.2 M $MgCl_2$ and 2.7 μL of a biotinylating reagent. This reagent contained 11.7 mg/mL of ARP (N-(aminooxyacetyl)-N'-(D-biotinoyl) hydrazine, trifluoroacetic acid salt) (Molecular Probes, Eugene, OR) dissolved in a 22.5 mM solution of dibasic sodium phosphate. All tubes were then incubated at 50°C for 30 minutes, then 10 μL of 1 M Tris, pH 8.5 was added to each tube.

[0243] The contents of each tube were purified using a single CentriSep size exclusion column (Princeton Separations, Adelphia, N. J.) following the manufacturer's instructions. Concentration of recovered product was estimated from A_{260} assuming that 33 μg/mL of DNA gives $A_{260} = 1.0$. It was noted that the A_{260}/A_{280} ratio for the product heated at 95 °C was 1.75, while all other products gave ratios > 1.9. Recoveries were 60-69%.

[0244] Extent of fragmentation and labeling was determined using a gel shift assay. Two aliquots of product, each containing 50 ng DNA (3.1-3.5 μL), were mixed in PCR tubes with 0.5 μL of 10x TE buffer (0.1 M Tris, 10 mM EDTA, pH 7.5). To one aliquot was added 3 μL of a 2.5 mg/mL solution of streptavidin (Sigma-Aldrich, St. Louis, MO) in water. After about 3 minutes incubation, 3 μL of 30% glycerol/ bromophenol blue was added to each tube, and products were loaded and run on Novex 4-20% TBE gels (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Bands were visualized by staining with Sybr Green II (Molecular Probes, Eugene, OR) diluted 1:5,000 in gel buffer for 6 minutes, followed by imaging with an AlphaImager. Unreacted cDNA product and molecular weight markers were included as controls.

[0245] An image of the gel is shown in Figure 1. Lane 1 is molecular weight markers, lane 2 is unreacted starting material, and subsequent lanes are paired products without or with streptavidin treatment. Lane 3 (5 min at 95°C, pH 3.0) shows extensive fragmentation of product compared to control in Lane 2. Average apparent size appears to be reduced to 400-500 bases, and the high molecular weight material in Lane 2 is virtually absent. Lane 4 shows that nearly all of the fragmented DNA is retarded by streptavidin,

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appearing as a high molecular weight smear, much of which does not enter the gel. The other extreme was represented by lanes 11 and 12 (5 min at 65°C, pH 3.5), where the length without streptavidin appeared identical to control, and some but not all of the product was shifted to higher molecular weight by streptavidin. The other pairs of lanes represented intermediate conditions in which little fragmentation was evident, but various extents of labeling were seen, as measured by streptavidin shift. Compared to lanes 11 and 12, longer time (30 min in Lanes 9 and 10), lower pH (pH 3.0 in Lanes 5 and 6), or both lower pH and longer time (30 min at pH 3.0 in Lanes 7 and 8) resulted in increased labeling.

[0246] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entireties for all purposes.

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CLAIMS

We claim:

1. A method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) cleaving a base portion of the methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated;

(b) cleaving the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and

(c) labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

2. A method according to claim 1, wherein the agent capable of cleaving the base portion of the methylated nucleotide is selected from the group consisting of an enzyme, a chemical agent, and acidic conditions.

3. A method according to claim 1, wherein cleavage of the phosphodiester backbone is performed with an agent selected from the group consisting of an enzyme, a chemical agent, acidic conditions, basic conditions, and heat.

4. A method according to claim 1, wherein steps (a), (b), and (c) are performed simultaneously.

5. A method according to claim 1, wherein steps (a) and (b) are performed simultaneously.

6. A method according to claim 1, wherein steps (b) and (c) are performed simultaneously.

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7. A method according to claim 1, wherein step (b) is performed before step (c).

8. A method according to claim 1, wherein step (c) is performed before step (b).

9. A method according to claim 1, wherein the labeled polynucleotide fragment comprises a detectable label.

10. A method according to claim 1, further comprising hybridizing the labeled polynucleotide fragment to a probe.

11. A method for labeling a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) cleaving a base portion of the methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated; and

(b) labeling at the abasic site, whereby a labeled polynucleotide is generated.

12. A method according to claim 11, further comprising hybridizing the labeled polynucleotide to an array.

13. A method for fragmenting a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) cleaving a base portion of the methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated; and

(b) cleaving the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site, whereby polynucleotide fragments are generated.

14. A method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) incubating a reaction mixture, said reaction mixture comprising:

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(i) a polynucleotide comprising a methylated nucleotide; and

(ii) an agent capable of specifically cleaving a base portion of a methylated nucleotide;

wherein the incubation is under conditions that permit cleavage of the base portion of the methylated nucleotide, whereby a polynucleotide comprising an abasic site is generated;

(b) incubating a reaction mixture, said reaction mixture comprising:

(i) the polynucleotide comprising the abasic site; and

(ii) an agent capable of effecting cleavage of phosphodiester backbone at the abasic site,

wherein the incubation is under conditions that permit cleavage of the phosphodiester backbone at the abasic site, whereby fragments of the polynucleotide are generated; and

(c) incubating a reaction mixture, said reaction mixture comprising:

(i) a polynucleotide fragment comprising an abasic site; and

(ii) an agent capable of labeling at the abasic site,

wherein the incubation is under conditions that permit labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

15. A method for labeling and fragmenting a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving a methylated nucleotide;

(b) cleaving the phosphodiester backbone of the polynucleotide comprising the abasic site at the abasic site; and

(c) labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

16. A method according to claim 15, wherein the agent capable of cleaving a base portion of an unmethylated nucleotide comprises an enzyme.

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17. A method according to claim 16, wherein the unmethylated nucleotide is cytosine and said enzyme comprises cytosine deaminase in conjunction with uracil DNA glycosylase.

18. A method according to claim 15, wherein cleavage of the phosphodiester backbone is performed with an agent selected the group consisting of an enzyme, a chemical agent, acidic conditions, basic conditions, and heat.

19. A method according to claim 15, wherein the polynucleotide comprising a methylated nucleotide is contacted with a methyl binding agent prior to or during step (a).

20. A method according to claim 15, wherein steps (a), (b), and (c) are performed simultaneously.

21. A method according to claim 15, wherein steps (a) and (b) are performed simultaneously.

22. A method according to claim 15, wherein steps (b) and (c) are performed simultaneously.

23. A method according to claim 15, wherein step (b) is performed before step (c).

24. A method according to claim 15, wherein step (c) is performed before step (b).

25. A method according to claim 15, wherein the labeled polynucleotide fragment comprises a detectable label.

26. A method according to claim 15, further comprising immobilizing the labeled polynucleotide fragment to a substrate.

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27. A method for labeling a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving the base portion of a methylated nucleotide; and

(b) labeling at the abasic site, whereby a labeled polynucleotide is generated.

28. A method according to claim 27, further comprising hybridizing the labeled polynucleotide fragment to an array.

29. A method for fragmenting a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving the base portion of a methylated nucleotide; and

(b) cleaving the phosphodiester backbone of the polynucleotide comprising the abasic site, whereby polynucleotide fragments are generated.

30. A method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, said method comprising:

(a) incubating a reaction mixture, said reaction mixture comprising:

(i) a polynucleotide comprising a methylated nucleotide; and

(ii) an agent capable of cleaving a base portion of an unmethylated nucleotide, wherein the agent is not capable of cleaving a methylated nucleotide, and whereby a polynucleotide comprising an abasic site is generated;

(b) incubating a reaction mixture, said reaction mixture comprising:

(i) the polynucleotide comprising the abasic site; and

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(ii) an agent capable of effecting cleavage of a phosphodiester backbone at the abasic site, wherein the incubation is under conditions that permit cleavage of the phosphodiester backbone at the abasic site, whereby fragments of the polynucleotide are generated; and

(c) incubating a reaction mixture, said reaction mixture comprising:

(i) a polynucleotide fragment comprising an abasic site; and

(ii) an agent capable of labeling at the abasic site,

wherein the incubation is under conditions that permit labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

31. A method for fragmenting and labeling a polynucleotide comprising a canonical nucleotide, said method comprising:

(a) cleaving a base portion of a canonical nucleotide present in a polynucleotide comprising the canonical nucleotide with an agent capable of cleaving a base portion of the canonical nucleotide, whereby an abasic site is generated;

(b) cleaving the phosphodiester backbone at the abasic site; and

(c) labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

32. A method according to claim 31, wherein the agent capable of cleaving a base portion of a canonical nucleotide comprises an enzyme.

33. A method according to claim 32, wherein the canonical nucleotide is cytosine and said enzyme comprises cytosine deaminase in conjunction with uracil DNA glycosylase.

34. A method according to claim 31, wherein cleavage of the phosphodiester backbone is performed with an agent selected from the group consisting of an enzyme, a chemical agent, acidic conditions, basic conditions, and heat.

35. A method according to claim 31, wherein steps (a), (b), and (c) are performed simultaneously.

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36. A method according to claim 31, wherein steps (a) and (b) are performed simultaneously.

37. A method according to claim 31, wherein steps (b) and (c) are performed simultaneously.

38. A method according to claim 31, wherein step (b) is performed before step (c).

39. A method according to claim 31, wherein step (c) is performed before step (b).

40. A method according to claim 31, wherein the labeled polynucleotide fragment comprises a detectable label.

41. A method according to claim 31, further comprising immobilizing the polynucleotide fragment to a substrate.

42. A method for fragmenting and labeling a polynucleotide comprising a canonical nucleotide, said method comprising:

(a) incubating a reaction mixture, said reaction mixture comprising:

(i) a polynucleotide comprising a canonical nucleotide; and

(ii) an agent capable of specifically cleaving a base portion of a canonical nucleotide, wherein the incubation is under conditions that permit cleavage of the base portion of the canonical nucleotide, whereby a polynucleotide comprising an abasic site is generated;

(b) incubating a reaction mixture, said reaction mixture comprising:

(i) the polynucleotide comprising the abasic site; and

(ii) an agent capable of effecting cleavage of the phosphodiester backbone at the abasic site, wherein the incubation is under conditions that permit cleavage

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of the phosphodiester backbone at the abasic site, whereby fragments of the polynucleotide are generated; and

(c) incubating a reaction mixture, said reaction mixture comprising:

(i) a polynucleotide fragment comprising an abasic site; and

(ii) an agent capable of labeling at the abasic site, wherein the incubation is under conditions that permit labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

43. A method for characterizing a methylated polynucleotide, said method comprising detecting a polynucleotide fragment, wherein said polynucleotide fragment is prepared according to the method of any of claims 1, 13, 14, 15, 29, or 30, wherein detection of said polynucleotide fragment correlates with presence, absence, sequence, or amount of the methylated polynucleotide.

44. A method according to claim 43, wherein said detection comprises hybridizing the polynucleotide fragment to a probe.

45. A method for characterizing a methylated polynucleotide, said method comprising detecting a labeled polynucleotide, wherein said labeled polynucleotide is prepared according to the method of any of claims 1, 11, 14, 15, 27, or 30, wherein detection of said labeled polynucleotide correlates with presence, absence, sequence, or amount of the methylated polynucleotide.

46. A method according to claim 45, wherein said detection comprises hybridizing the labeled polynucleotide to a probe.

47. A composition comprising an agent capable of cleaving a base portion of a methylated nucleotide to produce an abasic site on a polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide.

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48. A composition according to claim 47, further comprising an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site.

49. A composition comprising an agent capable of cleaving a base portion of a canonical nucleotide to produce an abasic site on a polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide.

50. A composition according to claim 49, further comprising an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site.

51. A composition according to any of claims 47-50, further comprising a composite primer comprising a DNA portion and a 5' RNA portion.

52. A composition comprising a population of labeled polynucleotides produced by the method of any of claims 1, 11, 14, 15, 27, 30, 31, or 42.

53. A kit comprising an agent capable of cleaving a base portion of a methylated nucleotide to produce an abasic site on a polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide.

54. A kit according to claim 53, further comprising an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site.

55. A kit comprising an agent capable of cleaving a base portion of a canonical nucleotide to produce an abasic site on a polynucleotide and an agent capable of labeling at an abasic site on a polynucleotide.

56. A kit according to claim 55, further comprising an agent capable of cleaving a phosphodiester backbone of a polynucleotide at an abasic site.

57. A kit according to any of claims 53-56, further comprising a composite primer comprising a DNA portion and a 5' RNA portion.

58. A method according to any of claims 14, 15, 27, 30, 31, or 42, further comprising hybridizing the labeled polynucleotide to a probe.

59. A method according to claim 13 or 29, further comprising hybridizing the polynucleotide fragments to a probe.

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60. A method for fragmenting a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated.

61. A method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, and labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

62. A method for labeling a polynucleotide comprising a methylated nucleotide, said method comprising labeling at the abasic site of a polynucleotide comprising an abasic site, whereby a labeled polynucleotide is generated, wherein the abasic site is generated by cleaving a base portion of a methylated nucleotide with an agent capable of cleaving the base portion of the methylated nucleotide, whereby an abasic site is generated.

63. A method for fragmenting a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving a methylated nucleotide.

64. A method for fragmenting and labeling a polynucleotide comprising a methylated nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is

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generated, wherein the agent is not capable of cleaving a methylated nucleotide, and labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

65. A method for labeling a polynucleotide comprising a methylated nucleotide, said method comprising labeling at the abasic site of a polynucleotide comprising an abasic site, whereby a labeled polynucleotide is generated, wherein the abasic site is generated by cleaving a base portion of an unmethylated nucleotide with an agent capable of cleaving the base portion of the unmethylated nucleotide, whereby an abasic site is generated, wherein the agent is not capable of cleaving a methylated nucleotide.

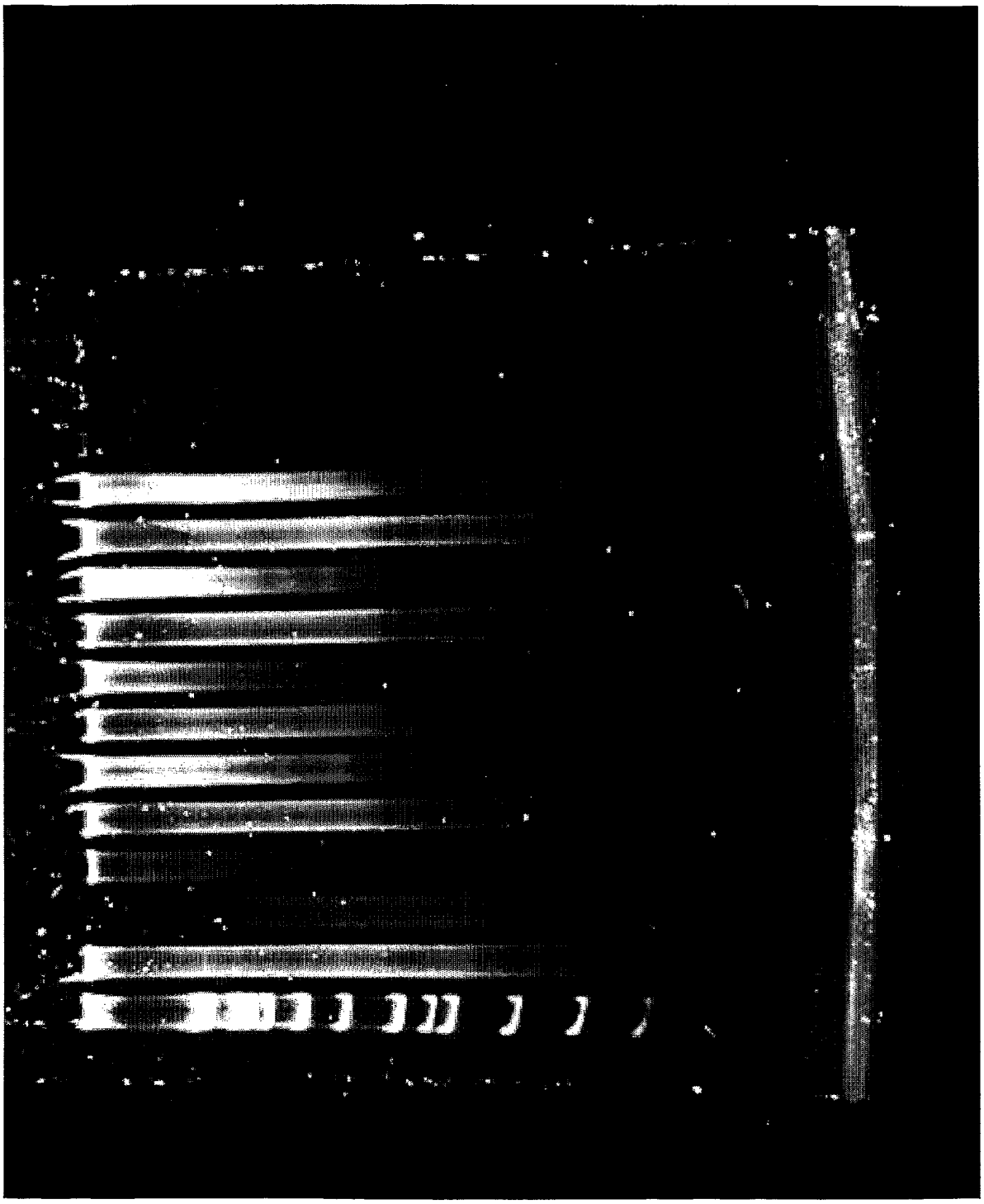
66. A method for fragmenting a polynucleotide comprising a canonical nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a canonical nucleotide with an agent capable of cleaving the base portion of the canonical nucleotide, whereby an abasic site is generated.

67. A method for fragmenting and labeling a polynucleotide comprising a canonical nucleotide, said method comprising cleaving the phosphodiester backbone of a polynucleotide comprising an abasic site at the abasic site, wherein the abasic site is generated by cleaving a base portion of a canonical nucleotide with an agent capable of cleaving the base portion of the canonical nucleotide, whereby an abasic site is generated, and labeling at the abasic site, whereby a labeled polynucleotide fragment is generated.

68. A method for labeling a polynucleotide comprising a canonical nucleotide, said method comprising labeling at the abasic site of a polynucleotide comprising an abasic site, whereby a labeled polynucleotide is generated, wherein the abasic site is generated by cleaving a base portion of a canonical nucleotide with an agent capable of cleaving the base portion of the canonical nucleotide, whereby an abasic site is generated.

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Figure 1



Espacenet

Bibliographic data: WO2004092418 (A2) — 2004-10-28

GLOBAL AMPLIFICATION USING A RANDOMLY PRIMED COMPOSITE PRIMER

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Classification: **- international:** ***C12P19/34; C12Q1/68;*** (IPC1-7): C12Q1/68
- cooperative: **C12P19/34**; **C12Q1/6844**; **C12Q1/6853** more

Application number: WO2004US12779 20040414 Global Dossier

Priority number (s): US20030462962P 20030414 ; US20030462965P 20030414

Also published as: WO2004092418 (A3) AU2004230494 (A1) CA2521084 (A1) EP1613778 (A2) EP1613778 (B1) more

Abstract of WO2004092418 (A2)

The invention relates to the field of polynucleotide amplification. More particularly, the invention provides methods, compositions and kits for amplification of (i.e., making multiple copies of) a multiplicity of different polynucleotide template sequences using a randomly primed RNA/DNA composite primer.

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 October 2004 (28.10.2004)

PCT

(10) International Publication Number
WO 2004/092418 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/US2004/012779

(22) International Filing Date: 14 April 2004 (14.04.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/462,962 14 April 2003 (14.04.2003) US
60/462,965 14 April 2003 (14.04.2003) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: GLOBAL AMPLIFICATION USING A RANDOMLY PRIMED COMPOSITE PRIMER

(57) Abstract: The invention relates to the field of polynucleotide amplification. More particularly, the invention provides methods, compositions and kits for amplification of (i.e., making multiple copies of) a multiplicity of different polynucleotide template sequences using a randomly primed RNA/DNA composite primer.

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GLOBAL AMPLIFICATION USING A RANDOMLY PRIMED COMPOSITE PRIMER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Applications Nos. 60/462,962, filed April 14, 2003, and 60/462,965, filed April 14, 2003, each of which is incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] The invention relates to the field of polynucleotide amplification. More particularly, the invention provides methods, compositions and kits for amplifying (i.e., making multiple copies of) a multiplicity of different polynucleotide template sequences using a randomly primed RNA/DNA composite primer.

BACKGROUND ART

[0003] The quality and quantity of nucleic acid (e.g. genomic DNA) sample is important for many studies. High-throughput genomic analysis requires large amounts of template for testing, yet typically the yield of nucleic acids from individual patient samples is limited. Forensic and paleoarcheology work also can be severely limited by nucleic acid sample size. The limitation of starting material impacts the ability to carry out large scale analysis of multiple parameters, as is required for, for example, the genotyping of multiple loci in the study of complex diseases. Moreover, it is well accepted that molecular analysis determination of genomic instability in various pathological condition such as cancer, is most precisely carried out in well defined cell populations, such as that obtained by laser capture micro-dissection or cell sorting. Nucleic acid amplification technologies that provide global amplification of very small polynucleotide samples, for example, from one or a very few cells, may provide a solution to the limited starting materials generally available for analysis.

[0004] Likewise, the ability to amplify ribonucleic acid (RNA) is an important aspect of efforts to elucidate biological processes. Total cellular mRNA represents gene expression activity at a defined time. Gene expression is affected by cell cycle progression,

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developmental regulation, response to internal and external stimuli and the like. The profile of expressed genes for any cell type in an organism reflects normal or disease states, response to various stimuli, developmental stages, cell differentiation, and the like. Non-coding RNAs have been shown to be of great importance in regulation of various cellular functions and in certain disease pathologies. Such RNAs are often present in very low levels. Thus, amplification methods capable of amplifying low abundance RNAs, including RNAs that are not polyadenylated, are of great importance.

[0005] Various methods for global amplification of DNA target molecules (e.g., whole genome amplification) have been described, including methods based on the polymerase chain reaction (PCR). *See, e.g.*, U.S. Patent Nos. 5,731,171; 6,365,375; Daigo et al., (2001) Am. J. Pathol. 158 (5):1623-1631; Wang et al, (2001); Cancer Res. 61:4169-4174; Zheng et al, (2001) Cancer Epidemiol. 10:697-700; Dietmaier et al (1999) Am. J. Pathol. 154 (1) 83-95; Stoecklein et al (2002) Am. J. Pathol. 161 (1):43-51; U.S. Patent Nos. 6,124,120; 6,280,949; Dean et al (2002) PNAS 99 (8):5261-5266. However, PCR-based global amplification methods, such as whole genome amplification (WGA), may generate non-specific amplification artifacts, give incomplete coverage of loci, or generate DNA of insufficient length that cannot be used in many applications. PCR-based methods also suffer from the propensity of the PCR reaction to generate products that are preferentially amplified, and thus resulting in biased representation of genomic sequences in the products of the amplification reaction.

[0006] Additionally, a number of methods for the analysis of gene expression have been developed in recent years. See, for example, U.S. Pat. Nos. 6,251,639, 6,692,918, 6,686,156, 5,744,308; 6,143,495; 5,824,517; 5,829,547; 5,888,779; 5,545,522; 5,716,785; 5,409,818; EP 0971039A2; EP0878553A2; and U.S. published patent applications nos. 2002/0115088, 2003/0186234, 2003/0087251, and 2004/0023271. These include quantification of specific mRNAs, and the simultaneous quantification of a large number of mRNAs, as well as the detection and quantification of patterns of expression of known and unknown genes. RNA amplification is most commonly performed using the reverse transcriptase-polymerase chain reaction (RT-PCR) method and variations thereof. These methods are based on replication of RNA by reverse transcriptase to form single stranded DNA complementary to the RNA (cDNA), which is followed by polymerase chain reaction (PCR) amplification to produce multiple copies of double stranded DNA. Although these methods are most commonly used, they have some significant drawbacks: a) the reactions

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require thermocycling; b) the products are double stranded, thus rendering them less accessible to binding to probes; and c) the reactions are prone to contamination with products of prior amplification, thus requiring strict containment of reaction mixtures. Other current RNA amplification methods use initiation of replication of mRNA from the poly-A tail at their 3' ends. However, not all RNA transcripts have a mRNA tail (for example, prokaryotic RNAs and non-coding RNAs). In addition, due to sample preparation procedures, the RNA transcript structural integrity is compromised. Thus, it may be desirable in certain circumstances to use RNA amplification methods that do not require initiation of replication at the defined poly-A tail. Although analysis of non-amplified RNA is feasible, a significant amount of starting RNA would be required. However, the total amount of sample RNA that is available is frequently limited by the amount of biological sample from which it is derived. Biological samples are often limited in amount and precious. Moreover, the amount of the various RNA species is not equal; some species are more abundant than others are, and these are more likely and easier, to analyze. The ability to amplify RNA sequences enables the analysis of less abundant, rare RNA species. The ability to analyze small samples, by means of nucleic acid amplification, is also advantageous for design parameters of large scale screening of effector molecule libraries, for which reduction in sample volume is a major concern both for the ability to perform very large scale screening or ultra high throughput screening, and in view of the limiting amounts of library components.

[0007] Therefore, there is a need for improved amplification methods, particularly methods which can globally amplify DNA or RNA polynucleotide targets. The invention described herein fulfills this need.

[0008] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

DISCLOSURE OF THE INVENTION

[0009] The invention provides methods, compositions, and kits for isothermal global amplification using a randomly hybridized RNA/DNA composite primer, as well as applications of the amplification methods.

[0010] Accordingly, in one aspect, the invention provides methods for amplification of a template polynucleotide, said methods comprising: (a) incubating a

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reaction mixture, said reaction mixture comprising: (i) a template polynucleotide; (ii) a first primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) a DNA-dependent DNA polymerase ; and (iv) an RNA-dependent DNA polymerase (which may be present as a separate enzyme or as an enzyme comprising both DNA-dependent DNA polymerase and RNA-dependent DNA polymerase activities); wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and, in some embodiments, displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising (i) the reaction products generated according to step (a) (or an aliquot thereof); (ii) a composite primer (which may be the same as the first primer, or may be a different primer), wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) an DNA-dependent DNA polymerase; and (iv) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit RNA cleavage from an RNA/DNA heteroduplex, primer hybridization, primer extension, and displacement of the primer extension product from the complex of (a) when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide (generally, DNA) amplification product are generated. In embodiments wherein the template polynucleotide is RNA, the reaction mixture of step (a) further comprises (v) an agent (such as an enzyme)that cleaves RNA from an RNA/DNA hybrid, whereby template RNA is cleaved form the complex comprising template RNA and first primer extension product. In some embodiments, the reaction mixture of step (b) comprises the reaction mixture according to step (a) (or an aliquot thereof). In other embodiments, step (b) is initiated by the addition of an agent that cleaves RNA from a partial RNA/DNA heteroduplex (such as RNase H), and optionally, a DNA-dependent DNA polymerase, to the reaction mixture of step (a). In some embodiments, the reaction mixture of step (a) and/or (b) further comprises auxiliary primers. In some embodiments (generally embodiments in which the template polynucleotide is DNA), the RNA-dependent DNA polymerase may be omitted from reaction mixture (a).

[0011] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture, said reaction mixture

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comprising: (a) a complex comprising a RNA/DNA partial heteroduplex, wherein the complex is generated by incubating a first reaction mixture, said first reaction mixture comprising: (i) a polynucleotide template; (ii) a first primer; wherein the first primer is a composite primer, the composite primer comprising a RNA portion and a 3' DNA portion; and wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites; (iii) a DNA-dependent DNA polymerase; and (iv) an RNA-dependent DNA polymerase; wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; (b) a composite primer, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated. In some embodiments, the reaction mixture and/or first reaction mixture further comprises auxiliary primers. In some embodiments wherein the template polynucleotide is RNA, template RNA in step (a) is cleaved following primer extension via conditions or agents promoting cleavage. In some embodiments, the first reaction mixture further comprises: (v) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid, whereby template RNA is cleaved from the complex comprising template RNA and composite primer extension product. In some embodiments (generally those in which the template polynucleotide is DNA), the complex comprising a RNA/DNA partial heteroduplex may be generated without the use of RNA-dependent DNA polymerase.

[0012] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture, said reaction mixture comprising: (a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a randomly primed first primer hybridized to target polynucleotide with a DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites, and wherein the second primer extension product is generated by

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extension of a second primer hybridized to the first primer extension product; (b) a composite primer that is hybridizable to the second primer extension product, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated. In some embodiments wherein the template polynucleotide is RNA, the first primer extension product is generated by extension of a randomly primed first primer hybridized to target RNA with a RNA-dependent DNA polymerase. In some embodiments, the first primer extension product and/or the second primer extension product are generated in the presence of auxiliary primers.

[0013] In another aspect, the invention provides methods for amplification of a polynucleotide template comprising: (a) random priming of polynucleotide template strand with a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion, and wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites; and (b) incubating template strand in the presence of a DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and an agent that cleaves RNA from an RNA/DNA, whereby multiple copies of polynucleotide amplification product are generated via primer extension and strand displacement. In some embodiments, random priming occurs in the presence of a DNA polymerase. In some embodiments, auxiliary primers are included in step (a) and/or step (b). In some embodiments (generally those in which the polynucleotide template is DNA), the RNA-dependent DNA polymerase is omitted from the incubation.

[0014] In another aspect, the invention provides methods for amplification of RNA template polynucleotides which operate as follows: a multiplicity of template polynucleotide sequences are amplified by incubating a reaction mixture, the reaction mixture comprising: (a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a first primer hybridized to template RNA strand with an RNA-dependent DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of sites on

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template RNA; wherein the second primer extension product is generated by extension of a second primer hybridized to the first primer extension product; and wherein RNA from the complex of first and second primer extension product is cleaved (using e.g., an enzyme that cleaves RNA from an RNA/DNA hybrid or conditions permitting cleavage, such as heat and/or alkaline conditions); (b) a composite primer that is hybridizable to the second primer extension product, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds to the second primer extension product and is extended, whereby multiple copies of polynucleotide amplification product are generated. In some embodiments, the complex of step (a) is generated in the presence of auxiliary primers. In some embodiments, the second primer comprises fragment(s) of cleaved RNA template.

[0015] In another aspect, the invention provides methods for amplification of a template polynucleotide by (a) randomly priming a template polynucleotide with a first primer, wherein said first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises a RNA portion and a 3' DNA portion; (b) extending the first primer with a DNA polymerase; (c) cleaving RNA from the first primer with an agent that cleaves RNA from a RNA/DNA heteroduplex; (d) hybridizing an amplification primer to the template polynucleotide, wherein said amplification primer is a composite primer comprising a RNA portion and a 3' DNA portion; (e) extending the hybridized amplification primer by strand displacement DNA synthesis; and (f) cleaving RNA from the amplification primer with an agent that cleaves RNA from a RNA/DNA heteroduplex, such that another amplification primer can hybridize and be extended, whereby multiple copies of a polynucleotide amplification product are generated.

[0016] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture including: (a) a polynucleotide template strand; (b) a first primer, wherein said first primer is a composite primer comprising a RNA portion and a 3' DNA portion, and wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites; (c) a DNA-dependent DNA

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polymerase; (d) a RNA-dependent DNA polymerase; and (e) an agent that cleaves RNA from a RNA/DNA heteroduplex, whereby multiple copies of polynucleotide amplification product are generated by primer extension and strand displacement. In some embodiments (generally those embodiments in which the polynucleotide template is DNA), the RNA-dependent DNA polymerase is omitted from the reaction mixture.

[0017] As is clear to one skilled in the art, reference to production of copies of a polynucleotide (*e.g.*, DNA or RNA) template or copies of a polynucleotide sequence complementary to a polynucleotide template refers to products that may contain, comprise or consist of such sequences. As is evident to one skilled in the art, aspects that refer to combining and incubating the resultant mixture also encompasses method embodiments which comprise incubating the various mixtures (in various combinations and/or subcombinations) so that the desired products are formed.

[0018] Various embodiments of the composite primer(s) used in the methods of the invention are described herein. For example, in some embodiments, the RNA portion of a composite primer is 5' with respect to the 3' DNA portion. In still other embodiments, the 5' RNA portion is adjacent to the 3' DNA portion. In other embodiments, the RNA portion of the composite primer consists of 7 to about 20 nucleotides and the DNA portion of the composite primer consists of about 5 to about 20 nucleotides. In still other embodiments, the RNA portion of the composite primer consists of about 10 to about 20 nucleotides and the DNA portion of the composite primer consists of about 7 to about 20 nucleotides. In some embodiments the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0019] In some embodiments, the composite primer comprises random sequence or partially randomized sequence, although certain embodiments (such as certain embodiments wherein the template polynucleotide is RNA) exclude the use of primers comprising random or partially random sequence. In embodiments utilizing a composite primer with random or partially random sequence, the composite primer may be a population or pool of different primers comprising at least 2, at least 3, at least 4, at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, or at least 100 different sequences. In other embodiments, the composite primer contains one or more "degenerate"

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nucleotides that is able to hybridize to multiple different nucleotide bases (*e.g.*, inosine, which is able to hybridize to all four canonical bases).

[0020] In some embodiments, the composite primer that hybridizes to target polynucleotide (such as mRNA or genomic DNA) and the composite primer used during single primer isothermal amplification (i.e., phase (b) of the methods) are the same. In some embodiments, the composite primer that hybridizes to target polynucleotide (such as mRNA or genomic DNA) and the composite primer used during single primer isothermal amplification are different. In some embodiments, two (or more) different composite primers that hybridize to target polynucleotide are used in the methods of the invention.

[0021] The methods are applicable to amplifying any target polynucleotide, including, for example, DNA (such as genomic DNA, including human and other mammalian genomic DNA) and RNA (such as total RNA, mRNA, noncoding RNA and ribosomal RNA). One or more steps may be combined and/or performed sequentially (often in any order, as long as the requisite product(s) are able to be formed), and, as is evident, the invention includes various combinations of the steps described herein. It is also evident, and is described herein, that the invention encompasses methods in which the initial, or first, step is any of the steps described herein. For example, the methods of the invention do not require that the first step be random hybridization of composite primer. Methods of the invention encompass embodiments in which later, "downstream" steps are an initial step.

[0022] The enzymes which may be used in the methods and compositions are described herein. For example, the agent (such as an enzyme) that cleaves RNA may be an RNase H, and the RNA-dependent DNA polymerase may be reverse transcriptase. The RNA-dependent DNA polymerase may comprise an RNase H enzyme activity, or separate enzymes may be used. Similarly, a DNA polymerase may comprise both RNA-dependent and DNA-dependent DNA polymerase enzyme activities, or separate enzymes may be used. A DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and the enzyme that cleaves RNA can also be the same enzyme, or separate enzymes comprising each of these activities may be used.

[0023] In some embodiments, methods of the invention are used to generate labeled polynucleotide products (generally DNA products). In some embodiments of methods for generating labeled DNA products, at least one type of dNTP used is a labeled dNTP. In

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other embodiments of methods for generating labeled DNA products, a labeled composite primer is used.

[0024] The invention also provides methods which employ (usually, analyze) the products of the amplification methods of the invention, such as detection of sequence alteration(s) (*e.g.*, genotyping, nucleic acid mutation detection, analysis of splice variants, and the like); determining presence or absence of a sequence of interest; quantifying a sequence of interest; gene expression profiling; subtractive hybridization; preparation of subtractive hybridization probe; differential amplification; preparation of libraries (including genomic, cDNA and differential expression libraries); preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray, preparing labeled probes for analysis on arrays (including high density arrays) for the detection and quantification of sequences of interest, including, for example, sequence determination, detecting sequence variation and genotyping; comparative genome hybridization; detection and/or identification of novel RNAs; and characterizing nucleic acids using the amplification nucleic acid products generated by the methods of the invention.

[0025] Any of the methods of the invention can be used to generate polynucleotide products that are suitable for characterization of a polynucleotide sequence of interest in a sample. In one embodiment, the invention provides methods for characterizing (for example, detecting (presence or absence) and/or quantifying) a polynucleotide sequence of interest comprising: (a) amplifying a target polynucleotide by any of the methods described herein; and (b) analyzing the amplification products. Step (b) of analyzing the amplification products can be performed by any method known in the art or described herein, for example by detecting and/or quantifying amplification products that are hybridized to a probe. These amplification products may or may not be labeled. Any of the methods of the invention can be used to generate polynucleotide (such as DNA) products that are labeled by incorporating labeled nucleotides and/or labeled composite primers into appropriate step(s) of the methods. These labeled products are particularly suitable for quantification and/or identification by methods known in the art, which include the use of arrays such as cDNA microarrays and oligonucleotide arrays. In one aspect, the invention provides a method of characterizing a polynucleotide sequence of interest, comprising (a) amplifying a target polynucleotide by a method described herein to generate labeled polynucleotide products; and (b) analyzing the labeled polynucleotide products. In

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some embodiments, the step of analyzing polynucleotide products comprises determining amount of said products, whereby the amount of the polynucleotide sequence of interest present in a sample is quantified.

[0026] The amplification products can also serve as template for further analysis such as sequence analysis, polymorphism detection (including multiplex SNP detection) using, e.g., oligonucleotide ligation-based assays, analysis using Invader, Cleavase or limited primer extension, and other methods known in the art. For methods that generally require larger volumes of input material, the methods of the invention may be used to "pre" amplify a pool of polynucleotides to generate sufficient input material for subsequent analysis.

[0027] In another embodiment, the polynucleotide products can be analyzed by, for example, contacting them with at least one probe. In some embodiments, the at least one probe is provided as a microarray. The microarray can comprise at least one probe immobilized on a solid or semi-solid substrate fabricated from a material selected from the group consisting of paper, glass, ceramics, plastic, polypropylene, polystyrene, nylon, polyacrylamide, nitrocellulose, silicon, other metals, and optical fiber. A probe can be immobilized on the solid or semi-solid substrate in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and cylinders.

[0028] In another aspect, the invention provides methods of determining a gene expression profile in a sample, the methods comprising (a) amplifying RNA template in a sample using any of the methods described herein; and (b) determining an amount of amplification products of each RNA sequence of interest in the sample, whereby the gene expression profile of the sample is determined. The invention further provides methods of determining a gene expression profile by determining an amount of amplification products of each RNA sequence of interest in a sample, the sample comprising multiple copies of RNA template amplified by any of the methods described herein, whereby the gene expression profile of the sample is determined.

[0029] Additionally, the invention also provides methods for archiving polynucleotide templates. Because the amplification methods of the invention provide representative amplification of the sequences of the template polynucleotide, amplified product produced by the instant methods may be used as an archival source for the original template polynucleotide. Accordingly, the invention provides methods for archiving a

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polynucleotide template by storing the amplification products produced by the methods of the invention. The archived amplification products may be analyzed as described herein, or may be subjected to further amplification in accordance with the methods of the invention.

[0030] In another aspect, the invention provides products (*e.g.*, multiple copies of a template polynucleotide) produced by the methods disclosed herein.

[0031] The invention also provides compositions, kits, complexes, reaction mixtures and systems comprising various components (and various combinations of the components) used in the amplification methods described herein.

[0032] In another aspect, the invention provides compositions comprising any of the complexes (which are generally considered as intermediates with respect to the final amplification products) described herein.

[0033] In another aspect, the invention includes any one or more products (including intermediates) and compositions comprising the products (including intermediates) produced by any aspect of the methods of the invention.

[0034] In another aspect, the invention provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein.

[0035] In another aspect, the invention provides kits for conducting the methods described herein. These kits, in suitable packaging and generally (but not necessarily) containing suitable instructions, contain one or more components used in the amplification methods.

[0036] In another aspect, the invention provides systems for effecting the amplification methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] Figure 1 illustrates one embodiment of a composite primer useful in the methods of the present invention. As illustrated in the Figure, the composite primer comprises a DNA portion at its 3' end and an RNA portion at its 5' end. As discussed herein, it is also possible to employ a composite primer in which the 3' DNA portion is followed, in the direction of its 5', by an RNA portion, which is followed by a portion which is DNA.

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[0038] Figure 2 illustrates a composite primer that hybridizes to multiple sites on a template polynucleotide where differing portions of the composite primer are hybridized to template polynucleotide depending on the site at which it is hybridized.

[0039] Figure 3 illustrates primer extension from composite primers that are hybridized at multiple sites on a template strand, where a composite primer extension products is being displaced by primer extension from a composite primer hybridized at a downstream site on the template strand.

[0040] Figure 4 shows a collection of composite primer extension products comprising composite primer 1 linked (via extension) to sequences corresponding to a multiplicity of target polynucleotide sequences.

[0041] Figure 5 shows generation of second primer extension product that is randomly primed by the composite primer.

[0042] Figure 6 shows single primer isothermal amplification using the complex comprising a RNA/DNA partial heteroduplex as a template for further composite-primer dependent amplification.

[0043] Figure 7 illustrates primer extension from composite primers and auxiliary primers that are hybridized at multiple sites on a template strand.

[0044] Figure 8 illustrates generation of a second primer extension product primed by auxiliary primers hybridized to composite primer extension product.

[0045] Figure 9 shows a photograph of a gel showing amplified reaction product generated using a single randomly primed composite primer to amplify a multiplicity of template polynucleotide sequences from human genomic DNA.

MODES FOR CARRYING OUT THE INVENTION

Overview of the Invention and Its Advantages

[0046] The invention discloses novel methods, compositions and kits for global amplification. The methods provide for amplification using a composite primer that is capable of binding to multiple sites within template polynucleotide (e.g., mRNA or genomic DNA), whereby a large multiplicity of template polynucleotide sequences (for example, essentially all genomic DNA) is amplified. The methods are suitable for use with either DNA or RNA template. They generate polynucleotide (generally, DNA) products, which are readily suitable for a variety of uses including comparative genome

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hybridization, expression profiling, and multiple genotype determinations, e.g., by multiplex analysis by microarray technologies. The methods are amenable to automation and do not require thermal cycling. Thus, one of the major advantages of the methods of the invention is the ability to amplify an entire pool of sequences (or a subset thereof, depending on the desired extent of amplification), which is essential for application such as comparative genome hybridization, generation of cDNA libraries, generation of subtractive hybridization probes, and array based assays, including multiple genotype determinations.

[0047] The amplification methods of the invention involve composite primer random hybridization, primer extension and displacement of composite primer extension product by strand displacement, whereby a complex comprising a RNA-DNA heteroduplex is generated, followed by composite primer-dependent isothermal amplification using the complex as a substrate for further amplification, an aspect that permits rapid amplification and distinguishes the invention from other strand displacement amplification methods, such as MDA.

[0048] In one aspect, the methods of the invention involve two phases: (a) composite primer random hybridization to template polynucleotide, primer extension and displacement of composite primer extension product by strand displacement DNA synthesis, whereby a complex comprising a RNA/DNA partial heteroduplex is generated, and (b) composite primer-dependent single primer isothermal amplification using the complex comprising a RNA/DNA partial heteroduplex as a substrate for further amplification.

[0049] The methods generally comprise using specially-designed primers, generally a RNA/DNA composite primer, to randomly prime template polynucleotide (such as genomic DNA template, mRNA or noncoding RNA). By "randomly prime" or "random hybridization", as used herein, it is meant that the composite primer hybridizes to multiple sites within template polynucleotide. In some embodiments, an aspect of the invention is displacement of primer extension product from template polynucleotide(s) during primer extension by strand displacement DNA synthesis (*e.g.*, by primer extension with a DNA polymerase having strand displacement activity) of primers hybridized at a downstream position(s) on the template.

[0050] Thus, the invention provides methods of incubating a reaction mixture, said reaction mixture comprising: (a) a polynucleotide template; (b) a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion, and

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wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites; (c) a DNA-dependent DNA polymerase with strand displacement activity; and (d) an RNA-dependent DNA polymerase; wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and displacement of the primer extension product from template polynucleotide, whereby a population of intermediate complexes are generated that generally includes (a) copies of template polynucleotide and/or copies of the complement of polynucleotide sequence appended (via extension) to composite primer sequences; and (b) copies of template polynucleotide and copies of the complement of template polynucleotide appended (via extension) to the complement of composite primer sequences. The intermediate complexes may be double-stranded or may be partially double stranded. By virtue of the presence of composite primer sequences in the intermediate complexes, the complexes comprise a RNA/DNA heteroduplex partial heteroduplex. The RNA portion of the RNA/DNA partial heteroduplex generally is introduced by the RNA portion of the composite primer, and the DNA portion of the heteroduplex is made of the complement of the RNA portion of the composite primer. For simplicity, this population of intermediate complexes is termed "a complex comprising an RNA/DNA partial heteroduplex."

[0051] Generally, the composite primer comprises at least a 3' DNA portion that is capable of randomly priming template polynucleotide. Thus, and as the description makes clear, reference to a primer that hybridizes to a sequence encompasses embodiments in which at least a portion of the primer is hybridized, embodiments in which two (or more portions) of the primer are hybridized (separated by unhybridized (looped out) portions of the primer), and embodiments in which the entire primer is hybridized.

[0052] The complex comprising a RNA/DNA partial heteroduplex is substrate for further amplification as follows: an enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNase H) cleaves RNA sequence from the hybrid, leaving a 3' single stranded DNA sequence available for binding by a composite primer (which may or may not be the same as the first composite primer). Extension of a bound composite primer by DNA-dependent DNA polymerase produces a primer extension product, which displaces the previously bound cleaved first primer extension product, whereby polynucleotide (generally, DNA) product accumulates. It is understood that amplified product generally is a mixture of sense and antisense copies of a given template polynucleotide. For example, if the template polynucleotide is double stranded DNA, the amplification product will

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correspond to each strand. If the template polynucleotide is single stranded, amplification product will generally be produced that is the copy of template polynucleotide (sense copy) and the complement of the template polynucleotide (antisense copy).

[0053] The methods disclosed herein are applicable to the amplification of any target polynucleotide, including both DNA (*e.g.*, genomic DNA) and RNA (*e.g.*, mRNA and ribosomal RNA) targets. As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

[0054] In another aspect of the invention, auxiliary primers are present in the reaction mixture comprising template polynucleotide, composite primer, DNA-dependent DNA polymerase and RNA-dependent DNA polymerase. As used herein, "auxiliary primers" refers to a population of random and/or partially randomized primers. Inclusion of a population of random primers during the amplification is believed to enhance the efficiency of production of and/or global (template-wide, *e.g.*, providing representative amplification of the template, whether the template is DNA or RNA) coverage of the amplification product.

[0055] In certain aspects, global amplification of genomic DNA is exemplified herein. It is understood, however, that the amplification methods of the invention are suitable for amplification of any pool or subset (or polynucleotides representing a significant proportion of a pool or subset, depending on desired extent of amplification) of polynucleotides.

[0056] Accordingly, in one aspect, the invention provides methods for amplification of a template polynucleotide, said methods comprising: (a) incubating a reaction mixture, said reaction mixture comprising: (i) a template polynucleotide; (ii) a first primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) a DNA-dependent DNA polymerase ; and (iv) an RNA-dependent DNA polymerase (which may be present as a separate enzyme or as an enzyme comprising both DNA-dependent DNA polymerase and RNA-dependent DNA polymerase activities); wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture

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comprising (i) the reaction products generated according to step (a) (or an aliquot thereof); (ii) a composite primer (which may be the same as the first primer, or may be a different primer), wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) an DNA-dependent DNA polymerase; and (iv) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product (*i.e.*, strand displacement DNA synthesis) from the complex of (a) when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide (generally, DNA) amplification product are generated. In some embodiments, the reaction mixture of step (b) comprises the reaction mixture according to step (a) (or an aliquot thereof). In other embodiments, step (b) is initiated by the addition of an agent that cleaves RNA from an RNA/DNA heteroduplex (such as RNase H), and optionally, a DNA-dependent DNA polymerase, to the reaction mixture of step (a). In some embodiments, the reaction mixture of step (a) and/or (b) further comprises auxiliary primers.

[0057] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture, said reaction mixture comprising: (a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a randomly primed first primer hybridized to target polynucleotide with a DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites, and wherein the second primer extension product is generated by extension of a second primer hybridized to the first primer extension product; (b) a composite primer that is hybridizable to the second primer extension product, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated. In some embodiments, the first primer extension product and/or the second primer extension product are generated in the presence of auxiliary primers.

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[0058] In another aspect-of the invention wherein the template polynucleotide is RNA, template RNA is cleaved following random composite primer hybridization and primer extension. Template RNA can be cleaved using methods well-known in the art, including cleavage with an agent (such as an enzyme, such as RNase H) that cleaves RNA from an RNA/DNA hybrid, cleavage resulting from heat treatment, and cleavage due to chemical treatment (e.g., treatment under alkaline conditions). In some embodiments, the invention provides methods of incubating a reaction mixture, said reaction mixture comprising: (a) an RNA template; (b) a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion; and wherein the composite primer is capable of hybridizing to a multiplicity of sites in template RNA; (c) a DNA-dependent DNA polymerase; (d) an RNA-dependent DNA polymerase; and (e) an enzyme capable of cleaving RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit composite primer random hybridization, primer extension, template RNA cleavage from an RNA/DNA heteroduplex, whereby a complex comprising an RNA/DNA partial heteroduplex is generated. The complex comprising an RNA/DNA partial heteroduplex is the substrate for further amplification as described above (i.e., single primer isothermal amplification). In some embodiments, an auxiliary primer is included in the reaction mixture.

[0059] Template polynucleotide may also be prepared from RNA template by synthesis of cDNA. cDNA synthesis may be accomplished using standard methods, such as priming with random primers (*e.g.*, random hexamer deoxyoligonucleotides) and primer extension with a RNA-dependent DNA polymerase (*e.g.*, reverse transcripatase) in the presence of dNTPs and appropriate reaction conditions (*e.g.*, temperature, pH and ionic conditions). Only the first strand cDNA synthesis need be performed, as first strand cDNA synthesis will produce a DNA polynucleotide that can be amplified in accordance with the methods of the invention.

[0060] The methods of the invention include methods using the amplification products (so-called "applications"). The invention also provides methods which employ (usually, analyze) the products of the amplification methods of the invention, such as methods of nucleic acid mutation detection (including methods of genotyping), determining the presence or absence of a sequence of interest, quantitating a sequence of interest, preparation of an immobilized nucleic acid, comparative genomic hybridization, discovery

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of novel nucleic acid sequences, and characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention.

[0061] Any of the methods of the invention can be used to generate polynucleotide products that are suitable for characterization of a polynucleotide sequence of interest in a sample. In one embodiment, the invention provides methods for characterizing (for example, detecting (presence or absence) and/or quantifying) a polynucleotide sequence of interest comprising: (a) amplifying a target polynucleotide by any of the methods described herein; and (b) analyzing the amplification products. Step (b) of analyzing the amplification products can be performed by any method known in the art or described herein, for example by detecting and/or quantifying amplification products that are hybridized to a probe. These amplification products may or may not be labeled. Any of the methods of the invention can be used to generate polynucleotide (such as DNA) products that are labeled by incorporating labeled nucleotides and/or labeled composite primers into appropriate step(s) of the methods. These labeled products are particularly suitable for quantification and/or identification by methods known in the art, which include the use of arrays such as cDNA microarrays and oligonucleotide arrays.

[0062] The invention provides methods to characterize (for example, detect presence or absence of and/or quantify) an polynucleotide sequence of interest by generating polynucleotide products using amplification methods of the invention, and analyzing the products by detection/quantification methods such as those based on array technologies or solution phase technologies. These amplification products may be labeled or unlabeled.

[0063] The methods of the invention may be used to amplify a pool of polynucleotides (or polynucleotides representing a significant proportion of a pool, depending on desired extent of amplification) to generate sufficient input material for subsequent analysis. Thus, and as is described herein, amplification products can also serve as template for further analysis such as sequence, polymorphism detection (including multiplex SNP detection) using, e.g., oligonucleotide ligation-based assays, analysis using Invader, Cleavase or limited primer extension, and the like. Amplification product may also serve as template for further amplification by the methods of the invention or other amplification method known in the art.

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[0064] In yet another embodiment, the invention provides methods for immobilizing nucleic acids, including methods for generating microarrays of nucleic acids, using amplification products of the amplification methods of the invention.

[0065] In another embodiment, the invention provides methods of generating cDNA libraries, methods of generating subtractive hybridization probes, and methods of generating subtractive hybridization libraries.

Advantages of the invention

[0066] Various methods for global amplification of nucleic acids have been developed. PCR-based methods, such as PEP, may generate non-specific amplification artifacts, give incomplete coverage of loci, or generate DNA product of insufficient length that cannot be used in many applications. PCR-based methods also suffer from the propensity of the PCR reaction to generate products that are preferentially amplified, and thus resulting in biased representation of genomic sequences in the products of the amplification reaction. Also, PCR-based methods require cumbersome thermal cycling.

[0067] Additionally, a number of methods for the detection and quantification of gene expression levels have been developed in recent years. For example, microarrays, in which either defined cDNAs or oligonucleotides are immobilized at discrete locations on, for example, solid or semi-solid substrates, or on defined particles, enable the detection and/or quantification of the expression of a multitude of genes in a given specimen. Using these previously known methods to detect presence of absence of and/or quantify multiple RNA species in a sample, which in turn is used as a measure of gene expression profiling, generally requires direct labeling of cDNA, which requires a large amount of input total RNA. Thus, when using total RNA preparations from a given cell or tissue sample to analyze mRNA, the analysis of gene expression in the sample using methods such as arrays requires a substantial amount of input RNA, which generally ranges from 50 to 200 μg. Similarly, 2 to 5 μg of mRNA purified from a total RNA preparation would generally be required for a single analysis of gene expression profiling using array technologies. This is a clear limitation of methods such as those based on array technology, insofar as the number of cells, or size of tissue specimen required is very large, and these cells or tissue specimens are often scarcely available for testing or are too precious. This limitation is especially severe in the study of clinical specimens, where the cells to be studied are rare and/or difficult to cultivate in vitro, and in high throughput screening of libraries of effector

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molecules. Also, previous transcription-based methods of amplification of mRNA (described in, for example, Lockhart et al, Nature Biotechnology (1996), 14, 1675-1680); van Gelder et al., U.S. Patent No. 5,716,785), are limited to the amplification efficiency of DNA-dependent RNA polymerases and some of these methods require multiple steps. Moreover, the process by which the polymerase promoter sequence is incorporated is prone to result in non-specific amplification.

[0068] The methods of the invention offer the ability to efficiently amplify template polynucleotides (including both RNA and DNA). Thus, the utility of detection/quantification methods which ca be used with the amplification products of the invention, such as those based on array technology, real time PCR, quantitative TaqMan, quantitative PCR using molecular beacons, and the like, should be greatly enhanced.

[0069] The methods of the invention do not require thermocycling and all of the steps can be performed isothermally, although the various steps may be carried out a different temperatures. This feature provides numerous advantages, including facilitating automation and adaptation for high through-put procedures. The isothermal reaction is generally faster than that afforded by thermal cycling, and is suitable for performing the methods of the invention in miniaturized devices.

[0070] The ability to efficiently amplify pools of template polynucleotide sequences (such as genomic DNA) under conditions that will generally not alter the representation of the nucleic acid sequences in the starting sample, will greatly enhance the utility of the detection/quantification methods such as those based on the powerful array technology.

[0071] The ability to efficiently amplify RNA using the random initiation of replication according the methods of the invention provides means for representing all sequences in the pool of sequences (or representing a significant proportion of a pool depending on desired extent of the amplification) in the sample, for example, sequences representing the full length of mRNA species in a sample. The methods of the invention do not rely on oligo-dT primers (to bind the poly-A tail of mRNA) to initiate amplification; thus, the methods may be used to amplify non-poly-A tailed RNAs, such as noncoding RNAs and RNAs of non-eukaryotic species. The methods of the invention do not require prior knowledge of the sequences in the sample and are thus suitable for discovery of novel transcripts, even when present in low abundance and/or representing non-coding transcripts. The ability to efficiently amplify RNA under conditions that will generally not

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alter the representation of the nucleic acid sequences in the preparation, will greatly enhance the utility of the detection/quantification methods such as those based on the powerful array technology.

General Techniques

[0072] The practice of the invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

[0073] Primers, oligonucleotides and polynucleotides employed in the invention can be generated using standard techniques known in the art.

Definitions

[0074] A "template," "template strand," "template polynucleotide," "template DNA," target sequence," "target nucleic acid," or "target DNA," "target polynucleotide," "template RNA," or "target RNA," as used herein, is a polynucleotide for which amplification is desired. The template polynucleotide can comprise DNA or RNA. The template sequence may be known or not known, in terms of its actual sequence. Generally, the terms "target," "template ," and variations thereof, are used interchangeably.

[0075] "Amplification," or "amplifying", as used herein, generally refers to the process of producing multiple copies of a desired sequence. "Multiple copies" mean at least 2 copies. A "copy" does not necessarily mean perfect sequence complementarity or identity to the template sequence. For example, copies can include nucleotide analogs such as deoxyinosine, intentional sequence alterations (such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the template, or a non-target sequence introduced through a primer), and/or sequence errors that occur during amplification. "Amplifying" a sequence may generally refer to making copies of a sequence or its complement, with the understanding that, as stated above,

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copying does not necessarily mean perfect sequence complementarity or identity with respect to the template sequence.

[0076] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping groups moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'--O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S

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("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0077] A "labeled dNTP," or "labeled rNTP," as used herein, refers, respectively, to a dNTP or rNTP, or analogs thereof, that is directly or indirectly attached with a label. For example, a "labeled" dNTP or rNTP, may be directly labeled with, for example, a dye and/or a detectable moiety, such as a member of a specific binding pair (such as biotin-avidin). A "labeled" dNTP or rNTP, may also be indirectly labeled by its attachment to, for example, a moiety to which a label is/can be attached. A dNTP or rNTP, may comprise a moiety (for example, an amine group) to which a label may be attached following incorporation of the dNTP or rNTP into an extension product. Useful labels in the present invention include fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, green fluorescent protein and the like), radioisotopes (e.g., ^{3}H, ^{35}S, ^{32}P, ^{33}P, ^{125}I, or ^{14}C), enzymes (e.g., LacZ, horseradish peroxidase, alkaline phosphatase,), digoxigenin, and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Various anti-ligands and ligands can be used (as labels themselves or as a means for attaching a label). In the case of a ligand that has a natural anti-ligand, such as biotin, thyroxine and cortisol, the ligand can be used in conjunction with labeled anti-ligands.

[0078] The "type" of dNTP or rNTP, as used herein, refers to the particular base of a nucleotide, namely adenine, cytosine, thymine, uridine, or guanine.

[0079] "Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. Oligonucleotides in the invention include the composite primer and auxiliary primer. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

[0080] A "primer," as used herein, refers to a nucleotide sequence, generally with a free 3'-OH group, that hybridizes with a template sequence (such as a target polynucleotide, target DNA, or a primer extension product) and is capable of promoting

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polymerization of a polynucleotide complementary to the template. A "primer" can be, for example, an oligonucleotide.

[0081] "Auxiliary primers" as used herein, are a population of primers comprising randomized and/or partially-randomized sequences. Auxiliary primers are a polynucleotide as described herein, though generally, auxiliary primers are made of DNA. Random primers are known in the art and are commercially available. An example of auxiliary primers is the population of randomized hexamer primers shown in Example 1.

[0082] To "inhibit" is to decrease or reduce an activity, function, and/or amount as compared to a reference.

[0083] By "randomly prime" or "random hybridization", as used herein, it is meant that the composite primer hybridizes to multiple sites within the template polynucleotide.

[0084] As used herein, "complex comprising an RNA/DNA partial heteroduplex" generally refers to a population of intermediate complexes that generally includes (a) copies of template polynucleotide and/or copies of the complement of template polynucleotide sequence appended to composite primer sequences; and (b) copies of template polynucleotide and/or copies of the complement of template polynucleotide appended to the complement of composite primer sequences. By virtue of the presence of composite primer sequence in the intermediate complexes, the complexes comprise **at least** a RNA/DNA partial heteroduplex. The RNA portion of the partial heteroduplex generally is introduced (via extension) by the RNA portion of the composite primer, and the DNA portion of the partial heteroduplex comprises the complement of the RNA portion of the composite primer. As discussed herein, the complex comprising an RNA/DNA partial heteroduplex functions as a substrate for further amplification during the single primer isothermal amplification phase of the methods. Generally, RNA in the RNA/DNA partial heteroduplex is cleaved, generating a 3' single stranded portion with sequences that are complementary to RNA in a composite primer (and thus forming a binding site for a composite primer). Thus, reference to "a complex comprising a 3' single stranded portion" generally refers to the complex comprising an RNA/DNA partial heteroduplex when its RNA is cleaved.

[0085] A "complex" is an assembly of components. A complex may or may not be stable and may be directly or indirectly detected. For example, as is described herein, given certain components of a reaction, and the type of product(s) of the reaction, existence of a complex can be inferred. For purposes of this invention, a complex is generally an

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intermediate with respect to the final amplification product(s). An example of a complex is a complex of composite primer extension product and second composite primer extension product, as described herein.

[0086] A "portion" or "region," used interchangeably herein, of a polynucleotide or oligonucleotide is a contiguous sequence of 2 or more bases. In other embodiments, a region or portion is at least about any of 3, 5, 10, 15, 20, 25 or more contiguous nucleotides.

[0087] A region, portion, or sequence which is "adjacent" to another sequence directly abuts that region, portion, or sequence. For example, an RNA portion which is adjacent to a 5' DNA portion of a composite primer directly abuts that region.

[0088] A "reaction mixture" is an assemblage of components, which, under suitable conditions, react to form a complex (which may be an intermediate) and/or a product(s).

[0089] "A", "an" and "the", and the like, unless otherwise indicated include plural forms. "A" fragment means one or more fragments.

[0090] Conditions that "allow" an event to occur or conditions that are "suitable" for an event to occur, such as hybridization, strand extension, and the like, or "suitable" conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event. Such conditions, known in the art and described herein, depend upon, for example, the nature of the nucleotide sequence, temperature, and buffer conditions. These conditions also depend on what event is desired, such as hybridization, cleavage, and/or strand extension.

[0091] Sequence "mutation," as used herein, refers to any sequence alteration in a sequence of interest in comparison to a reference sequence. A reference sequence can be a wild type sequence or a sequence to which one wishes to compare a sequence of interest. A sequence mutation includes single nucleotide changes, or alterations of more than one nucleotide in a sequence, due to mechanisms such as substitution, transversion, deletion or insertion. Single nucleotide polymorphism (SNP) is also a sequence mutation as used herein.

[0092] "Microarray" and "array," as used interchangeably herein, comprise a surface with an array, preferably ordered array, of putative binding (e.g., by hybridization) sites for a biochemical sample (target) which often has undetermined characteristics. In a preferred embodiment, a microarray refers to an assembly of distinct polynucleotide or oligonucleotide probes immobilized at defined locations on a substrate. Arrays are formed

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on substrates fabricated with materials such as paper, glass, plastic (e.g., polypropylene, nylon, polystyrene), polyacrylamide, nitrocellulose, silicon, optical fiber or any other suitable solid or semi-solid support, and configured in a planar (e.g., glass plates, silicon chips) or three-dimensional (e.g., pins, fibers, beads, particles, microtiter wells, capillaries) configuration. Probes forming the arrays may be attached to the substrate by any number of ways including (i) *in situ* synthesis (e.g., high-density oligonucleotide arrays) using photolithographic techniques (see, Fodor et al., *Science* (1991), 251:767-773; Pease et al., *Proc. Natl. Acad. Sci. U.S.A.* (1994), 91:5022-5026; Lockhart et al., *Nature Biotechnology* (1996), 14:1675; U.S. Pat. Nos. 5,578,832; 5,556,752; and 5,510,270); (ii) spotting/printing at medium to low-density (e.g., cDNA probes) on glass, nylon or nitrocellulose (Schena et al, *Science* (1995), 270:467-470, DeRisi et al, *Nature Genetics* (1996), 14:457-460; Shalon et al., *Genome Res.* (1996), 6:639-645; and Schena et al., *Proc. Natl. Acad. Sci. U.S.A.* (1995), 93:10539-11286); (iii) by masking (Maskos and Southern, *Nuc. Acids. Res.* (1992), 20:1679-1684) and (iv) by dot-blotting on a nylon or nitrocellulose hybridization membrane (see, e.g., Sambrook et al., Eds., 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, Cold Spring Harbor Laboratory (Cold Spring Harbor, N.Y.)). Probes may also be noncovalently immobilized on the substrate by hybridization to anchors, by means of magnetic beads, or in a fluid phase such as in microtiter wells or capillaries. The probe molecules are generally nucleic acids such as DNA, RNA, PNA, and cDNA but may also include proteins, polypeptides, oligosaccharides, cells, tissues and any permutations thereof which can specifically bind the target molecules.

[0093] The term "3'" generally refers to a region or position in a polynucleotide or oligonucleotide 3' (downstream) from another region or position in the same polynucleotide or oligonucleotide.

[0094] The term "5'" generally refers to a region or position in a polynucleotide or oligonucleotide 5' (upstream) from another region or position in the same polynucleotide or oligonucleotide.

[0095] The term "3'-DNA portion," "3'-DNA region," "3'-RNA portion," and "3'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 3' end of the polynucleotide or oligonucleotide, and may or may not include the 3' most nucleotide(s) or moieties attached to the 3' most nucleotide of the same polynucleotide or oligonucleotide. The 3' most nucleotide(s) can be preferably from about

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1 to about 50, more preferably from about 10 to about 40, even more preferably from about 20 to about 30 nucleotides.

[0096] The term "5'-DNA portion," "5'-DNA region," "5'-RNA portion," and "5'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 5' end of the polynucleotide or oligonucleotide, and may or may not include the 5' most nucleotide(s) or moieties attached to the 5' most nucleotide of the same polynucleotide or oligonucleotide. The 5' most nucleotide(s) can be preferably from about 1 to about 50, more preferably from about 10 to about 40, even more preferably from about 20 to about 30 nucleotides.

[0097] "Absent" or "absence" of product, and "lack of detection of product" as used herein includes insignificant, or *de minimus* levels, generally due to lack of significant accumulation of product.

[0098] In accordance with well established principles of patent law "comprising" means "including."

Amplification methods of the invention

[0099] The following are examples of the amplification methods of the invention. It is understood that various other embodiments may be practiced, given the general description provided above. For example, reference to using a composite primer means that any of the composite primers described herein may be used.

Amplification using a composite primer that hybridizes to a multiplicity *of template polynucleotide sites*

[0100] The invention provides methods for global amplification using a composite primer that is capable of binding to multiple sites within template polynucleotide, including DNA and RNA template polynucleotides.

[0101] Generally, the methods of the invention involve two phases. (a) composite primer random hybridization, primer extension and displacement of composite primer extension product by strand displacement, whereby a complex comprising a RNA/DNA partial heteroduplex is generated, and (b) composite-primer dependent single primer isothermal amplification.

[0102] Thus, the methods of the invention work as follows: (a) incubating a reaction mixture, said reaction mixture comprising: (i) a template polynucleotide; (ii) a first

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primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) a DNA-dependent DNA polymerase ; and (iv) an RNA-dependent DNA polymerase (which may be present as a separate enzyme or as an enzyme comprising both DNA-dependent DNA polymerase and RNA-dependent DNA polymerase activities); wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and, in some embodiments, displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising (i) the reaction products generated according to step (a) (or an aliquot thereof); (ii) a composite primer (which may be the same as the first primer or may different from the first primer); (iii) an DNA-dependent DNA polymerase; and (iv) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide (generally, DNA) amplification product are generated. In some embodiments, the reaction mixture of step (b) comprises the reaction mixture according to step (a) (or an aliquot thereof). In other embodiments, step (b) is initiated by the addition of an agent that cleaves RNA from an RNA/DNA heteroduplex (such as RNase H) to the reaction mixture of step (a). In embodiments in which the template polynucleotide is RNA, the reaction mixture of step (a) further comprises an agent (such as an enzyme) that that cleaves RNA from an RNA/DNA heteroduplex.

(a) Composite primer random hybridization, primer extension and displacement of composite primer extension product by strand displacement

[0103] The methods generally comprise using specially-designed primers, generally a RNA/DNA composite primer. In a first phase of the amplification methods, composite primer is used to randomly prime template polynucleotide (such as genomic DNA). By "randomly prime" or "random hybridization", as used herein, it is meant that the composite primer hybridizes to multiple sites within template polynucleotide. We have discovered that certain composite primers bind a multiplicity of sites within template polynucleotide

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(generally, under conditions promoting random primer hybridization) and are thus particularly suitable for use in the methods of the invention. Generally, suitable composite primers show partial homology to a multiplicity of template nucleic acid sequences, particularly in the 3' sequences of the composite primer, and thus, the composite primer comprises at least a 3' DNA portion that is capable of randomly priming template polynucleotide (particularly under conditions permitting random primer hybridization). Selection and design of composite primers is described further below.

[0104] Various embodiments of the composite primer and used in the methods of the invention are described herein. For example, Figure 1 illustrates one embodiment of a composite primer useful in the methods of the present invention. As illustrated in the Figure, the composite primer comprises a DNA portion at its 3' end and an RNA portion at its 5' end. As discussed herein, it is also possible to employ a composite primer in which the 3' DNA portion is followed, in the direction of its 5', by an RNA portion, which is followed by a portion which is DNA. The length of each of these sections is generally determined for maximum efficiency of the amplification. In some embodiments, the composite primer that hybridizes to target polynucleotide and the composite primer used during single primer isothermal amplification are the same. In some embodiments, the composite primer that hybridizes to target polynucleotide and the composite primer used during single primer isothermal amplification are different.

[0105] Reference to a primer that binds (hybridizes to) a sequence (or template) encompasses embodiments in which at least a portion of the primer is hybridized, as well as those embodiments in which an entire primer is hybridized. Thus, and as the description makes clear, reference to a primer that hybridizes to a sequence encompasses embodiments in which at least a portion of the primer is hybridized as well as embodiments in which two (or more portions) of the primer are hybridized, separated by unhybridized (looped out) portions of the primer, and embodiments in which the entire primer is hybridized. For example, Figure 2 illustrates a single composite primer that hybridizes to multiple positions on a template polynucleotide where differing portions of the composite primer are hybridized to template polynucleotide depending on the site (sequence) at which it is hybridized. Thus, according to the methods of the invention, only a portion of the 3'-end of the composite primer must be hybridized in order for initiation of primer extension by DNA polymerase. In some embodiments, for example, only 2, 3, 4, 5, 6, 7, 8 or more nucleotides of the 3' end of the primer need to hybridize in order for primer extension to be

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initiated. It is understood that hybridization of the 3'-most portion of the composite primer may be stabilized to various extents by further hybridization of another portion of the primer (with or without looping out of intervening primer portions). A DNA polymerase can be included during primer hybridization to enhance (e.g., stabilize) hybridization of composite primer by initiation of primer extension, and thus, stabilization of primer hybridization.

[0106] Random hybridization of the composite primer to template polynucleotide generally occurs under conditions permitting random (nonspecific) primer hybridization. Such conditions are well known in the art and include: decreased stringency during primer hybridization and/or primer extension (including reduced temperature and/or buffer conditions of reduced stringency); composite primer selection and/or design (discussed further herein); and composite primer and template concentration. It is understood that stringency of hybridization, composite primer selection and/or primer concentration may be used to control the frequency of composite primer hybridization, and thus to control coverage and/or representation of template sequences in amplification product. As noted above, an aspect of the invention is displacement of intervening primer extension product during primer extension of composite primers hybridized at downstream position(s) on the template, whereby primer extension products are displaced from template polynucleotide. Preferably, a DNA polymerase is used that possesses stand displacement activity.

[0107] Composite primer random hybridization, primer extension and displacement of primer extension product by strand displacement results in generation of a multiplicity of complexes comprising a RNA/DNA partial heteroduplex. The complexes comprise (a) copies of template polynucleotide and/or copies of the complement of polynucleotide sequence appended (via extension) to composite primer sequences; and (b) copies of template polynucleotide and copies of the complement of template polynucleotide appended (via extension) to the complement of composite primer sequences. Generally, the RNA portion of the complex is introduced by the composite primer.

[0108] In some embodiments, generation of complexes comprising a RNA/DNA partial heteroduplex involves the following steps: (i) formation of a composite primer extension product; and (ii) formation of a second primer extension product by primer extension along the first primer extension product. For example, in some embodiments, complex comprising an RNA/DNA partial heteroduplex is generated as follows: following random hybridization of the composite primer at multiple sites on template polynucleotide

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strands, a DNA polymerase extends the composite primer along the template strand generating a composite primer extension product that is complementary to the polynucleotide template strand. Primer extension extends to and displaces strands being extended from primers hybridized at upstream sites on the template. Thus, as noted above, an aspect of the invention is displacement of intervening primer extension product during primer extension of composite primers hybridized at downstream site(s) on the template, whereby composite primer extension products are displaced from template polynucleotide. Figure 3 illustrates primer extension from composite primers that are hybridized at multiple sites on a template strand, where a composite primer extension products is being displaced by primer extension from a composite primer hybridized at a downstream site(s) on template strand.

[0109] Displaced composite primer product comprises the composite primer sequence at the 5' end, including the 5' RNA portion. Although for convenience, reference is made only to "a" composite primer extension product, it is understood that a multiplicity of composite primer extension products are generated the complement of a multiplicity of template polynucleotide sequences appended (via extension) to the sequence of the composite primer. Figure 4 shows a collection of composite primer extension products comprising composite primer 1 linked (by extension) to sequences comprising the complement of a multiplicity of target polynucleotide sequences.

[0110] Using displaced composite primer extension product as a template, a second primer extension product complementary to the first primer extension product is generated by extension by a DNA-dependent DNA polymerase along the DNA portion of the composite primer extension product, and extension by a RNA-dependent DNA polymerase along the 5' RNA portion of the composite primer extension product, generating a double stranded complex comprising a RNA/DNA complex at the end. Generation of second primer extension product may be random primed using the composite primer, as depicted in Figure 5. Alternatively, second primer extension product may be primed by the 3' end of a different composite primer extension product. Additional embodiments in which second strand production is primed by exogenous (added) primers and/or by fragments of template RNA (endogenous primers) are described below.

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(b) Single primer isothermal amplification using a complex comprising an RNA/DNA partial heteroduplex as a template

[0111] In a second phase of the methods, termed single primer isothermal amplification, the complex comprising an RNA/DNA partial heteroduplex is a substrate for further amplification as follows: an enzyme which cleaves RNA sequence from an RNA/DNA hybrid (such as RNase H) cleaves RNA from the partial heteroduplex, leaving a partially double stranded polynucleotide complex comprising a 3' single stranded DNA sequence. The 3' single stranded sequence (formed by cleavage of RNA in the complex comprising an RNA/DNA partial heteroduplex) is generally the complement of the RNA in the composite primer, and thus forms a specific binding site for a composite primer (which may or may not be the same as the first composite primer). Extension of a bound composite primer by a DNA-dependent DNA polymerase produces a primer extension product, which displaces the previously bound cleaved primer extension product, whereby polynucleotide (generally, DNA) product accumulates. See, for example, U.S. Patents Nos. 6,251,639 and 6,692,918. Figure 6 shows amplification of DNA product using a composite primer and a complex comprising a RNA/DNA partial heteroduplex as a template for further amplification.

[0112] Amplification using a complex comprising an RNA/DNA partial heteroduplex as a template for further amplification (also termed single primer isothermal amplification) generally occurs under conditions permitting composite primer hybridization, primer extension, cleavage of RNA from an RNA/DNA hybrid and strand displacement. In so far as the composite primer hybridizes to the 3' single stranded portion (of the partially double stranded polynucleotide which is formed by cleaving RNA in the complex comprising an RNA/DNA partial heteroduplex) comprising, generally, the complement of at least a portion of the composite primer sequence, composite primer hybridization may be under conditions permitting specific hybridization. Thus, in some embodiments, the reactions conditions permit stringent hybridization (i.e., hybridization of sequences that are generally complementary). As is evident from the description herein, in other embodiments, the reaction conditions are less stringent (i.e., permit hybridization of sequences that are less than fully complementary).

[0113] Generally, the methods of the invention result in amplification of a multiplicity, a large multiplicity, or a very large multiplicity of template polynucleotide sequences. In some embodiments, essentially all of the template polynucleotide present in

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the initial sample (*e.g.*, all of the mRNA or all of the genomic DNA) is amplified. In other embodiments, at least 50, at least 100, at least 200, at least 300, or more distinct sequences (such as a gene or other subsegment of a polynucleotide, a marker (such as a SNP or other polymorphism) are amplified, as assessed, e.g., by analysis of marker sequences known to be present in the template sample under analysis, using methods known in the art. Template polynucleotide sequences that are amplified may be present on the same polynucleotide (e.g., a chromosome or portion of a chromosome for genomic DNA template or on the same RNA for RNA template) or on different template polynucleotides (e.g., different chromosome or portions of chromosomes for DNA template, or different RNAs for RNA template). Although, amplification of genomic DNA is exemplified herein, it will be understood by those of skill in the art, however, that the global amplification methods of the invention are suitable for amplification of any pool or subset of polynucleotides.

[0114] For convenience, reference is made to a polynucleotide (generally, DNA) product. It is understood that amplified product generally is a mixture of sense and antisense copies of a given template polynucleotide. For example, if the template polynucleotide is double stranded DNA, the amplification product will correspond to each strand. If the template polynucleotide is single stranded (*e.g.*, RNA or single stranded DNA), amplification product will generally be produced that is the copy of template polynucleotide (sense copy) and the complement of the template polynucleotide (antisense copy). The amplification product of different senses can be annealed to form a double stranded (or partially double stranded) complex, or can be prevented from annealing (or subsequently denatured) to produce a mixture of single stranded amplification products. The amplified products may be of differing lengths.

[0115] As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

[0116] As illustrated in these embodiments, all steps are isothermal (in the sense that thermal cycling is not required), although the temperatures for each of the steps may or may not be the same. It is understood that various other embodiments may be practiced, given the general description provided above. For example, as described and exemplified herein, certain steps may be performed as temperature is changed (e.g., raised, or lowered).

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[0117] For simplicity, the methods of the invention are described as two distinct steps or phases, above. It is understood that the two phases may occur simultaneously in some embodiments (for example, if the enzyme that cleaves RNA from RNA/DNA hybrid is included in the first reaction mixture). In other embodiments, step (b) may be initiated by addition of an enzyme that cleaves RNA from an RNA/DNA hybrid (e.g., ribonuclease, such as RNase H), and optionally, a DNA-dependent DNA polymerase, as shown in Example 1. In this embodiment, addition of an enzyme that cleaves RNA from an RNA/DNA hybrid permits further amplification using the complex comprising an RNA/DNA partial heteroduplex as a template (i.e., step (b), above). It is understood, however, that primer extension (and strand displacement) along template polynucleotide strand from random primed composite primer(s) may continue during single primer isothermal amplification.

[0118] Although generally only one composite primer is described above, it is further understood that the amplification methods may be performed in the presence of two or more different composite primers that randomly prime template polynucleotide. In addition, the amplification polynucleotide products of two or more separate amplification reactions conducted using two or more different composite primers that randomly prime template polynucleotide can be combined. In addition, it is understood that different composite primers can be used in step (a) (i.e., random priming of template polynucleotide) and step (b) (i.e., single primer isothermal amplification). In this instance, the different composite primer comprises sequences hybridizable to the 3' single stranded DNA portion of the partially double stranded complex (which is generated by cleaving RNA from the complex comprising a RNA/DNA partial heteroduplex). Generally, the second composite primer comprises sequences overlapping with the first composite primer.

Amplification using a composite primer that hybridizes to a multiplicity of template polynucleotide sites and auxiliary primers

[0119] In another aspect of the invention, auxiliary primers are present in the reaction mixture comprising template polynucleotide, composite primer, DNA-dependent DNA polymerase and RNA-dependent DNA polymerase. As used herein, "auxiliary primers" refers to a population of random or partially randomized primers. An example of auxiliary primers is the random hexamer primers used in Example 1. Inclusion of auxiliary primers (i.e., population of different random primers) during the amplification is believed

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to enhance the efficiency of production of and/or target coverage of the amplification product.

[0120] In some embodiments, the methods of the invention work as follows: (a) incubating a reaction mixture, said reaction mixture comprising a composite primer as described herein; auxiliary primers; a template polynucleotide, DNA-dependent DNA polymerase, and RNA-dependent DNA polymerase (which may be present as a single enzyme comprising both activities), wherein the incubation is under conditions suitable for random composite primer hybridization, auxiliary primer hybridization, primer extension, and strand displacement, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising the reaction products from step (a) (or an aliquot thereof); a composite primer (which may be the same as the composite primer of step (a) or may be a different composite primer); a DNA-dependent DNA polymerase; optionally, auxiliary primers; and an enzyme that cleaves RNA from a RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product from the complex when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide template sequence are generated.

[0121] Inclusion of auxiliary primers (i.e., a population of different random primers) during the amplification is believed to enhance the efficiency of production of and/or coverage of template polynucleotide. Without being bound by theory, it is believed that primer extension of auxiliary primers increases displacement of composite primer extension product from template polynucleotide and/or primes generation of second primer extension product. Figure 7 illustrates primer extension from composite primers and auxiliary primers that are hybridized at multiple sites on a template strand. Figure 8 illustrates generation of a second primer extension product primed by auxiliary primers hybridized to composite primer extension product.

[0122] Although for simplicity, use of auxiliary primers is described only in the first phase, random composite primer hybridization (i.e., step (a)), it is evident that auxiliary primers may be present in the reaction mixture for the second phase of the methods, single primer isothermal amplification (i.e., step (b)).

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[0123] As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

Amplification using a composite primer that hybridizes to a multiplicity of template RNA sites and auxiliary primers

[0124] In another aspect of the invention, auxiliary primers are present in the reaction mixture comprising template RNA, composite primer, DNA-dependent DNA polymerase and RNA-dependent DNA polymerase. As used herein, "auxiliary primers" refers to a population of random or partially randomized primers. Inclusion of auxiliary primers (i.e., population of different random primers) during the amplification is believed to enhance the efficiency of production of and/or target coverage of the amplification product.

[0125] In some embodiments, the methods of the invention operate as follows: (a) incubating a reaction mixture, said reaction mixture comprising a composite primer as described herein; auxiliary primers; a template RNA, DNA-dependent DNA polymerase, and RNA-dependent DNA polymerase (which may be present as a single enzyme comprising both activities), wherein the incubation is under conditions suitable for random composite primer hybridization, auxiliary primer hybridization, primer extension, and strand displacement, whereby a complex comprising a RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising the reaction products from step (a) (or an aliquot thereof); a composite primer (which may be the same as the composite primer of step (a) or may be a different composite primer); a DNA-dependent DNA polymerase; optionally, auxiliary primers; and an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product from the complex when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide template sequence are generated.

[0126] Inclusion of auxiliary primers (i.e., a population of different random primers) during the amplification is believed to enhance the efficiency of production of and/or coverage of template RNA. Without being bound by theory, it is believed that primer extension of auxiliary primers increases displacement of composite primer

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extension product from template RNA and/or primes generation of second primer extension product. Figure 7 illustrates primer extension from composite primers and auxiliary primers that are hybridized at multiple sites on a template strand. Figure 8 illustrates generation of a second primer extension product primed by auxiliary primers hybridized to composite primer extension product.

[0127] Although for simplicity, use of auxiliary primers is described only in the first phase, random composite primer hybridization (i.e., step (a)), it is evident that auxiliary primers may be present in the reaction mixture for the second phase of the methods, single primer isothermal amplification (i.e., step (b)).

[0128] As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

Components and reaction conditions used in the methods of the invention

Template nucleic acid

[0129] The nucleic acid (NA) target to be amplified includes nucleic acids from any source in purified or unpurified form, which can be DNA (dsDNA and ssDNA) or RNA, including tRNA, mRNA, rRNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, humans, and fragments thereof. Preferred target polynucleotide includes DNA (*e.g.*, genomic DNA, including human genomic DNA, and mammalian genomic DNA (such as mouse, rat,))and RNA (*e.g.*, mRNA, ribosomal RNA, and total RNA). It should be understood that template RNA includes coding and non-coding RNA. The sequences can be naturally occurring or recombinant nucleic acid targets, including cloned nucleic fragments of interest.

[0130] The target nucleic acid can be only a minor fraction of a complex mixture such as a biological sample and can be obtained from various biological material by procedures well known in the art. Nucleic acid can be obtained from sources containing very small quantities of nucleic acid, such a single cells, small numbers of cells, patient samples, forensic samples, and archeological samples. Obtaining and purifying nucleic acids use standard techniques in the art, including methods designed to isolate one or a very

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small number of cells, such a cell sorting or laser capture micro-dissection. The methods of the invention are particularly suited for use with genomic DNA (*e.g.*, human and other mammalian genomic DNA), as well as RNA (*e.g.*, total RNA or mRNA samples). Amplification of an RNA target may be accomplished by initial cDNA synthesis, as known in the art, followed by amplification from the cDNA template.

[0131] The target polynucleotide(s) can be known or unknown and may contain more than one desired specific nucleic acid sequence of interest, each of which may be the same or different from each other. If the target polynucleotide is double stranded (*e.g.*, double stranded DNA or a double stranded DNA/RNA hybrid, such as is produced by first strand cDNA synthesis), the target may first be treated to render it single stranded (*e.g.*, by denaturation or by cleavage of the RNA portion of a DNA/RNA hybrid). Denaturation may also be carried out to remove secondary structure present in a single stranded target molecule (*e.g.*, RNA). In some cases, double stranded DNA target polynucleotide may be first cleaved by one or more restriction endonuclease enzymes.

[0132] When the target polynucleotide is DNA, the initial step of the amplification of a target nucleic acid sequence is rendering the target single stranded. If the target nucleic acid is a double stranded (ds) DNA, the initial step can be target denaturation. The denaturation step may be thermal denaturation or any other method known in the art, such as alkali treatment. If the target nucleic acid is present in an DNA-RNA hybrid, the initial step can be denaturation of the hybrid to obtain a DNA, or removal of the RNA strand using other means known in the art, such as thermal treatment, digestion with an enzyme that cleaves RNA from an RNA/DNA hybrid (such as RNase H) or alkali treatment, to generate single stranded DNA. When the target is RNA, the initial step may be the synthesis of a single stranded cDNA. Techniques for the synthesis of cDNA from RNA are known in the art, and include reverse transcription of RNA strand using a primer that binds to a specific target, such as the poly-A tail of eukaryotic mRNAs or other specific or consensus sequences. In addition, reverse transcription can be primed by a population of degenerate or partially degenerate primers. First strand cDNA can be separated from the complex of RNA and first strand cDNA as described herein.

[0133] RNAs can be from any source in purified or unpurified form, which can be RNA such as total RNA, tRNA, mRNA, rRNA, mitochondrial RNA, chloroplast RNA, DNA-RNA hybrids, or mixtures thereof, from any source and/or species, including human, animals, plants, and microorganisms such as bacteria, yeasts, viruses, viroids, molds, fungi,

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plants, and fragments thereof. It is understood that the RNA can be coding or noncoding RNA (such as untranslated small RNAs). RNAs can be obtained and purified using standard techniques in the art. Use of a DNA target (including genomic DNA target) would require initial transcription of the DNA target into RNA form, which can be achieved using methods disclosed in Kurn, U.S. Patent No. 6,251,639 B1, and by other techniques (such as expression systems) known in the art. Thus, RNA template can be itself generated from a DNA source (such as genomic DNA), using methods known in the art, including Kurn, U.S. Patent No. 6,251,639. RNA copies of genomic DNA would generally include untranscribed sequences generally not found in mRNA, such as introns, regulatory and control elements, etc. RNA targets may also be generated from cloned genomic DNA sequences that can be subjected to in vitro transcription. Use of a DNA-RNA hybrid would require denaturation of the hybrid to obtain a single stranded RNA, denaturation followed by transcription of the DNA strand to obtain an RNA, or other methods known in the art such as digestion with an RNAse H to generate single stranded DNA.

Composite primer

[0134] The methods of the invention employ a composite primer that is composed of RNA and DNA portions. We have observed that suitable composite primers show partial nucleic acid sequence homology to a multiplicity of genomic DNA sequences, particularly in the 3' sequences of the composite primer, when analyzed using standard nucleic acid comparison algorithms. For example, composite primer sequence can be used as a query sequence in Blast, to search the human genomic DNA database (or other suitable database, such as a mammalian genomic DNA database). Generally, the search is performed using search parameters suitable for identification of partial or "low stringency" alignments, generally the least stringent conditions provided by the program. Such parameters are known in the art and include use of the NCBI Blast program for searching "short, nearly exact matches", with word size = 7 (conditions permitting as few as 7 consecutive nucleotide perfect matches at any position in the primer sequence). *See, e.g.,* http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?ALIGNMENTS=50&ALIGNMENT_VIEW=Pairwise&AUTO_FORMAT=Semiauto&CLIENT=web&DATABASE=nr&DESCRIPTIONS=100&ENTREZ_QUERY=>(none)&EXPECT=1000&FORMAT_BLOCK_ON_RESPAGE=None&FORMAT_ENTREZ_QUERY=(none)&FORMAT_OBJECT=Alignment&F

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ORMAT_TYPE=HTML&LAYOUT=TwoWindows&NCBI_GI=on&PAGE=Nucleotides &PROGRAM=blastn&SERVICE=plain&SET_DEFAULTS.x=16&SET_DEFAULTS.y=8 &SHOW_OVERVIEW=on&WORD_SIZE=7&END_OF_HTTPGET=Yes. Composite primers useful in the methods of the invention (i.e., that randomly hybridize to template polynucleotide) generally exhibit high partial homology rate with genomic DNA sequences, for example homology of stretches of 7 nucleotides with about 100 genomic DNA sequences, with about 70% of the hits located at the 3' end of the composite primer. A composite primer with a very unique sequence (i.e., low levels of homology with target genomic DNA sequences) did not function efficiently in the methods of the invention when used with genomic DNA template.

[0135] As is evident from the discussion above, reference to a primer that binds (hybridizes to) a sequence (or template) encompasses embodiments in which at least a portion of the primer is hybridized, embodiments in which two (or more portions) of the primer are hybridized (separated by unhybridized (looped out) portions of the primer), and embodiments in which the entire primer is hybridized. In certain embodiments, a 5'-portion, commonly the 5'-most portion, of the composite primer is designed such that the particular 5'-portion it is not expected to randomly hybridize to template polynucleotide (composite primers of this configuration are referred to as "tailed" primers, in reference to the 'tail' of unhybridized primer). In some embodiments, the tail portion of the composite primer is the entire 5' RNA portion of the composite primer. Thus, according to the methods of the invention, only a portion of the 3'-end of the composite primer must be hybridized in order for initiation of primer extension by DNA polymerase. In some embodiments, for example, only 2, 3, 4, 5, 6, 7 or more nucleotides of the 3' end of the primer must hybridize in order for primer extension to be initiated. It is understood that hybridization of the 3'-most portion of the composite primer may be stabilized to various extents by further hybridization of another portion of the primer (with or without looping out of intervening primer portions). A DNA polymerase can be included during primer hybridization to enhance (e.g., stabilize) hybridization of composite primer by initiation of primer extension, and thus, stabilization of primer hybridization.

[0136] We have also observed that composite primers that are suitable for use in the present methods can be identified by conducting single primer isothermal amplification as described in Kurn, U.S. Patent No. 6,251,639, using the composite primer under high stringency conditions using a genomic DNA template, and observing the presence of a

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smear of reaction products as visualized, for example, on a gel. Preferably, the genomic DNA does not contain a sequence(s) that is complementary to the composite primer. Production of a "smear" of reaction products, i.e., generation of a complex mixture of product of multiple molecular weights, visible on a gel as a smear, indicates that the composite primer is randomly priming genomic DNA amplification.

[0137] In another example, single primer isothermal amplification of a specific synthetic target oligonucleotide (e.g., a target oligonucleotide comprising a specific target for composite primer hybridization) is conducted at high stringency in the presence or absence of genomic DNA template (e.g., 1-100 ng of human genomic DNA). Composite primers that are suitable for the methods of the invention will demonstrate a strong effect of genomic DNA on the efficiency of the amplification of the specific synthetic target, resulting in about a 100-fold or greater reduction of amplification efficiency as compared with amplification efficiency conducted in the absence of genomic DNA.

[0138] Random composite primer hybridization and/or generation of composite primer extension product is promoted by use of conditions designed to permit random (non-specific) primer hybridization. Such conditions are well known in the art, and are further discussed below, and include: decreased stringency during primer hybridization and/or first strand synthesis (including reduced temperature and/or buffer conditions of reduced stringency, such as reduced ionic strength); composite primer selection and/or design (discussed further herein); composite primer and template concentration, presence or absence of an agent that stabilizes a 3' hybridized primer (such as a DNA polymerase), and presence or absence of agents such as DMSO that lower the temperature requirements for stable hybridization. It is understood that the selection of reaction conditions may be used to control the frequency of composite primer hybridization, and thus control coverage and/or representation of template polynucleotide sequences in amplification product.

[0139] Generally, the composite primer is also designed so that there is no primer-dimer formation capability, as determined using software routinely available to one of ordinary skill in the art, e.g. Oligo Primer Analysis Software from Molecular Biology Insight, and references therein. One of skill in the art will understand that other factors affect nucleic acid hybridization affinities. For example, any and all of the guanosine-cytosine content of the primer-target and primer-primer duplexes, minor groove binders, O-methylation or other modification of nucleotides, temperature, and salt are potentially important factors in constructing primers with the requisite differences in binding energies.

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Another factor in designing and constructing primers is the free energy parameters of hybridization of given sequences under a given set of hybridization conditions. The free energy parameters for the formation of a given hybrid may be calculated by methods known in the art (see, e.g., Tinoco et al. *Nature* (1973) 246:40-41 and Freier et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:9373-9377; computer programs, e.g., Oligo Primer Analysis Software from Molecular Biology Insight, and references therein), and it is possible to predict, for a given oligonucleotide template, primer sequences for which the required free energy changes for formation of various complexes will be met.

[0140] The primers should be extendable by DNA polymerase. Generation of primers suitable for extension by polymerization is well known in the art, such as described in PCT Pub. No. WO99/42618 (and references cited therein). Generally, the primer should permit high efficiency of amplification of a synthetic target that contains a specific primer target binding site (e.g., the complementary sequence to the primer), for example, permitting amplification of about 10^6 to 10^9 using methods described in Kurn, U.S. Patent 6,251,639. The composite primer is designed such that subsequent displacement of the primer extension product by binding of a new (additional) composite primer and the extension of the new primer by the polymerase can be achieved. In addition, cleavage of the RNA portion of the primer extension product leads to generation of amplification product which is not a substrate for amplification by the composite primer. It is understood that, in the following section that generally describes aspects of the composite primers used in the methods of the invention, characteristics described may be applicable to the primers if used for hybridizing and initiating the polynucleotide amplification (production of composite extension product) and/or for single primer isothermal amplification as described herein.

[0141] In some embodiments, a first composite primer is used in the methods of the invention, including those steps which involve single primer isothermal amplification (i.e., phase (b)). In other embodiments, a first and second, different, composite primer are used in the methods of the invention. The second composite primer is used for the single primer isothermal amplification step, and may comprise some or all of the sequence of the first composite primer, and the first composite primer may comprise some or all of the sequence of the second composite primer. In some embodiments, the second composite primer comprises a different sequence than the first composite primer.

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[0142] For use in single primer isothermal amplification and/or composite primer extension product formation, a composite primer comprises at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence on the single stranded portion of the complex (formed by cleavage of RNA in the complex comprising a RNA/DNA partial heteroduplex) (in some embodiments, on second primer extension product) independent of hybridization of the DNA portion(s) to a sequence on the same single stranded portion; and (b) being cleaved with an agent such as a ribonuclease when hybridized to the single stranded portion. The composite primers bind to the single stranded portion, and are extended by DNA polymerase to form a RNA/DNA partial heteroduplex in which only the RNA portion of the primer is cleaved upon contact with an agent which cleaves RNA in an RNA/DNA hybrid, such as an enzyme, such as a ribonuclease (such as RNase H), while the composite primer extension product remains intact, thus enabling annealing of another composite primer.

[0143] When used for the single primer isothermal amplification described herein, the composite primers also comprise a 3' DNA portion that is capable of hybridization to a sequence on the 3' single stranded portion of the complex such that its hybridization to the 3' single stranded portion is favored over that of the nucleic acid strand that is displaced from the complex by the DNA polymerase. Such primers can be rationally designed based on well known factors that influence nucleic acid binding affinity, such as sequence length and/or identity, as well as hybridization conditions. In a preferred embodiment, hybridization of the 3' DNA portion of the composite primer to its complementary sequence in the complex (e.g., in the second primer extension product) favored over the hybridization of the homologous sequence in the 5' end of the displaced strand to the composite primer extension product.

[0144] The composite primer comprises a combination of RNA and DNA (see definition above), with the 3'-end nucleotide being a nucleotide suitable for nucleic acid extension. The 3'-end nucleotide can be any nucleotide or analog that when present in a primer, is extendable by a DNA polymerase. Generally, the 3'-end nucleotide has a 3'-OH. Suitable primers include those that comprise at least one portion of RNA and at least one portion of DNA. For example, composite primers can comprise a 5'-RNA portion and a 3'-DNA portion (in which the RNA portion is adjacent to the 3'-DNA portion); or 5'- and 3'-DNA portions with an intervening RNA portion. Accordingly, in one embodiment, the composite primer comprises a 5' RNA portion and a 3'-DNA portion, preferably wherein

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the RNA portion is adjacent to the 3'-DNA portion. In another embodiment, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion (i.e., an RNA portion between the two DNA portions). In yet another embodiment, the composite primer of the invention comprises a 3'-DNA portion and at least one intervening RNA portion (i.e., an RNA portion between DNA portions).

[0145] The length of an RNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 50, more preferably from about 3 to about 20, even more preferably from about 4 to about 15, and most preferably from about 5 to about 10 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and an RNA portion, an RNA portion can be at least about any of 1, 3, 4, 5 nucleotides, with an upper limit of about any of 10, 14, 15, 20, 25, 3, 50 nucleotides. In certain embodiments, the composite primer has an RNA portion of about 14 or about 20 nucleotides.

[0146] The length of the 5'-RNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 3 to about 50 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In other embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 14, 15, 17, 18, 20, 50 nucleotides. In certain embodiments, the composite primer has an RNA portion of about 14 or about 20 nucleotides.

[0147] In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In certain embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be at least about any of 1, 2, 3, 5, with an upper limit of about any of 5, 6, 7, 10 nucleotides.

[0148] In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 5'-RNA portion can be preferably from about 3 to about 50 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to

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about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In certain embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 14, 15, 17, 18, 20, 50 nucleotides. In certain embodiments, the composite primer has an RNA portion of about 14 or about 20 nucleotides.

[0149] The length of an intervening RNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. The length of an intervening RNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. In a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, further comprising a 5'-RNA portion, the 5'-RNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, further comprising a 5'-RNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 nucleotides.

[0150] The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and an RNA portion, the 3'-DNA portion

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can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. In one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0151] The length of the 3'-DNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 3' DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. In one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0152] In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

[0153] In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In certain embodiments of the primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. In one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0154] The length of a non-3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, a non-3'-

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DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

[0155] The length of the 3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. In one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0156] The length of a non-3'-DNA portion (i.e., any DNA portion other than the 3'-DNA portion) in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, a non-3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides. The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. In one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides. It is understood that the lengths for the various portions can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

[0157] In some embodiments, the 5'-DNA portion of a composite primer includes the 5'-most nucleotide of the primer. In some embodiments, the 5'-RNA portion of a composite primer includes the 5' most nucleotide of the primer. In other embodiments, the 3'-DNA portion of a composite primer includes the 3' most nucleotide of the primer. In other embodiments, the 3'-DNA portion is adjacent to the 5'-RNA portion and includes the

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3' most nucleotide of the primer (and the 5'-RNA portion includes the 5' most nucleotide of the primer).

[0158] The total length of the composite primer can be preferably from about 10 to about 50 nucleotides, more preferably from about 15 to about 30 nucleotides, and most preferably from about 20 to about 25 nucleotides. In some embodiments, the length can be at least about any of 10, 15, 20, 25 nucleotides, with an upper limit of about any of 25, 30, 50, 60 nucleotides. In certain embodiments, the composite primer is about 21 or about 27 nucleotides in length. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

[0159] As described herein, one or more different composite primers may be used in an amplification reaction.

Auxiliary primers

[0160] "Auxiliary primer" as used herein, are a population of primers comprising randomized and/or partially-randomized sequences. Auxiliary primers are a polynucleotide as described herein, though generally, auxiliary primers are made of DNA. Such random primers are known in the art. An example of auxiliary primers is the population of randomized hexamer primers shown in Example 1. In some embodiments, the random primers may contain natural or non-natural nucleotide(s) that permit non-specific hybridization in order to increase the number of sequences to which the random primers may bind. Similarly, abasic sites can be introduced randomly within the population of random primers, which can permit non-specific hybridization by stabilizing mismatches between primer and template. The primers should be extendable by DNA polymerase. Generation of primers suitable for extension by polymerization is well known in the art, such as described in PCT Pub. No. WO 99/42618 (and references cited therein).

[0161] In some embodiments, auxiliary primers can be at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 15, at least 18, at least 20, or more nucleotides in length. In some embodiments, a population of primers of differing lengths is used.

DNA polymerase, and an agent capable of cleaving an RNA-DNA hybrid

[0162] The amplification methods of the invention employ the following enzymes: an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase, and an agent

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capable of cleaving an RNA strand of an RNA-DNA hybrid (for example, a ribonuclease such as RNase H). One or more of these activities may be found and used in a single enzyme. For example, RNase H activity may be supplied by an RNA-dependent DNA polymerase (such as reverse transcriptase) or may be provided in a separate enzyme. Reverse transcriptases useful for this method may or may not have RNase H activity. Many reverse transcriptases, such as those from avian myeloblastosis virus (AMV-RT), and Moloney murine leukemia virus (MMLV-RT) comprise more than one activity (for example, polymerase activity and ribonuclease activity) and can function in the formation of the double stranded cDNA molecules. However, in some instances, it is preferable to employ a reverse transcriptase which lacks the RNase H activity. Reverse transcriptase devoid of RNase H activity are known in the art, including those comprising a mutation of the wild type reverse transcriptase where the mutation eliminates the RNase H activity. In these cases, the addition of an RNase H from other sources, such as that isolated from *E. coli*, can be employed for the formation of the double stranded cDNA. The RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity may be provided by the same enzyme (for example, Bst polymerase), or these activities may be provided in separate enzymes.

[0163] One aspect of the invention is the formation of a complex comprising an RNA/DNA partial heteroduplex. This process generally utilizes the enzymatic activities of an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase. Generally, RNA in the RNA/DNA partial heteroduplex is cleaved by an agent (such as an enzyme, such as a ribonuclease) capable of cleaving RNA from an RNA/DNA hybrid, generating a 3' single stranded portion with sequences that are complementary to RNA in a composite primer (and thus forming a binding site for a composite primer).

[0164] RNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of a primer according to the methods of the invention. Accordingly, a preferred RNA-dependent DNA polymerase is one that is capable of extending a nucleic acid primer along a nucleic acid template that is comprised at least predominantly of ribonucleotides. Suitable RNA-dependent DNA polymerases for use in the methods and compositions of the invention include reverse transcriptase and, for example, a DNA polymerase that possesses both DNA-dependent and RNA-dependent DNA polymerase activity, such as Bst DNA polymerase.

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[0165] DNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of the composite primer according to the methods of the invention. Accordingly, a preferred polymerase is one that is capable of extending a nucleic acid primer along a nucleic acid template that is comprised at least predominantly of deoxynucleotides. The formation of the complex comprising the RNA/DNA partial heteroduplex can be carried out by a DNA polymerase which comprises both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities (such as Bst DNA polymerase, or a reverse transcriptase). Amplification of an RNA sequence according to methods of the invention involves the use of a DNA polymerase that is able to displace a nucleic acid strand from the polynucleotide to which the displaced strand is bound, and, generally, the more strand displacement capability the polymerase exhibits (i.e., compared to other polymerases which do not have as much strand displacement capability) is preferable. Preferably, the DNA polymerase has high affinity for binding at the 3'-end of an oligonucleotide hybridized to a nucleic acid strand. Preferably, the DNA polymerase does not possess substantial nicking activity. Generally, the DNA polymerase preferably has little or no 5'->3' exonuclease activity so as to minimize degradation of primer, or primer extension polynucleotides. Generally, this exonuclease activity is dependent on factors such as pH, salt concentration, whether the template is double stranded or single stranded, and so forth, all of which are familiar to one skilled in the art. Mutant DNA polymerases in which the 5'->3' exonuclease activity has been deleted, are known in the art and are suitable for the amplification methods described herein. Mutant DNA polymerases which lack both 5' to 3' nuclease and 3' to 5' nuclease activities have also been described , for example, exo$^{-/-}$Klenow DNA polymerase. It is preferred that the DNA polymerase displaces primer extension products from the template nucleic acid in at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, of the incidence of contact between the polymerase and the 5' end of the primer extension product. In some embodiments, the use of thermostable DNA polymerases with strand displacement activity is preferred. Such polymerases are known in the art, such as described in U.S. Pat. No. 5744312 (and references cited therein). Preferably, the DNA polymerase has little to no proofreading activity

[0166] Suitable DNA polymerases for use in the methods and compositions of the invention include those disclosed in U.S. Pat. Nos. 5648211 and 5744312, which include

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exo⁻ Vent (New England Biolabs), exo⁻ Deep Vent (New England Biolabs), Bst (BioRad), exo⁻ Pfu (Stratagene), Bca (Panvera), sequencing grade Taq (Promega), exo$^{-/-}$Klenow DNA polymerase, and thermostable DNA polymerases from thermoanaerobacter thermohydrosulfuricus.

[0167] The ribonuclease for use in the methods and compositions of the invention is capable of cleaving ribonucleotides in an RNA/DNA hybrid. Preferably, the ribonuclease cleaves ribonucleotides in an RNA/DNA hybrid regardless of the identity and type of nucleotides adjacent to the ribonucleotide to be cleaved. It is preferred that the ribonuclease cleaves independent of sequence identity. Examples of suitable ribonucleases for the methods and compositions of the invention are well known in the art, including ribonuclease H (RNase H), e.g., Hybridase.

[0168] As is well known in the art, DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity, and the ability to cleave RNA from a RNA/DNA hybrid may be present in different enzymes, or two or more activities may be present in the same enzyme. Accordingly, in some embodiments, the same enzyme comprises RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, different enzymes comprise RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity. In some embodiments, different enzymes comprise RNA -dependent DNA polymerase activity and cleave RNA from an RNA/DNA hybrid. In some embodiments, different enzymes comprise DNA-dependent DNA polymerase activity and cleave RNA from an RNA/DNA hybrid.

[0169] In general, the enzymes used in the methods and compositions of the invention should not produce substantial degradation of the nucleic acid components of said methods and compositions.

Reaction conditions and detection

[0170] Appropriate reaction media and conditions for carrying out the methods of the invention are those that permit nucleic acid amplification according to the methods of the invention. Such media and conditions are known to persons of skill in the art, and are

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described in various publications, such as U.S. Pat. Nos. 5,554,516; 5,716,785; 5,130,238; 5,194,370; 6,090,591; 5,409,818; 5,554,517; 5,169,766; 5,480,784; 5,399,491; 5,679,512; and PCT Pub. No. WO99/42618. For example, a buffer may be Tris buffer, although other buffers can also be used as long as the buffer components are non-inhibitory to enzyme components of the methods of the invention. The pH is preferably from about 5 to about 11, more preferably from about 6 to about 10, even more preferably from about 7 to about 9, and most preferably from about 7.5 to about 8.5. The reaction medium can also include bivalent metal ions such as Mg^{2+} or Mn^{2+}, at a final concentration of free ions that is within the range of from about 0.01 to about 15 mM, and most preferably from about 1 to 10 mM. The reaction medium can also include other salts, such as KCl or NaCl, that contribute to the total ionic strength of the medium. For example, the range of a salt such as KCl is preferably from about 0 to about 125 mM, more preferably from about 0 to about 100 mM, and most preferably from about 0 to about 75 mM. The reaction medium can further include additives that could affect performance of the amplification reactions, but that are not integral to the activity of the enzyme components of the methods. Such additives include proteins such as BSA or acetylated BSA, single strand binding proteins (for e.g., T4 gene 32 protein), and non-ionic detergents such as NP40 or Triton. Reagents, such as DTT, that are capable of maintaining enzyme activities can also be included. Such reagents are known in the art. Where appropriate, an RNase inhibitor (such as Rnasin) that does not inhibit the activity of the RNase employed in the method can also be included. Any aspect of the methods of the invention can occur at the same or varying temperatures. Preferably, the amplification reactions (particularly, primer extension other than the composite and second primer extension product synthesis steps, and strand displacement) are performed isothermally, which avoids the cumbersome thermocycling process. The amplification reaction is carried out at a temperature that permits hybridization of the oligonucleotides (primer) of the invention to the template polynucleotide and primer extension products, and that does not substantially inhibit the activity of the enzymes employed. The temperature can be in the range of 0° C to about 85° C, about 25° C to about 85° C, about 30°C to about 80°C, and about 37°C to about 75°C.

[0171] Random priming and/or primer extension and/or isothermal amplification can be conducted under conditions of reduced stringency (i.e., permitting hybridization of sequences that are not fully complementary). For a given set of reaction conditions, the ability of two nucleotide sequences to hybridize with each other is based on the degree of

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complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more stringent the conditions can be for hybridization and the more specific will be the binding of the two sequences. Conversely, the lower the stringency of the conditions for hybridization, the lower the complementarity necessary for binding between the hybridizing and/or partially hybridizing composite primer and template polynucleotide. Decreased stringency is achieved by any one or more of the following: reducing the temperature, decreasing the ratio of cosolvents, lowering the salt concentration, and the like. Conditions that increase or reduce the stringency of a hybridization reaction are widely known and published in the art. See, for example, Sambrook et al. (1989), and in Ausubel (1987), supra. Useful hybridization conditions are also provided in, e.g., Tijessen, 1993, Hybridization With Nucleic Acid Probes, Elsevier Science Publishers B.V. and Kricka, 1992, Nonisotopic DNA Probe Techniques, Academic Press San Diego, Calif. The hybridization conditions chosen depend on a variety of factors known in the art, for example the length and type (e.g., RNA, DNA, PNA) of primer and primer binding region of the oligonucleotide template, as well as the concentration of primer and template polynucleotides.

[0172] Insofar as it is convenient to use buffer conditions that are compatible with DNA polymerase activity and/or ribonuclease activity, stringency of hybridization of composite primers can be controlled by altering temperature of the reaction. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of approximately 15°C, 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 60°C, or more. Accordingly, in some embodiments, composite primer random hybridization occurs at a reduced temperature, for example at 25°C - 37°C, followed at incubation at increased temperature(s) suitable for the isothermal amplification phase of the methods (such as about 50°C). In some embodiments, temperature is increased at 5°C increments. In other embodiments, temperature is shifted from low to high temperature.

[0173] Nucleotide and/or nucleotide analogs, such as deoxyribonucleoside triphosphates, that can be employed for synthesis of the primer extension products in the methods of the invention are provided in the amount of from preferably about 50 to about 2500 μM, more preferably about 100 to about 2000 μM, even more preferably about 200 to about 1700 μM, and most preferably about 250 to about 1500 μM. In some embodiments, a nucleotide or nucleotide analog whose presence in the primer extension strand enhances

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displacement of the strand (for example, by causing base pairing that is weaker than conventional AT, CG base pairing) is included. Such nucleotide or nucleotide analogs include deoxyinosine and other modified bases, all of which are known in the art.

[0174] The oligonucleotide components of the amplification reactions of the invention are generally in excess of the number of target nucleic acid sequence to be amplified. They can be provided at about or at least about any of the following: 10, 10^2, 10^4, 10^6, 10^8, 10^{10}, 10^{12} times the amount of target nucleic acid. Composite primers can each be provided at about or at least about any of the following concentrations: 50 nM, 100 nM, 500 nM, 1 uM, 2.5 uM , 5uM, 10 uM. Composite primer concentration also impacts frequency and/or position of composite primer hybridization. Generally, increased primer concentrations increased frequency of primer hybridization. Auxiliary primers can be provided at about or at least about any of the following concentrations: about 25 nM, about 50nM, about 100 nM, about 500 nM, about 1 uM, about 2.5 uM, about 5 uM, about 10 uM, or more.

[0175] In one embodiment, the foregoing components are added simultaneously at the initiation of the amplification process. In another embodiment, components are added in any order prior to or after appropriate timepoints during the amplification process, as required and/or permitted by the amplification reaction. Such timepoints, some of which are noted below, can be readily identified by a person of skill in the art. The enzymes used for nucleic acid amplification according to the methods of the invention can be added to the reaction mixture either prior to the target nucleic acid denaturation step, following the denaturation step, or following hybridization of the primer to the target polynucleotide, as determined by their thermal stability and/or other considerations known to the person of skill in the art. In these embodiments, the reaction conditions and components may be varied between the different reactions.

[0176] The amplification process can be stopped at various timepoints, and resumed at a later time. Said timepoints can be readily identified by a person of skill in the art. One timepoint is at the end of random composite primer hybridization. Another timepoint is at the end of random composite primer hybridization and composite primer extension product synthesis. Another timepoint (in some embodiments) is following cleavage of template RNA. Another timepoint is immediately prior to initiation of single primer isothermal amplification (which in some embodiments, may be initiated by addition of an enzyme (such as RNase H) that cleaves RNA from RNA/DNA heteroduplex, and

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optionally, DNA polymerase). Another timepoint is at the end of second primer extension product synthesis. Methods for stopping the reactions are known in the art, including, for example, cooling the reaction mixture to a temperature that inhibits enzyme activity or heating the reaction mixture to a temperature that destroys an enzyme. Methods for resuming the reactions are also known in the art, including, for example, raising the temperature of the reaction mixture to a temperature that permits enzyme activity, replenishing a destroyed (depleted) enzyme, or adding reagent(s) necessary for initiation of a step (for example, addition of RNase H and/or DNA polymerase to initiate the single primer isothermal amplification phase of the methods). In some embodiments, one or more of the components of the reactions is replenished prior to, at, or following the resumption of the reactions. For example, it may be necessary to replenish the composite primer prior to beginning the single primer isothermal amplification reaction if the same composite primer is being used. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

[0177] The reaction can be allowed to proceed without purification of intermediate complexes, for example, to remove primer. Products can be purified at various timepoints, which can be readily identified by a person of skill in the art. One timepoint is at the end of formation of the complex comprising an RNA/DNA partial heteroduplex. Another timepoint is at the end of random composite primer hybridization.

[0178] The detection of the amplification product is indicative of the presence of the target sequence. Quantitative analysis is also feasible. Direct and indirect detection methods (including quantitation) are well known in the art. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of a polynucleotide containing a target sequence to the product of amplification of a reference sample that has a known quantity of a polynucleotide that contains the target sequence, the amount of target sequence in the test sample can be determined.

Compositions and kits of the invention

[0179] The invention also provides compositions and kits used in the methods described herein. The compositions may be any component(s), reaction mixture and/or intermediate described herein, as well as any combination.

[0180] In one embodiment, the invention provides a composition comprising a composite primer as described herein. In some embodiments, the composite primer

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comprises an RNA portion adjacent to the DNA portion. In another embodiment, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion. In other embodiments, the RNA portion of the composite primer consists of 7 to about 20 nucleotides and the DNA portion of the composite primer consists of about 5 to about 20 nucleotides. In still other embodiments, the RNA portion of the composite primer consists of about 10 to about 20 nucleotides and the DNA portion of the composite primer consists of about 7 to about 20 nucleotides. In some embodiments, the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0181] In other examples, the invention provides a composition comprising a composite primer as described herein, and auxiliary primers (for example, a population of randomized hexamer primers). In some embodiments, the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0182] In other examples, the invention provides a composition comprising a composite primer that is derivatized by attachment of a moiety capable of effecting attachment of a polynucleotide comprising the composite primer to a solid substrate used in preparing nucleic acid microarrays. In some embodiments, the composite primer is further by attachment of a positively charged moiety such as an amine. In other embodiments, the composite primer is labeled, for example by derivatizing the composite primer with a detectable moiety, such as a label, or a moiety that can be covalently or non-covalently attached to a label. Labeled composite primers are further described herein.

[0183] In other examples, the invention provides composition comprising a composite primer and one or more of: a DNA polymerase; an enzyme that cleaves RNA from an RNA/DNA duplex; and auxiliary primers (for example, a population of random hexamer primers). In some embodiments, the composition further comprises a labeled dNTP. In still other embodiments, the composition comprises a non-canonical nucleotide (such as dUTP), and reagents suitable for labeling and/or fragmenting abasic sites, as described in U.S. Patent Application Publication No. 2004/0005614 and Kurn et al, co-pending U.S. patent application No. 60/533,381.

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[0184] The compositions are generally in lyophilized or aqueous form, preferably in a suitable buffer.

[0185] The invention also provides compositions comprising the amplification products described herein. Accordingly, the invention provides a population of DNA which are copies or the complement of a target sequence, which are produced by any of the methods described herein (or compositions comprising the products). The invention also includes compositions and various configurations (such as arrays) of these populations, which may be homogeneous (same sequence) or heterogeneous (different sequence). These populations may be any assembly of sequences obtained from the methods described herein.

[0186] The compositions are generally in a suitable medium, although they can be in lyophilized form. Suitable media include, but are not limited to, aqueous media (such as pure water or buffers).

[0187] The invention provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for any one or more of the following uses: methods of amplification; genotyping, nucleic acid mutation detection (including methods of genotyping), determining the presence or absence of a sequence of interest, quantitating a sequence of interest, preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), comparative genomic hybridization, and characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention, methods of expression profiling, subtractive hybridization and the preparation of probes for subtractive hybridization, and methods of preparing libraries (which may be cDNA and/or differential hybridization libraries).

[0188] The kits of the invention comprise one or more containers comprising any combination of the components described herein, and the following are examples of such kits. A kit may comprise any of the composite primers described herein. In some embodiments, the kit comprises one or more composite primer selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides. In some embodiments, a kit further comprises auxiliary primers, which may or may not be

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separately packaged. The composite primer may be labeled or unlabeled. Kits may also optionally further include any of one or more of the enzymes described herein (for example, DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, a DNA polymerase that provides both DNA-dependent and RNA-dependent DNA polymerase activities, and an enzyme capable of cleaving RNA from an RNA/DNA hybrid, such as RNase H), as well as deoxynucleoside triphosphates (labeled or unlabeled or derivatized). Kits may also include one or more suitable buffers (for example, as described herein). Kits may also include a labeled dNTP(s) and/or a non-canonical nucleotide (such as dUTP), as described in Kurn et al, co-pending U.S. patent application No. 60/381,457.

[0189] One or more reagents in the kit can be provided as a dry powder, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentrations for performing any of the methods described herein. Each component can be packaged in separate containers or some components can be combined in one container where cross-reactivity and shelf life permit.

[0190] The kits of the invention may optionally include a set of instructions, generally written instructions, although electronic storage media (*e.g.*, magnetic diskette or optical disk) containing instructions are also acceptable, relating to the use of components of the methods of the invention for the intended nucleic acid amplification, and/or, as appropriate, for using the amplification products for purposes such as detection of sequence mutation. The instructions included with the kit generally include information as to reagents (whether included or not in the kit) necessary for practicing the methods of the invention, instructions on how to use the kit, and/or appropriate reaction conditions.

[0191] In another example, the kits of the invention comprise a complex of composite primer extension product and second primer extension product. In yet another example, any of these kits further comprises one or more controls (which can be, for example, template polynucleotide (*e.g.*, DNA template such as genomic DNA or RNA template such as total RNA or mRNA), composite primers, and/or auxiliary primer(s).

[0192] The component(s) of the kit may be packaged in any convenient, appropriate packaging. The components may be packaged separately, or in one or multiple combinations.

[0193] The relative amounts of the various components in the kits can be varied widely to provide for concentrations of the reagents that substantially optimize the

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reactions that need to occur to practice the methods disclosed herein and/or to further optimize the sensitivity of any assay.

[0194] The invention also provides systems for effecting the methods described herein. These systems comprise various combinations of the components discussed above.

[0195] Any of the systems embodiments may also comprise a template (target) sequence, as described herein. A system generally includes one or more apparatuses for performing the amplification methods of the invention. Such apparatuses include, for example, heating devices (such as heating blocks or water baths) and apparatuses which effect automation of one or more steps of the methods described herein. The methods of the invention are particularly suitable for use with miniaturized devices, as thermal cycling is not required for any of the steps. A non-limiting example of suitable devices includes the BioAnalyzer (Agilant and Caliper) and the eSensor.

[0196] The invention also provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. Examples of reaction mixtures have been described. In some embodiments, the invention provides reaction mixtures comprising (a) a target polynucleotide; (b) a composite primer comprising a 3' DNA portion and an RNA portion; (c) auxiliary primers; and (d) DNA polymerase. As described herein, any of the composite primers may be in the reaction mixture (or a plurality of composite primers), including a composite primer that comprises a 5' RNA portion which is adjacent to the 3' DNA portion. The reaction mixture could also further comprise an enzyme which cleaves RNA from an RNA/DNA hybrid, such as RNase H. In some embodiments, the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0197] Other reaction mixtures are described herein and are encompassed by the invention.

[0198] The invention also includes compositions comprising any of the complexes (which are intermediates in the methods described herein) described herein. Examples of such complexes are schematically depicted in Figures 1-8. As an example, one complex of the invention is a complex comprising: (a) a target polynucleotide strand; and (b) a composite primer, said composite primer comprising a 3' DNA portion and an RNA portion. The composite primer may have an RNA portion which is 5' and adjacent to the

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3" DNA portion. As another example, a complex of the invention is a complex comprising: (a) a composite primer extension product; and (b) a target polynucleotide.

[0199] In yet another example, a complex of the invention is a complex comprising a RNA/DNA partial heteroduplex, prepared by any of the methods described herein. In some embodiments, the complex further comprises a second RNA/DNA partial heteroduplex at a second end. In yet another example, the complex of the invention is a complex comprising a 3' single stranded DNA portion produced by any of the methods described herein. In some embodiments, the complex further comprises a second 3' single stranded region. In another example, the complex of the invention is (a) a complex comprising a 3' single stranded DNA portion, and (b) a composite primer hybridized to the 3' single stranded portion. .

Methods using the amplification methods and compositions of the invention

[0200] The methods and compositions of the invention can be used for a variety of purposes. For purposes of illustration, methods of nucleic acid mutation detection (including methods of genotyping), determining the presence or absence of a sequence of interest, quantitating a sequence of interest, preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), comparative genomic hybridization, and characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention, detecting and/or identifying novel nucleic acid sequences (such as novel coding or non-coding transcripts), and characterization of splice variant sequences, are described. Methods of expression profiling, methods of subtractive hybridization and the preparation of probes for subtractive hybridization, and methods of preparing libraries (which can be cDNA and/or differential hybridization libraries) are also described.

Method of preparing nucleic acids immoblized to a substrate, including a microarray of nucleic acids

[0201] The products of some of the amplification methods of the invention are suitable for immobilizing to a surface. In so far as the amplification products of the methods of the invention generally comprises a mixture of sequences corresponding to sense and antisense copies of template polynucleotide, it is useful to immobilize a population of sequences generated by amplification of template polynucleotide from a

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defined source (*e.g.*, DNA or RNA from a defined cell population or a single cell; organism-specific template (for example, the DNA or RNA of specific viruses or other pathogen(s) sufficient to identify the organism); or a disease-specific template. Immobilized amplification product may then be probed with different probes and the hybridization signals can be compared. For example, an immobilized array of genomic polynucleotides (DNA or RNA) from a known pathogen or non-pathogen(such as a virus, or group of viruses) may be used for assessment of the presence or identity of a pathogen within a sample of genetic material. Such arrays would be of use in disease surveillance and in identification of a pathogenic agent in the event of a disease outbreak. Polynucleotides may be isolated from a suspected sample, labeled using any method known in the art, and hybridized to such an array. The detection of signal due to hybridization to the array provides information as to the presence or identity of a pathogen present in sample polynucleotide.

[0202] Amplification products can be attached to a solid or semi-solid support or surface, which may be made, e.g., from glass, plastic (e.g., polystyrene, polypropylene, nylon), polyacrylamide, nitrocellulose, or other materials.

[0203] Several techniques are well-known in the art for attaching nucleic acids to a solid substrate such as a glass slide. One method is to incorporate modified bases or analogs that contain a moiety that is capable of attachment to a solid substrate, such as an amine group, a derivative of an amine group or another group with a positive charge, into the amplified nucleic acids. The amplification product is then contacted with a solid substrate, such as a glass slide, which is coated with an aldehyde or another reactive group which will form a covalent link with the reactive group that is on the amplification product and become covalently attached to the glass slide. Microarrays comprising the amplification products can be fabricated using a Biodot (BioDot, Inc. Irvine, CA) spotting apparatus and aldehyde-coated glass slides (CEL Associates, Houston, TX). Amplification products can be spotted onto the aldehyde-coated slides, and processed according to published procedures (Schena et al., *Proc. Natl. Acad. Sci. U.S.A.* (1995) 93:10614-10619). Arrays can also be printed by robotics onto glass, nylon (Ramsay, G., *Nature Biotechnol.* (1998), 16:40-44), polypropylene (Matson, et al., *Anal Biochem.* (1995), 224(1):110-6), and silicone slides (Marshall, A. and Hodgson, J., *Nature Biotechnol.* (1998), 16:27-31). Other approaches to array assembly include fine micropipetting within electric fields (Marshall and Hodgson, *supra*), and spotting the polynucleotides directly onto positively coated

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plates. Methods such as those using amino propyl silicon surface chemistry are also known in the art, as disclosed at http://www.cmt.corning.com and http://cmgm.stanford.edu/pbrown/.

[0204] One method for making microarrays is by making high-density polynucleotide arrays. Techniques are known for rapid deposition of polynucleotides (Blanchard et al., Biosensors & *Bioelectronics*, 11:687-690). Other methods for making microarrays, e.g., by masking (Maskos and Southern, *Nuc. Acids. Res.* (1992), 20:1679-1684), may also be used. In principle, and as noted above, any type of array, for example, dot blots on a nylon hybridization membrane, could be used. However, as will be recognized by those skilled in the art, very small arrays will frequently be preferred because hybridization volumes will be smaller.

[0205] The amplified polynucleotides may be spotted as a matrix on substrates comprising paper, glass, plastic, polystyrene, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, optical fiber or any other suitable solid or semi-solid (e.g., thin layer of polyacrylamide gel (Khrapko, et al., *DNA Sequence* (1991), 1:375-388) surface.

[0206] An array may be assembled as a two-dimensional matrix on a planar substrate or may have a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, cylinders and any other arrangement suitable for hybridization and detection of target molecules. In one embodiment the substrate to which the amplification products are attached is magnetic beads or particles. In another embodiment, the solid substrate comprises an optical fiber. In yet another embodiment, the amplification products are dispersed in fluid phase within a capillary which, in turn, is immobilized with respect to a solid phase.

[0207] Arrays may also be composed of particles, such as beads. The beads may be labeled with the amplified products alone, or may be labeled with both the amplified products and an additional label, such as defined dyes or other labels.

Characterization of nucleic acids

[0208] The amplification products obtained by the methods of the invention are amenable to further characterization. The products of the methods of the invention are particularly amenable to quantitative analysis, as sufficient DNA is produced which generally accurately reflect the representation of the various polynucleotides in the starting material.

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[0209] The amplified polynucleotide products (i.e., products of any of the amplification methods described herein), can be analyzed using, for example, probe hybridization techniques known in the art, such as Southern and Northern blotting, and hybridizing to probe arrays. They can also be analyzed by electrophoresis-based methods, such as differential display and size characterization, which are known in the art. In addition, the polynucleotide products may serve as starting material for other analytical and/or quantification methods known in the art, such as real time PCR, quantitative TaqMan, quantitative PCR using molecular beacons, methods described in U.S. Patents Nos. 6,251,639, 6,686,156, and 6,692,918; U.S. Patent Publication Nos. 2002/0115088 A1, 2003/0186234 A1, 2003/0087251A1, 2002/0164628, and 2003/0215926, and International Patent Application Publication WO 03/08343. Thus, the invention includes those further analytical and/or quantification methods as applied to any of the products of the methods herein.

[0210] In one embodiment, the amplification methods of the invention are utilized to generate multiple copies of polynucleotide products, and products are analyzed by contact with a probe.

[0211] In some embodiments, the amplification methods of the invention are utilized to generate multiple copies of single stranded polynucleotide (generally, DNA) products that are labeled by using composite primers that are labeled (in the portion(s) that is not cleaved). For example, the primer can be labeled with an aminoallyl labeled nucleotide. In other embodiments, the amplification methods of the invention are utilized to generate multiple copies of polynucleotide (generally, DNA) products that are labeled by the incorporation of labeled nucleotides during DNA. For example, amplification according to the methods of the invention can be carried out with suitable labeled dNTPs. These labeled nucleotides can be directly attached to a label, or can comprise a moiety which could be attached to a label. The label may be attached covalently or non-covalently to the amplification products. Suitable labels are known in the art, and include, for example, a ligand which is a member of a specific binding pair which can be detected/quantified using a detectable second member of the binding pair. Thus, amplification of template polynucleotide according to the methods of the invention in the presence of, for example, Cy3-dUTP or Cy5-dUTP results in the incorporation of these nucleotides into the amplification products. Amplification can also be in the presence of an

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aminoallyl-derivatized nucleotide, such as aminoallyl dUTP. Amplification product comprising aminoallyl dUTP can be coupled to a label, such as Cy3 or Cy5.

[0212] In other embodiments, the methods of the amplification are performed in he present of a non-canonical nucleotide, e.g., dUTP, and amplification comprising a non-canonical nucleotide is labeled and/or fragmented according to the methods disclosed in U.S. Patent Application Publication No. 2004/0005614. Briefly, non-canonical nucleotide (when incorporated into amplification product) is cleaved, generating an abasic site. The abasic site is then labeled by contacting with a reagent capable of labeling an abasic site. The polynucleotide comprising an abasic site can also be cleaved at the abasic site, generating fragments suitable for further analysis, e.g., hybridization to an array. The fragments can also be labeled as described above.

[0213] The labeled amplification products are particularly suitable for analysis (for example, detection and/or quantification) by contacting them with, for example, microarrays (of any suitable surface, which includes glass, chips, plastic), beads, or particles, that comprise suitable probes such as cDNA and/or oligonucleotide probes. Thus, the invention provides methods to characterize (for example, detect and/or quantify) an target polynucleotide of interest by generating labeled polynucleotide (generally, DNA) products using amplification methods of the invention, and analyzing the labeled products. Analysis of labeled products can be performed by, for example, hybridization of the labeled amplification products to, for example, probes immobilized at, for example, specific locations on a solid or semi-solid substrate, probes immobilized on defined particles, or probes immobilized on blots (such as a membrane), for example arrays, which have been described above. Other methods of analyzing labeled products are known in the art, such as, for example, by contacting them with a solution comprising probes, followed by extraction of complexes comprising the labeled amplification products and probes from solution. The identity of the probes provides characterization of the sequence identity of the amplification products, and thus by extrapolation the identity of the target polynucleotide present in a sample. Hybridization of the labeled products is detectable, and the amount of specific labels that are detected is proportional to the amount of the labeled amplification products of a specific target polynucleotide of interest.

[0214] The amount of labeled products (as indicated by, for example, detectable signal associated with the label) hybridized at defined locations on an array can be

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indicative of the detection and/or quantification of the corresponding target polynucleotide species in the sample.

[0215] Methods of characterization include sequencing by hybridization (see, e.g., Dramanac, U.S. Patent No. 6,270,961) and global genomic hybridization (also termed comparative genome hybridization) (*see, e.g.*,.Pinkel, U.S. Patent No. 6,159,685; Daigo et al (2001) Am. J. Pathol. 158 (5):1623-1631. Briefly, comparative genome hybridization comprises preparing a first population of labeled polynucleotides (which can be polynucleotide fragments) according to any of the methods described herein, wherein the template from which the first population is synthesized is total genomic DNA. A second population of labeled polynucleotides (to which the first population is desired to be compared) is prepared from a second genomic DNA template. The first and second populations are labeled with different labels. The hybridized first and second populations are mixed, and hybridized to an array or chromosomal spread. The different labels are detected and compared.

[0216] In another aspect, the invention provides a method of quantitating labeled and/or fragmented nucleic acids comprising use of an oligonucleotide (probe) of defined sequence (which may be immobilized, for example, on a microarray).

[0217] The amplification products generated according to the methods of the invention are also suitable for analysis for the detection of any alteration in the target nucleic acid sequence, as compared to a reference nucleic acid sequence which is identical to the target nucleic acid sequence other than the sequence alteration. When the target polynucleotide is genomic DNA or RNA, the sequence alterations may be sequence alterations present in the genomic sequence or may be sequence alterations which are not reflected in the genomic sequence, for example, alterations due to post transcriptional alterations, and/or mRNA processing, including splice variants. Sequence alterations (interchangeably called "mutations") include deletion, substitution, insertion and/or transversion of one or more nucleotide.

[0218] Other art recognized methods of analysis for the detection of any alteration in the target nucleic acid sequence, as compared to a reference nucleic acid sequence, are suitable for use with the nucleic acid products of the amplification methods of the invention. Such methods are well-known in the art, and include various methods for the detection of specific defined sequences including methods based on allele specific primer extension, allele specific probe ligation, differential probe hybridization, and limited primer

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extension. See, for example, Kurn et al, U.S. Patent No. 6,251,639 B1; U.S. Patent Nos. 5,888,819; 6,004,744; 5,882,867; 5, 854, 033; 5,710,028; 6,027,889; 6,004,745; 5,763,178; 5,011,769; 5,185,243; 4,876,187; 5,882,867; 5,731,146; WO US88/02746; WO 99/55912; WO 92/15712; WO 00/09745; WO 97/32040; WO 00/56925; and 5,660,988. Thus, the invention also provides methods for detection of a mutation in a target polynucleotide comprising a mutation (which can be a single nucleotide polymorphism), comprising: (a) amplifying a target polynucleotide using any of the methods described herein; and (b) analyzing the amplification products for presence of an alteration (mutation) as compared to a reference polynucleotide.

[0219] It is understood that the amplification products can also serve as template for further analysis such as sequence, polymorphism detection (including multiplex SNP detection) using, e.g., oligonucleotide ligation-based assays, analysis using Invader, Cleavase or limited primer extension, and the like. For methods that generally require larger volumes of input material, the methods of the invention may be used to "pre" amplify a pool of polynucleotides to generate sufficient input material for subsequent analysis.

Determination of gene expression profile

[0220] The amplification methods of the invention are particularly suitable for use in determining the levels of expression of one or more genes in a sample since the methods described herein are capable of amplifying a multiplicity, including a large multiplicity of target RNAs in the same sample. As described above, amplification products can be detected and quantified by various methods, as described herein and/or known in the art. Since RNA is a product of gene expression, the levels of the various RNA species, such as mRNAs, in a sample is indicative of the relative expression levels of the various genes (gene expression profile). Thus, determination of the amount of RNA sequences of interest present in a sample, as determined by quantifying amplification products of the sequences, provides for determination of the gene expression profile of the sample source.

[0221] Accordingly, the invention provides methods of determining gene expression profile in a sample, said method comprising: amplifying single stranded product from template RNA s in the sample, using any of the methods described herein ; and determining amount of amplification products of each RNA, wherein each said amount is indicative of amount of each RNA in the sample, whereby the expression profile in the

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sample is determined. Generally, labeled products are generated. In certain embodiments, the target RNA is mRNA. It is understood that amount of amplification product may be determined using quantitative and/or qualitative methods. Determining amount of amplification product includes determining whether amplification product is present or absent. Thus, an expression profile can includes information about presence or absence of one or more RNA sequence of interest. "Absent" or "absence" of product, and "lack of detection of product" as used herein includes insignificant, or *de minimus* levels.

[0222] The methods of expression profiling are useful in a wide variety of molecular diagnostic, and especially in the study of gene expression in essentially any mammalian cell (including a single cell) or cell population. A cell or cell population (e.g. a tissue) may be from, for example, blood, brain, spleen, bone, heart, vascular, lung , kidney, pituitary, endocrine gland, embryonic cells, tumors, or the like. Expression profiling is also useful for comparing a control (normal) sample to a test sample, including test samples collected at different times, including before , after, and/or during development, a treatment, and the like.

Method of preparing a library

[0223] The DNA products of the methods of the invention are useful in preparing libraries, including cDNA libraries and subtractive hybridization libraries. Using the methods of the invention, libraries may be prepared from limited amount of starting material, for example, mRNA extracted from limited amount of tissue or even single cells. Accordingly, in one aspect, the methods of the invention provides preparing a library from the DNA products of the invention. In still another aspect, the invention provides methods for making a library, said method comprising: preparing a subtractive hybridization probe using any of the methods described herein.

Methods of subtractive hybridization

[0224] The amplification methods of the invention are particularly suitable for use in subtractive hybridization methods, in which (at least) a first and second target polynucleotide population is compared, since the methods described herein are capable of amplifying multiple target polynucleotides in the same sample, and the methods of the invention are suitable for producing large amounts of single stranded antisense nucleic acid suitable for use as "driver" in subtractive hybridization. For example, two nucleic acid

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populations, one sense and one antisense, can be allowed to mix together with one population present in molar excess ("driver"). Sequence present in both populations will form hybrids, while sequences present in only one population remain single-stranded. Thereafter, various well known techniques are used to separate the unhybridized molecules representing differentially expressed sequences. See, e.g., Hamson et al., U.S. Patent No. 5,589,339; Van Gelder, U.S. Patent No. 6,291,170. The methods of subtractive hybridization provided herein are particularly suited for subtractive hybridization using amplified target RNAs.

[0225] Accordingly, the invention provides methods for performing subtractive hybridization, said methods comprising: (a) preparing multiple DNA copies of the complement of target polynucleotide from a first polynucleotide population using any of the amplification methods described herein; and (b) hybridizing the multiple copies to a second polynucleotide population, whereby a subpopulation of the second polynucleotide population forms a complex with DNA copies of the first polynucleotide population. The invention also provides methods for performing subtractive hybridization, said methods comprising: hybridizing multiple copies of the complement of at least one polynucleotide from a first polynucleotide population using any of the amplification methods described herein to a second polynucleotide population, whereby a subpopulation of the second population forms a complex with a copy from the copies of the first polynucleotide population. In preferred embodiments, the polynucleotide populations utilized in subtractive hybridization are RNA populations. In some embodiments, "driver" single stranded anti-sense DNA product of the methods of the invention is combined with tester (sense) RNA species. In some embodiments, "driver" single stranded antisense nucleic acid (generally, DNA) product is produced using the methods of the invention described herein.

[0226] In another aspect, the invention provides methods of differential amplification in which single stranded driver (antisense) DNA sequences that hybridize with tester RNA sequence are subjected to cleavage by an agent that cleaves RNA present in a DNA/RNA hybrid, such as RNase H. Cleavage of the RNA results in the inability to generate single stranded DNA product from the test RNA strands. Conversely, non-cleaved tester (i.e., tester RNA that did not hybridize to driver DNA molecules) may serve as a substrate for subsequent amplification. Amplified differentially expressed products have many uses, including as a differential expression probe, to produce differential

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expression libraries. Accordingly, the invention provides methods for differential amplification of one or more RNA template sequence, said method comprising: (a) preparing multiple polynucleotide (generally, DNA) copies of the complement of RNA from a first RNA population using any of the amplification methods described herein; (b) hybridizing the multiple copies to a second RNA population, whereby a subpopulation of the second RNA population forms a complex with a DNA copy; (c) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; and (d) amplifying an unhybridized subpopulation of the second RNA population, whereby multiple copies of single stranded DNA complementary to the unhybridized subpopulation of the second RNA population are generated. In some embodiments, step (d) is performed using any of the amplification methods described herein. In some embodiments, the methods comprise hybridizing multiple polynucleotide (generally, DNA) copies of the complement of at least one RNA sequences of interest from a first RNA population using any of the amplification methods described herein to a second RNA population, whereby a subpopulation of the second RNA population forms a complex with a DNA copy; (b) cleaving RNA in the complex of step (a) with an enzyme that cleaves RNA from an RNA/DNA hybrid; and (c) amplifying an unhybridized subpopulation of the second RNA population, whereby multiple copies of single stranded DNA complementary to the unhybridized subpopulation of the second RNA population are generated.

[0227] The following Examples are provided to illustrate, but not limit, the invention.

EXAMPLES

Example 1: Global amplification of human genomic DNA using a composite primer and random hexamer primers

[0228] Global amplification reactions were performed using composite primer IA20 and human genomic DNA as a template. The sequence of composite primer IA20 is as follows: IA20: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3' (SEQ ID NO:1), where italics denote ribonucleotides, and "d" denotes deoxyribonucleotides.

[0229] Human genomic DNA (Clontech, Cat. No. 6550-1) was diluted in TE buffer and denatured by heating to 99°C. In some samples, DNA and primers were mixed in amplification buffer and heated at 96°C for 2-4 minutes.

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[0230] The following reaction mixture was used.

[0231] 2 ng of pre-denatured human genomic DNA (approximately 600 copies)

[0232] 2 µl of random hexamer N6 (final concentration 2.5 µM) (Qiagen-Operon; item No.: PolyN (6-mer));

[0233] 0.5 µl (100 µM) composite primer IA20 (final concentration: 2.5 µM),

[0234] 0.1 µl Bst DNA polymerase (0.04 U/µl) (New England BioLabs, Catalog No. M0275);

[0235] 10 µl buffer (final concentration: 20 mM Tris-HCl, pH 8.5; 5 mM $MgCl_2$; RNasin, 0.3 U/µl; DTT, 0.5 mM; acetylated BSA, 0.1 µg/µl; T4 gp32 protein, 0.15 µg/µl)), and

[0236] RNase-free water to final volume of 15 µl.

[0237] The mixture was incubated at 30°C for 5 minutes, followed by 5 minutes at 40°C, and 2 minutes at 50°C.

[0238] 5 µl of enzyme mixture (RNase H, final concentration of 0.025 U/µl; and Bst DNA polymerase (large fragment), final concentration 0.2U/µl) was added, and the reaction was incubated at 50°C for 30 – 40 minutes. The reaction was stopped by incubation at 80°C for 5 minutes to inactivate the enzymes.

[0239] Control reactions were prepared in which either composite primer, N6 random primers, or RNase H were omitted. In control reactions, the corresponding volume (of the omitted reagent) was replaced by water.

[0240] Amplification reaction product was analyzed as follows. 0.5 µl of reaction mixture was loaded onto 4-20% gradient acrylamide gel and electrophoresed at 200-220 V constant voltage for 30 min. The gel was stained in 0.005 mg/ml Ethidium Bromide for 2 minutes and washed in water for 1 minute. The gel was then visualized and photographed on the AlphaImager 2200 system. The results are shown in Figure 9. Lanes correspond to the reaction mixtures containing the following components:

[0241] Lanes #1-2: Complete reaction mixture

[0242] Lanes #3-4: Reaction lacking N6 random primer.

[0243] Lanes #5-6: Reaction lacking composite primer.

[0244] Lanes #7-8: Reaction lacking RNase H.

[0245] Figure 9 shows that amplification product of varying molecular weight was produced in reactions containing the complete reaction mixture (described above), and in

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reaction lacking N6 random primers. However, reactions in which composite primer or RNase H were omitted did not show detectable reaction product.

[0246] Amplification product was quantified using the following procedure: reactions were prepared and processed as described above. Reaction mixture was diluted 100-fold and 2 µl of the diluted samples were used in Real Time PCR quantification with the following primer pairs that amplify a single copy sequence on Chromosome 7:

[0247] 221PF2 (5'-AGTATCTGGCACATCTT-3' (SEQ ID NO:_____)) and

[0248] 221PR2 (5'-GGGAGATATTATTTGGC-3' (SEQ ID NO:____)).

[0249] Amplification with primers 221PF2 and 221 PR2 was expected to yield a 62 base pair PCR product. The PCR reaction mixture contained: 1 µl of 10 µM of each primer, 6 µl of water, 10 µl of 2x SYBR Green PCR Master Mix (Applied Biosystems), and 2 µl diluted reaction mixture (diluted as described above). A control reaction was conducted using 2 µl human genomic DNA, instead of amplification reaction product as a template.

[0250] The thermal cycling program used was: one cycle of 94^{0}C for 10 min., followed by 45 cycles of 94^{0}C for 30 sec., 55^{0}C for 30 sec., and 72^{0}C for 30 sec. The Real Time PCR quantification data were presented as Ct values (threshold cycle). The values obtained for quantification of amplification products were compared with that obtained for 2 µl human genomic DNA (labeled "non-amplified Genomic DNA" in Table 1). The dilution factor between the diluted global amplification products and the original human genomic DNA input into the amplification reactions is 1000 fold. The data were summarized in Table 1.

Table 1: Quantification of amplification products and target human genomic DNA employing Real Time PCR with SYBR Green. A single copy sequence on Chromosome 7 of human genomic DNA (221) was used for determination of amplification efficiency. Amplification efficiency is expressed as the relative amount of this single copy sequence in starting genomic DNA sample and following global amplification.

Reaction components	**Ct**	**Efficiency**
Complete	29	4000 fold
No N6 random primers	36	40 fold
No Composite primer	None	None
No RNase H	None	None
None (non amplified Genomic DNA)	31	None

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[0251] In a second experiment, human genomic DNA was amplified using composite primer IA20 essentially as described above. Following amplification, several target sequences were quantified using Real Time PCR essentially as described above. Chromosomal location of target sequences and PCR primer pairs are shown in Table 2. Table 2 shows the relative amount of target sequence following global amplification, and the amplification efficiency. Real Time PCR quantification data were presented as Ct values (threshold cycle).

Table 2:

Target location	Forward PCR primer	Reverse PCR primer	Real time Delta C(t)	Amplification fold
chromosome #6	GGACGTGTGTTCCTGTTAA (SEQ ID NO___)	CACTTTGATCCTGAAAGACT (SEQ ID NO__)	3.5	2000
chromosome #7	AGTATCTGGCACATCTT (SEQ ID NO:__)	GGGAGATATTATTTGGC (SEQ ID NO:__)	4	3000
chromosome #11	AGGTTCCCAGCCTTGGTCC (SEQ ID NO__)	TGAGGCCATGTGTGTGGAAT (SEQ ID NO:__)	2	800
chromosome #12	AATAATGTCCAGATATCTTGGT (SEQ ID NO:__)	TCCCTACTCCAGCTACTTCT (SEQ ID NO:__)	2.5	1000
chromosome #16	CAGCAAGAACACAAGGGAC (SEQ ID NO__)	TCTTGAGAGCGAGGGCA (SEQ ID NO__)	2.5	1000

[0252] In a third experiment, human genomic DNA was amplified using composite primer BSCA-128F essentially as described above. The sequence of composite primer BSCA-128F is:

[0253] 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2) where italics denote ribonucleotides, and "d" denotes deoxyribonucleotides.

[0254] Amplification reaction product was analyzed as described above. Amplification product of varying molecular weights was generated, suggesting that the composite primer permitted amplification from a multiplicity of template sequences.

[0255] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the descriptions and examples should not be construed as limiting the scope of the invention.

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CLAIMS

We claim:

1. A method for amplification of a template polynucleotide, comprising:

(a) incubating a reaction mixture, said reaction mixture comprising:

(i) a template polynucleotide;

(ii) a first primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion;

(iii) a DNA-dependent DNA polymerase; and

(iv) an RNA-dependent DNA polymerase;

wherein the incubation is under conditions that permit first primer random hybridization to the template polynucleotide, and primer extension, whereby a complex comprising a RNA/DNA heteroduplex is generated; and

(b) incubating a reaction mixture, said reaction mixture comprising

(i) at least a portion of the reaction products generated according to step (a);

(ii) an amplification primer, wherein said amplification primer is a composite primer comprising an RNA portion and a 3' DNA portion;

(iii) auxiliary primers;

(iv) an DNA-dependent DNA polymerase; and

(v) an agent that cleaves RNA from an RNA/DNA hybrid;

wherein the incubation is under conditions that permit RNA cleavage, primer hybridization, primer extension, and displacement of the primer extension product when its RNA is cleaved and another amplification primer binds to the template and is extended by strand displacement, whereby multiple copies of a polynucleotide amplification product are generated.

2. The method of claim 1, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are the same enzyme.

3. The method of claim 1, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are different enzymes.

4. The method of claim 1, wherein said first primer and said amplification primer are the same primer.

5. The method of claim 1, wherein said first primer and said amplification primer are different primers.

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6. The method of claim 1, wherein step (b) is initiated by the addition of an agent that cleaves RNA from an RNA/DNA heteroduplex to the reaction mixture of step (a).

7. The method of claim 6, wherein said agent that cleaves RNA from an RNA/DNA heteroduplex is RNase H.

8. The method of claim 1, wherein the reaction mixture of step (a) further comprises auxiliary primers.

9. The method of claim 1, wherein said template polynucleotide is DNA.

10. The method of claim 9, wherein said first primer comprises a random sequence.

11. The method of claim 10, wherein said first primer is at least two different primers.

12. The method of claim 11, wherein said first primer is a plurality of different primers.

13. The method of claim 1, wherein said template polynucleotide is RNA.

14. The method of claim 13, wherein said template RNA is mRNA.

15. The method of claim 13, wherein the reaction mixture of step (a) further comprises an agent that cleaves RNA from a RNA/DNA heteroduplex to the reaction mixture of step (a).

16. The method of claim 15, wherein the agent in the reaction mixture of step (a) that cleaves RNA in a RNA/DNA heteroduplex is an enzyme.

17. The method of claim 16, wherein the enzyme that cleaves RNA in a RNA/DNA heteroduplex in the reaction mixture of step (a) is RNase H.

18. The method of claim 15, wherein said first primer and said amplification primer are different primers.

19. The method of claim 18, wherein step (b) is initiated by addition of the amplification primer to the reaction mixture of step (a).

20. The method of claim 15, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are the same enzyme.

21. The method of claim 15, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are different enzymes.

22. The method of claim 15, wherein the agent that cleaves RNA from an RNA/DNA heteroduplex is RNase H.

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23. The method of claim 15, wherein the reaction mixture of step (a) further comprises auxiliary primers.

24. The method of claim 15, wherein the reaction mixture of step (b) further comprises auxiliary primers.

25. The method of claim 1, wherein the RNA portion of the first primer is 5' with respect to the 3'-DNA portion.

26. The method of claim 25, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

27. The method of claim 26, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

28. The method of claim 25, wherein the 5' RNA portion of the first primer is adjacent to the 3' DNA portion.

29. The method of claim 28, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

30. The method of claim 29, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

31. The method of claim 1, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

32. The method of claim 31, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

33. The method of claim 1, wherein the RNA portion of the first primer consists of 7 to about 20 nucleotides.

34. The method of claim 33, wherein the DNA portion of the first primer consists of about 5 to about 20 nucleotides.

35. The method of claim 34, wherein the RNA portion of the first primer consists of about 10 to about 20 nucleotides.

36. The method of claim 35, wherein the DNA portion of the first primer consists of about 7 to about 20 nucleotides.

37. The method of claim 1, wherein the RNA portion of the amplification primer consists of 7 to about 20 nucleotides.

38. The method of claim 37, wherein the DNA portion of the amplification primer consists of about 5 to about 20 nucleotides.

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39. The method of claim 38, wherein the RNA portion of the amplification primer consists of about 10 to about 20 nucleotides.

40. The method of claim 39, wherein the DNA portion of the amplification primer consists of about 7 to about 20 nucleotides.

41. The method of claim 1, wherein the first primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

42. The method of claim 41, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

43. The method of claim 1, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

44. The method of claim 1, wherein the reaction mixture of step (b) further comprises a non-canonical nucleotide.

45. The method of claim 44, wherein the non-canonical nucleotide is dUTP.

46. The method of claim 1, wherein the reaction mixture of step (b) further comprises a labeled nucleotide.

47. A method for amplification of a template polynucleotide, comprising:

incubating a reaction mixture, said reaction mixture comprising:

(a) a complex comprising a RNA/DNA partial heteroduplex, wherein the complex is generated by incubating a first reaction mixture, said first reaction mixture comprising:

(i) a polynucleotide template;

(ii) a first primer; wherein the first primer is a composite primer, the composite primer comprises an RNA portion and a 3' DNA portion; and wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites;

(iii) a DNA-dependent DNA polymerase; and

(iv) an RNA-dependent DNA polymerase;

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wherein the incubation is under conditions that permit first primer random hybridization, and primer extension, whereby a complex comprising an RNA/DNA partial heteroduplex is generated;

(b) an amplification primer, wherein the amplification primer is a composite primer comprising an RNA portion and a 3' DNA portion;

(c) auxiliary primers;

(d) a DNA-dependent DNA polymerase; and

(e) an agent that cleaves RNA from an RNA/DNA hybrid;

wherein the incubation is under conditions that permit RNA cleavage, primer hybridization, primer extension, and displacement of primer extension product when its RNA is cleaved and another amplification primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated.

48. The method of claim 47, wherein the agent that cleaves RNA from an RNA/DNA hybrid is an enzyme.

49. The method of claim 48, wherein the enzyme that cleaves RNA from an RNA/DNA hybrid is RNase H.

50. The method of claim 48, wherein said DNA-dependent DNA polymerase and said enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

51. The method of claim 50, wherein said DNA-dependent DNA polymerase and said enzyme that cleaves RNA from an RNA/DNA hybrid are different enzymes.

52. The method of claim 47, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

53. The method of claim 52, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

54. The method of claim 47, wherein the RNA portion of the amplification primer consists of 7 to about 20 nucleotides.

55. The method of claim 54, wherein the DNA portion of the amplification primer consists of about 5 to about 20 nucleotides.

56. The method of claim 55, wherein the RNA portion of the amplification primer consists of about 10 to about 20 nucleotides.

57. The method of claim 56, wherein the DNA portion of the amplification primer consists of about 7 to about 20 nucleotides.

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58. The method of claim 47, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

59. The method of claim 47, wherein the reaction mixture further comprises a non-canonical nucleotide.

60. The method of claim 59, wherein the non-canonical nucleotide is dUTP.

61. The method of claim 47, wherein the reaction mixture further comprises a labeled nucleotide.

62. A method for amplification of a template polynucleotide, comprising:

incubating a reaction mixture, said reaction mixture comprising:

(a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a randomly primed first primer hybridized to target polynucleotide with a DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites, and wherein the second primer extension product is generated by extension of a second primer hybridized to the first primer extension product;

(b) an amplification primer, wherein the amplification is a composite primer comprises an RNA portion and a 3' DNA portion and is hybridizable to the second primer extension product;

(c) auxiliary primers;

(d) a DNA-dependent DNA polymerase; and

(e) an agent that cleaves RNA from an RNA/DNA hybrid;

wherein the incubation is under conditions that permit RNA cleavage, primer hybridization, primer extension, and displacement of composite primer extension product from the second primer extension product when the RNA portion of the composite primer is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated.

63. The method of claim 62, wherein said agent that cleaves RNA from a RNA/DNA hybrid is an enzyme.

64. The method of claim 63, wherein the enzyme that cleaves RNA from a RNA/DNA hybrid RNase H.

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65. The method of claim 63, wherein said DNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

66. The method of claim 62, wherein said DNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA hybrid are different enzymes.

67. The method of claim 62, wherein said template polynucleotide is RNA.

68. The method of claim 62, wherein said template polynucleotide is DNA.

69. The method of claim 62, wherein said reaction mixture further comprises auxiliary primers.

70. The method of claim 62, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

71. The method of claim 70, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

72. The method of claim 62, wherein the RNA portion of the amplification primer consists of 7 to about 20 nucleotides.

73. The method of claim 72, wherein the DNA portion of the amplification primer consists of about 5 to about 20 nucleotides.

74. The method of claim 73, wherein the RNA portion of the amplification primer consists of about 10 to about 20 nucleotides.

75. The method of claim 74, wherein the DNA portion of the amplification primer consists of about 7 to about 20 nucleotides.

76. The method of claim 62, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

77. The method of claim 62, wherein the reaction mixture further comprises a non-canonical nucleotide.

78. The method of claim 77, wherein the non-canonical nucleotide is dUTP.

79. The method of claim 62, wherein the reaction mixture further comprises a labeled nucleotide.

80. A method for amplification of a polynucleotide template, comprising:

(a) random priming of polynucleotide template strand with a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion, and wherein the composite primer is capable of hybridizing to a multiplicity of template

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polynucleotide sites, thereby producing a complex comprising a composite primer randomly hybridized to polynucleotide template; and

(b) incubating the complex in the presence of auxiliary primers, a DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and an agent that cleaves RNA from a RNA/DNA heteroduplex, whereby multiple copies of polynucleotide amplification product are generated by primer extension and strand displacement.

81. The method of claim 80, wherein step (a) further comprises auxiliary primers.

82. The method of claim 80, wherein step (a) further comprises a DNA-dependent DNA polymerase.

83. The method of claim 82, wherein step (a) further comprises a RNA-dependent DNA polymerase.

84. The method of claim 80, wherein the agent that cleaves RNA from a RNA/DNA heteroduplex is an enzyme.

85. The method of claim 84, wherein the enzyme that cleaves RNA from a RNA/DNA heteroduplex is RNase H.

86. The method of claim 84, wherein the RNA-dependent DNA polymerase, and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

87. The method of claim 86, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

88. The method of claim 84, wherein the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are different enzymes.

89. The method of claim 88, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are all different enzymes.

90. The method of claim 80, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are the same enzyme.

91. The method of claim 80, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are different enzymes.

92. The method of claim 80, wherein the RNA portion of the composite primer is 5' with respect to the 3'-DNA portion.

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93. The method of claim 92, wherein the 5' RNA portion of the composite primer is adjacent to the 3' DNA portion.

94. The method of claim 80, wherein the RNA portion of the composite primer consists of 7 to about 20 nucleotides.

95. The method of claim 94, wherein the DNA portion of the composite primer consists of about 5 to about 20 nucleotides.

96. The method of claim 95, wherein the RNA portion of the composite primer consists of about 10 to about 20 nucleotides.

97. The method of claim 96, wherein the DNA portion of the composite primer consists of about 7 to about 20 nucleotides.

98. The method of claim 80, wherein the composite primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

99. The method of claim 80, wherein the step of incubating is further in the presence of a non-canonical nucleotide.

100. The method of claim 99, wherein the non-canonical nucleotide is dUTP.

101. The method of claim 80, wherein the step of incubating is further in the presence of a labeled nucleotide.

102. A method for amplification of a polynucleotide template, comprising:

incubating a reaction mixture comprising:

(a) a polynucleotide template strand;

(b) a first primer, wherein said first primer is a composite primer comprising a RNA portion and a 3' DNA portion, and wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites;

(c) auxiliary primers;

(d) a DNA-dependent DNA polymerase;

(e) a RNA-dependent DNA polymerase; and

(f) an agent that cleaves RNA from a RNA/DNA heteroduplex,

whereby multiple copies of polynucleotide amplification product are generated by primer extension and strand displacement.

103. The method of claim 102, wherein the agent that cleaves RNA from a RNA/DNA heteroduplex is an enzyme.

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104. The method of claim 103, wherein the enzyme that cleaves RNA from a RNA/DNA heteroduplex is RNase H.

105. The method of claim 103, wherein the RNA-dependent DNA polymerase, and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

106. The method of claim 105, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

107. The method of claim 103, wherein the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are different enzymes.

108. The method of claim 107, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are all different enzymes.

109. The method of claim 102, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are the same enzyme.

110. The method of claim 102, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are different enzymes.

111. The method of claim 102, wherein said reaction mixture further comprises an amplification primer, wherein said amplification primer is a composite primer comprising a RNA portion and a 3' DNA portion.

112. The method of claim 111, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

113. The method of claim 112, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

114. The method of claim 102, wherein the RNA portion of the first primer consists of 7 to about 20 nucleotides.

115. The method of claim 114, wherein the DNA portion of the first primer consists of about 5 to about 20 nucleotides.

116. The method of claim 115, wherein the RNA portion of the first primer consists of about 10 to about 20 nucleotides.

117. The method of claim 116, wherein the DNA portion of the first primer consists of about 7 to about 20 nucleotides.

118. The method of claim 102, wherein the first primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-

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*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

119. The method of claim 102, wherein the reaction mixture further comprises a non-canonical nucleotide.

120. The method of claim 119, wherein the non-canonical nucleotide is dUTP.

121. The method of claim 102, wherein the reaction mixture further comprises a labeled nucleotide.

122. A method of making a polynucleotide array, comprising:

immobilizing polynucleotide amplification product onto a substrate, said polynucleotide amplification product produced according to any of methods 1, 47, 62, 80, or 102.

123. The method of claim 122, wherein said polynucleotide amplification products are generated by amplification of template polynucleotide from a defined source.

124. The method of claim 123, wherein the defined source is a defined cell population.

125. The method of claim 122, wherein said substrate is selected from the group consisting of paper, glass, plastic, nitrocellulose, silicon, and optical fiber.

126. The method of claim 122, wherein the substrate is a particle.

127. The method of claim 126, wherein the particle is a bead.

128. The method of claim 127, wherein the bead is labeled with a dye.

129. A method of characterizing a nucleic acid, comprising:

analyzing polynucleotide amplification product, said amplification product produced by the method of any of claims 1, 47, 62, 80, or 102.

130. The method of claim 129, wherein the analyzing is carried out by contacting the amplification product with a probe.

131. The method of claim 129, wherein the analyzing is carried out by quantifying a sequence of interest in the amplification product.

132. The method of claim 129, wherein the analyzing is carried out by sequencing the amplification product.

133. The method of claim 129, wherein the analyzing is carried out by detecting any alteration in a target nucleic acid sequence in the amplification product, as compared to a reference nucleic acid sequence.

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134. The method of claim 133, wherein detection of an alteration in a target nucleic acid sequence is carried out y a method selected from the group consisting of allele specific primer extension, allele specific probe ligation, differential probe hybridization, and limited primer extension.

135. A method for determining a gene expression profile, comprising:

quantifying amplification products of sequences of interest, wherein said amplification products are produced by any of the methods of claims 1, 47, 62, 80, or 102.

136. The method of claim 135, wherein said amplification products are amplified from RNA template.

137. A method of preparing a library, comprising:

preparing a library of polynucleotide amplification products, said amplification products produced by any of the methods of claims 1, 47, 62, 80, or 102.

138. A method for performing subtractive hybridization, comprising:

hybridizing a polynucleotide amplification product comprising multiple copies of target polynucleotide to a polynucleotide population, whereby a subpopulation of the polynucleotide population forms a complex with the polynucleotide amplification product, wherein said polynucleotide amplification product is produced according to the method of any of claims 1, 47, 62, 80, or 102.

139. A method for differential amplification, comprising:

hybridizing a DNA driver polynucleotide population to a RNA population, whereby a subpopulation of the RNA population forms a complex with the driver population;

cleaving RNA with an agent that cleaves RNA in an RNA/DNA heteroduplex; and

amplifying the remaining RNA population according to the method of any of claims 1, 47, 62, 80, or 102.

140. A method for archiving polynucleotide templates, comprising:

storing polynucleotide amplification product, wherein said polynucleotide amplification product is produced according to the method of any of claims 1, 47, 62, 80, or 102.

141. A kit for amplifying template polynucleotide, said kit comprising:

a composite primer that is capable of binding to multiple sites within template polynucleotide; and

instructions for carrying out the method according to any of claims 1, 47, 62, 80, or 102.

IDT Exhibit 1020.10343

142. The kit of claim 141, further comprising auxiliary primers.

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Figure 1

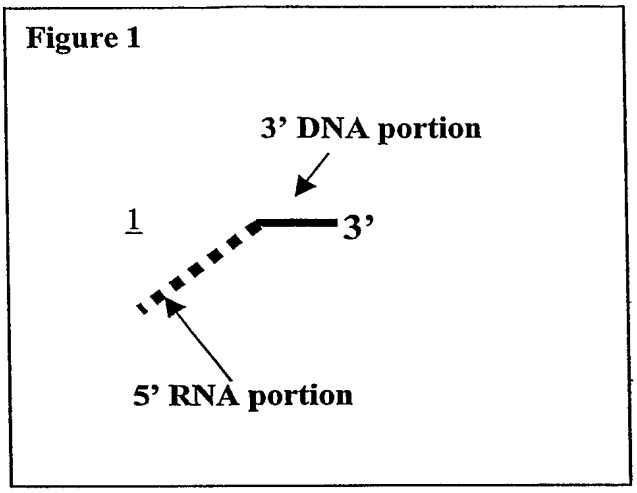


Figure 2

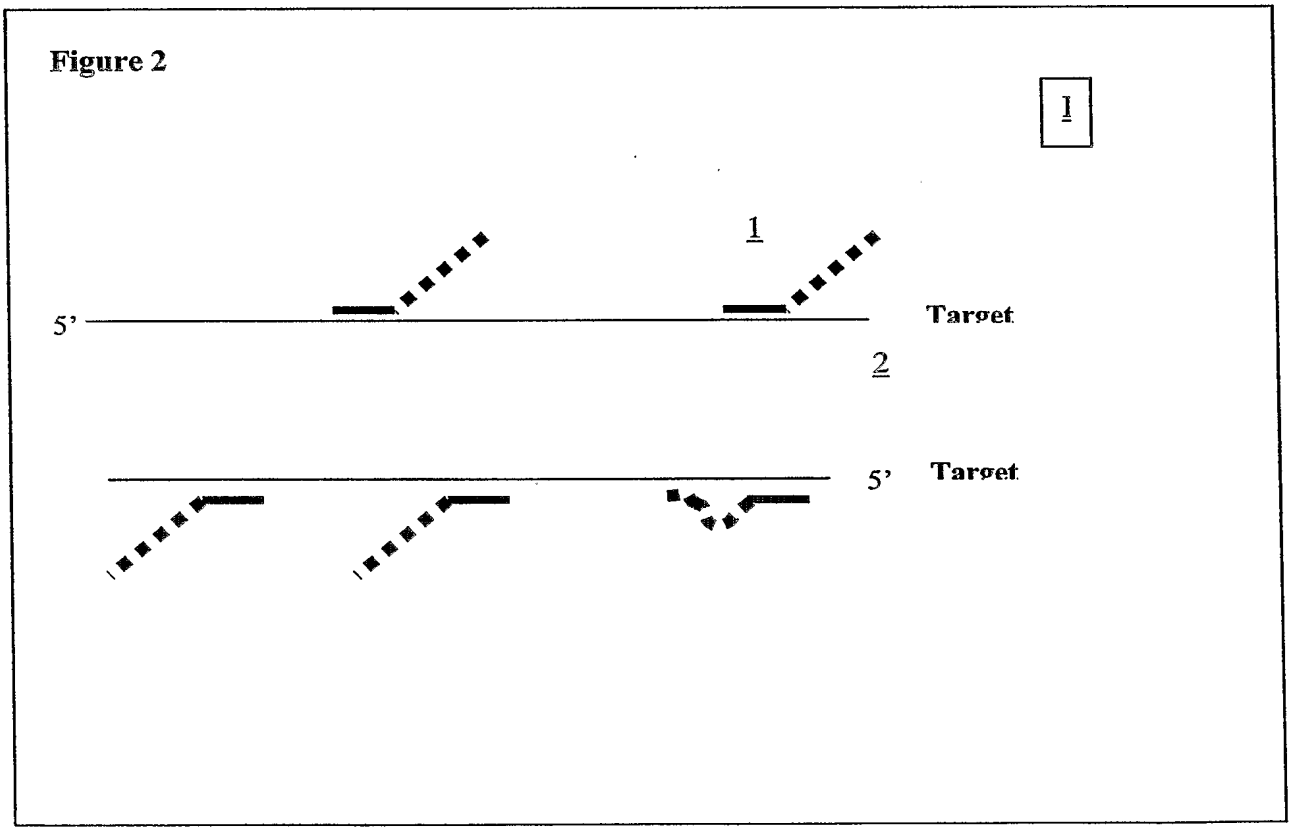


Figure 3

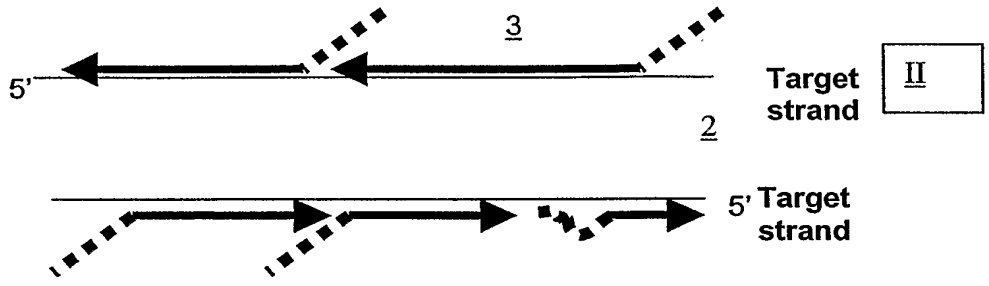


Figure 4

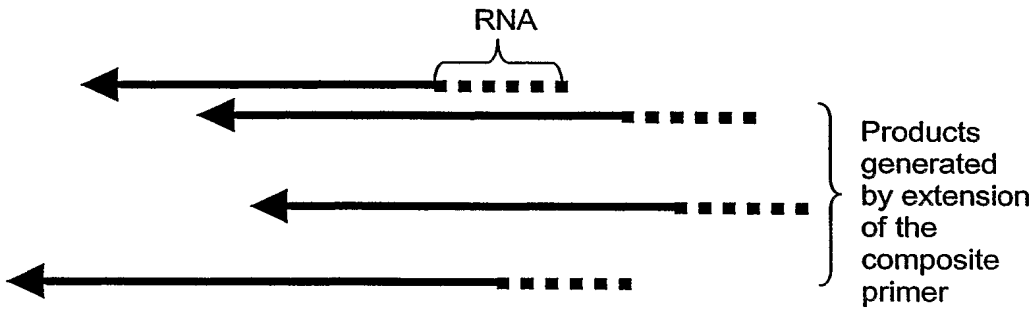

RNA
Products generated by extension of the composite primer

Figure 5

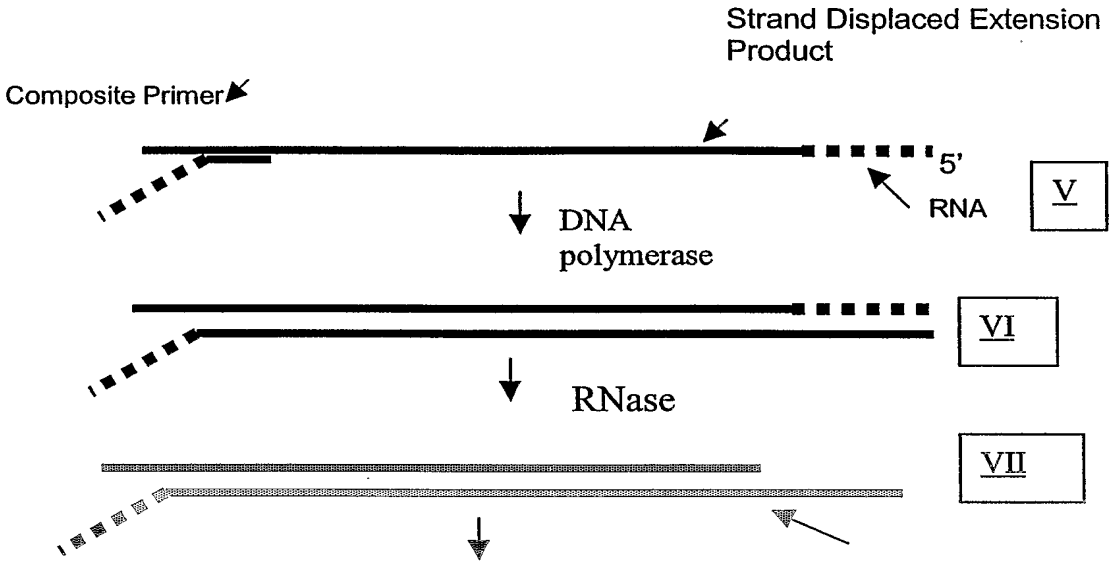

Strand Displaced Extension Product
Composite Primer
5'
RNA
V
DNA polymerase
VI
RNase
VII

Figure 6

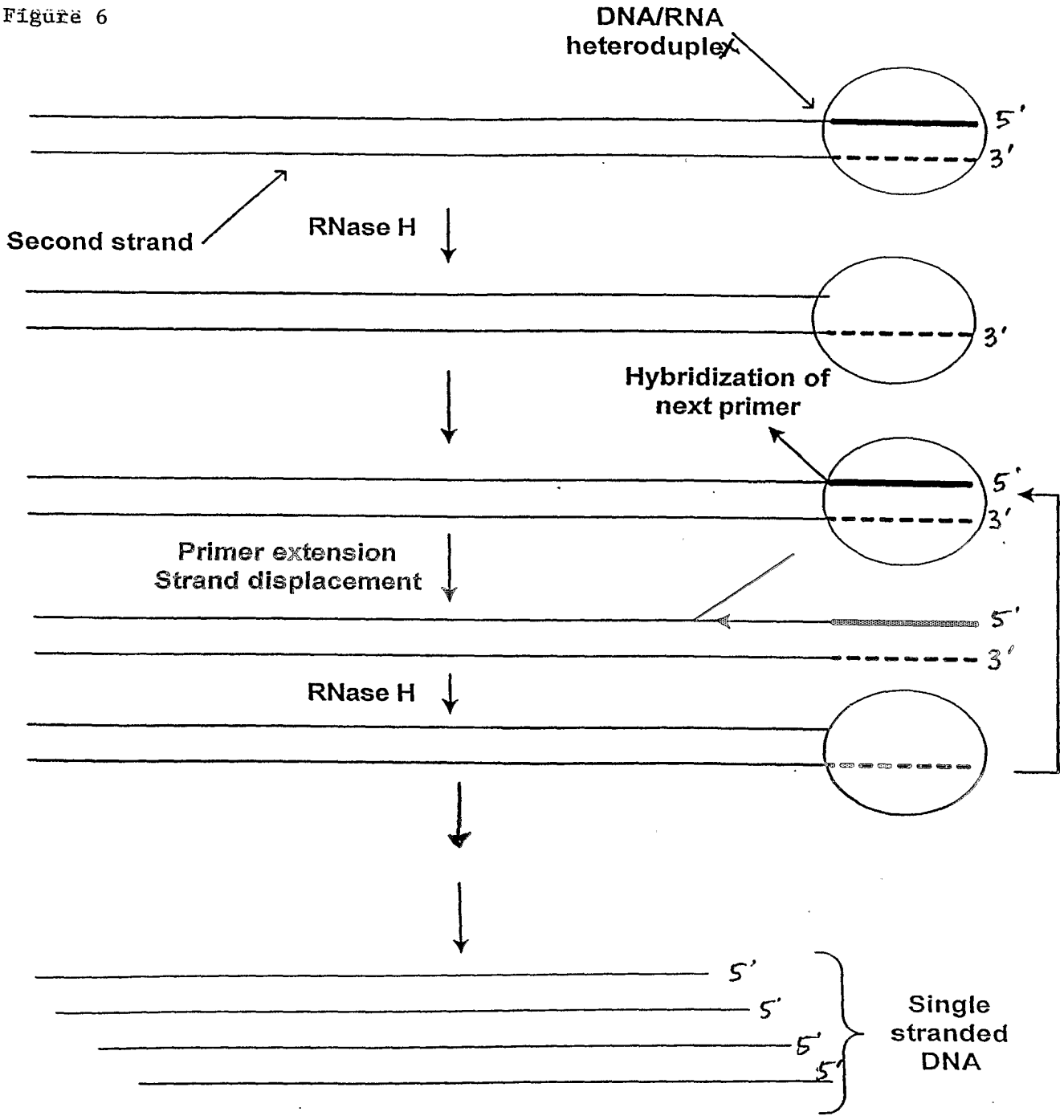
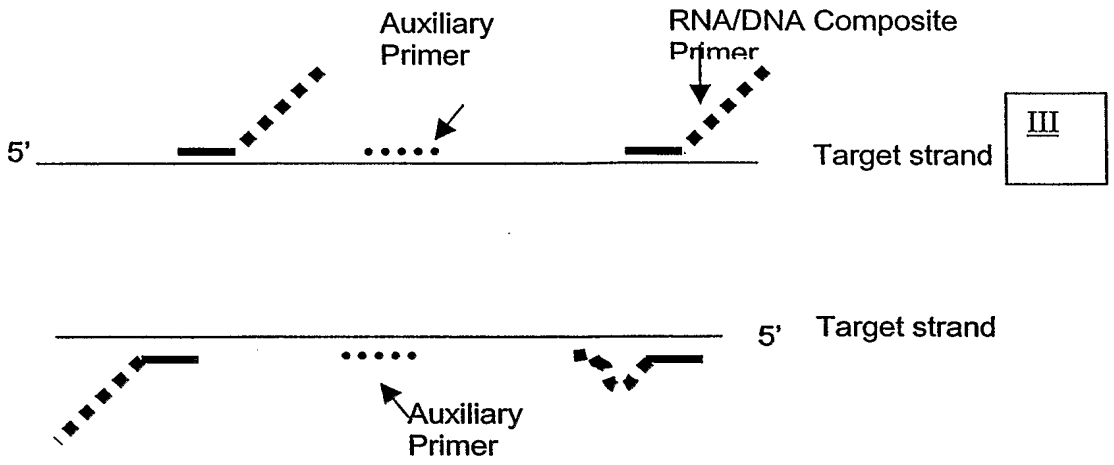

DNA/RNA
heteroduplex
5'
3'
Second strand
RNase H
3'
Hybridization of
next primer
5'
3'
Primer extension
Strand displacement
5'
3'
RNase H
5'
5'
5'
5'
Single
stranded
DNA

Figure 7A



5

Figure 7B

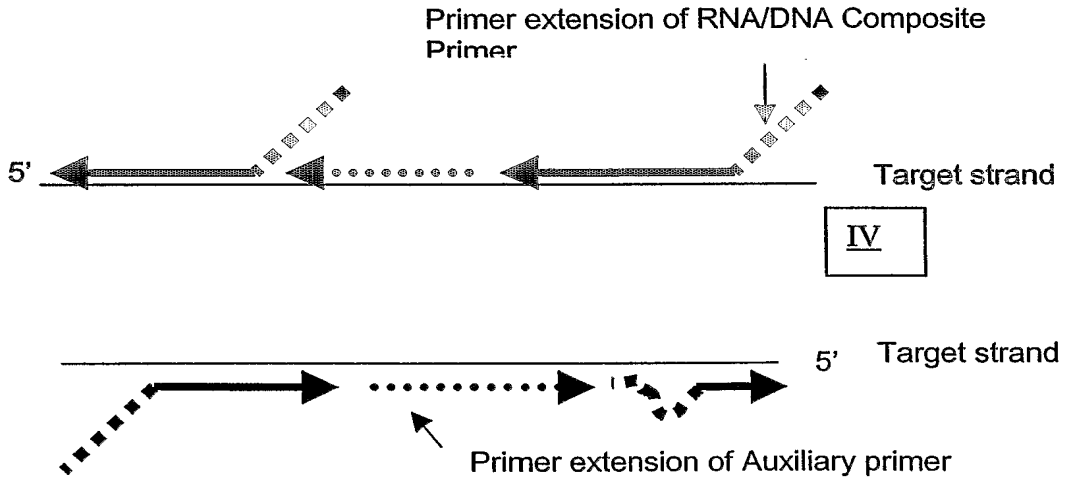


Figure 8

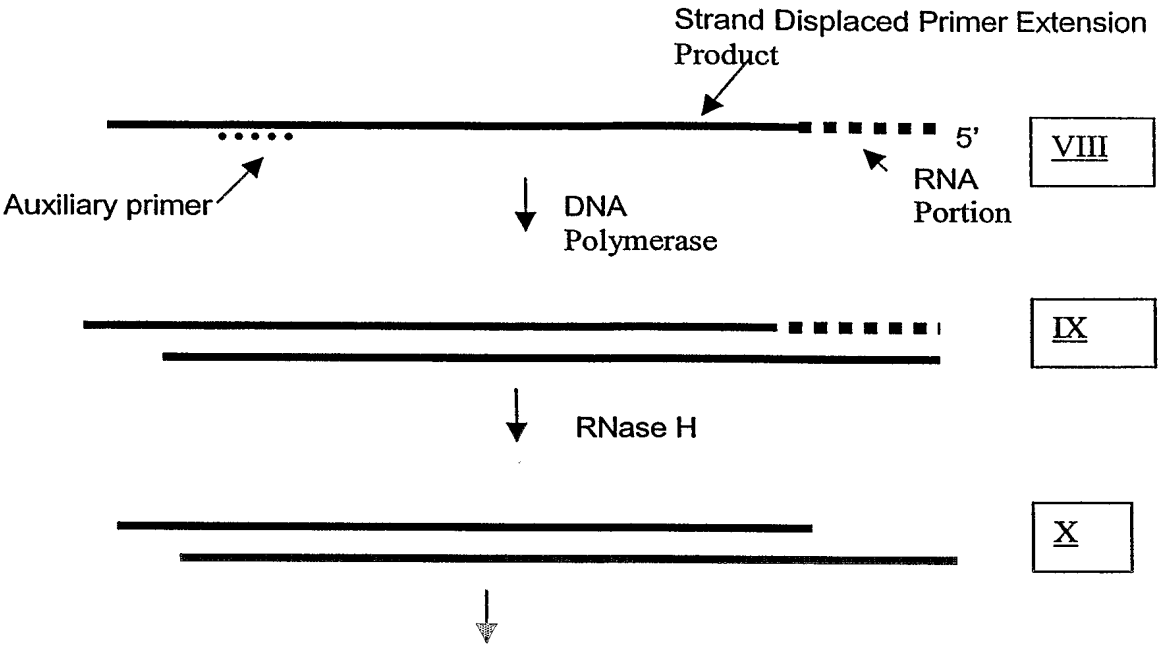
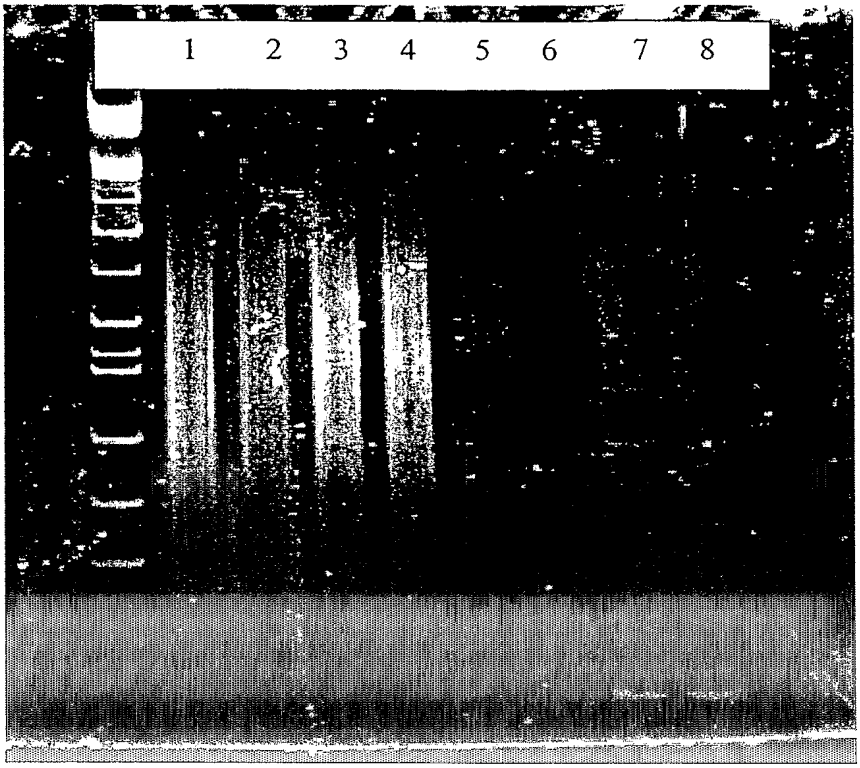

Strand Displaced Primer Extension Product
5'
VIII
RNA Portion
Auxiliary primer
DNA Polymerase
IX
RNase H
X

FIGURE 9



PTO/SB/08a (01-22)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17381888
Filing Date	2021-07-21
First Named Inventor	Douglas A. Amorese
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	NUGN-001/04US 34079/550

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	1	20090029385	A1	2009-01-29	Christians et al.	
	2	20090036663	A1	2009-02-05	Kurn	
	3	20090061425	A1	2009-03-05	Lo et al.	
	4	20090061439	A1	2009-03-05	Buzby	
	5	20090068645	A1	2009-03-12	Sibson	

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	6	20090068655	A1	2009-03-12	Williams	
	7	20090068709	A1	2009-03-12	Kurn et al.	
	8	20090105081	A1	2009-04-23	Rodesch et al.	
	9	20090117573	A1	2009-05-07	Fu et al.	
	10	20090117621	A1	2009-05-07	Boutell et al.	
	11	20090124514	A1	2009-05-14	Fu et al.	
	12	20090127589	A1	2009-05-21	Rothberg et al.	
	13	20090130721	A1	2009-05-21	Kurn et al.	
	14	20090203085	A1	2009-08-13	KURN et al.	
	15	20090203531	A1	2009-08-13	Kurn	
	16	20090233802	A1	2009-09-17	Bignell et al.	

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	17	20090233804	A1	2009-09-17	Kurn et al.	
	18	20090239232	A1	2009-09-24	Kurn	
	19	20090275486	A1	2009-11-05	Kurn et al.	
	20	20090280538	A1	2009-11-12	Patel et al.	
	21	20090298075	A1	2009-12-03	Travers et al.	
	22	20100015666	A1	2010-01-21	Brenner et al.	
	23	20100021973	A1	2010-01-28	Makarov et al.	
	24	20100022403	A1	2010-01-28	Kurn et al.	
	25	20100029511	A1	2010-02-04	Raymond et al.	
	26	20100105052	A1	2010-04-29	DRMANAC et al.	
	27	20100113296	A1	2010-05-06	Myerson	

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	28	20100129879	A1	2010-05-27	Ach et al.	
	29	20100137143	A1	2010-06-03	Rothberg et al.	
	30	20100159559	A1	2010-06-24	Kurn et al.	
	31	20100167954	A1	2010-07-01	Earnshaw et al.	
	32	20100173394	A1	2010-07-08	Colston, JR. et al.	
	33	20100203597	A1	2010-08-12	Chen et al.	
	34	20100267043	A1	2010-10-21	Braverman et al.	
	35	20100273219	A1	2010-10-28	May et al.	
	36	20100311066	A1	2010-12-09	Kurn	
	37	20100323348	A1	2010-12-23	Hamady et al.	
	38	20110015096	A1	2011-01-20	CHIU	

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	39	20110039732	A1	2011-02-17	Raymond et al.	
	40	20110104785	A1	2011-05-05	Vaidyanathan et al.	
	41	20110105364	A1	2011-05-05	Kurn	
	42	20110129827	A1	2011-06-02	Causey et al.	
	43	20110189679	A1	2011-08-04	Kurn et al.	
	44	20110224105	A1	2011-09-15	Kurn et al.	
	45	20110269647	A1	2011-11-03	ULE et al.	
	46	20110288780	A1	2011-11-24	Rabinowitz et al.	
	47	20110294132	A1	2011-12-01	Kurn	
	48	20110319290	A1	2011-12-29	Raymond et al.	
	49	20120003657	A1	2012-01-05	Myllykangas et al.	

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	50	20120028310	A1	2012-02-02	Kurn et al.	
	51	20120045797	A1	2012-02-23	Kurn et al.	
	52	20120071331	A1	2012-03-22	Casbon et al.	
	53	20120074925	A1	2012-03-29	Oliver	
	54	20120102054	A1	2012-04-26	POPESCU et al.	
	55	20120107811	A1	2012-05-03	Kelso et al.	
	56	20120122701	A1	2012-05-17	Ryan et al.	
	57	20120149068	A1	2012-06-14	Kurn	
	58	20120156728	A1	2012-06-21	Li et al.	
	59	20120157322	A1	2012-06-21	Myllykangas et al.	
	60	20120190587	A1	2012-07-26	Kurn et al.	

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	61	20120208705	A1	2012-08-16	Steemers et al.	
	62	20120220483	A1	2012-08-30	KURN et al.	
	63	20120220494	A1	2012-08-30	Samuels et al.	
	64	20120237943	A1	2012-09-20	Soldatov et al.	
	65	20120238738	A1	2012-09-20	Hendrickson	
	66	20120245041	A1	2012-09-27	Brenner et al.	
	67	20120270212	A1	2012-10-25	RABINOWITZ et al.	
	68	20120283145	A1	2012-11-08	Wang	
	69	20120289426	A1	2012-11-15	Roos et al.	
	70	20120309002	A1	2012-12-06	Link	
	71	20130005585	A1	2013-01-03	Anderson et al.	

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	72	20130059738	A1	2013-03-07	LEAMON et al.	
	73	20130231253	A1	2013-09-05	Amorese et al.	
	74	20140038188	A1	2014-02-06	Kurn	
	75	20140038236	A1	2014-02-06	Kurn et al.	
	76	20140065692	A1	2014-03-06	KURN et al.	
	77	20140274729	A1	2014-09-18	Kurn et al.	
	78	20140274731	A1	2014-09-18	RAYMOND et al.	
	79	20140274738	A1	2014-09-18	Amorese et al.	
	80	20140303000	A1	2014-10-09	Armour	
	81	20140378345	A1	2014-12-25	Hindson et al.	
	82	20150011396	A1	2015-01-08	Schroeder et al.	

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	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	83	20150017635	A1	2015-01-15	Myllykangas et al.	
	84	20150101595	A1	2015-04-16	Hancock et al.	
	85	20150133319	A1	2015-05-14	Fu et al.	
	86	20150284769	A1	2015-10-08	Schroeder	
	87	20150299767	A1	2015-10-22	Armour et al.	
	88	20150299784	A1	2015-10-22	Fan et al.	
	89	20150299812	A1	2015-10-22	Talasaz	
	90	20160122756	A1	2016-05-05	Armour	
	91	20160130576	A1	2016-05-12	Armour	
	92	20160153039	A1	2016-06-02	Amorese et al.	
	93	20160203259	A1	2016-07-14	SCOLNICK et al.	

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	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	94	20160220994	A1	2016-08-04	Wright	
	95	20160251711	A1	2016-09-01	AMORESE et al.	
	96	20160265042	A1	2016-09-15	Schroeder et al.	
	97	20160275240	A1	2016-09-22	HUELGA et al.	
	98	20160296930	A1	2016-10-13	Matear et al.	

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2006/086668	WO	A2	2006-08-17	Epict Technologies		
	2	2006/137733	WO	A1	2006-12-28	Keygene NV		
	3	2007/018601	WO	A1	2007-02-15	Rubicon Genomics, Inc		
	4	2007/019444	WO	A2	2007-02-15	Euclid Diagnostics LLC		

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	5	2007/030759	WO	A2	2007-03-15	Nugen Technologies, Inc		
	6	2007/037678	WO	A2	2007-04-05	Keygene NV		
	7	2007/052006	WO	A1	2007-05-10	Solexa Ltd		
	8	2007/057652	WO	A1	2007-05-24	Solexa Ltd		
	9	2007/073165	WO	A1	2007-06-28	Keygene NV		
	10	2007/136717	WO	A1	2007-11-29	Nugen Technologies, Inc		
	11	2008/005459	WO	A2	2008-01-10	Nugen Technologies, Inc		
	12	2008/015396	WO	A2	2008-02-07	Solexa Ltd		
	13	2008/033442	WO	A2	2008-03-20	Univ Leland Stanford Junior		
	14	2008/093098	WO	A2	2008-08-07	Solexa Ltd		
	15	2008/115185	WO	A2	2008-09-25	Nimblegen Systems, Inc		

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	16	2008150432	WO	A1	2008-12-11	454 Life Sciences Corp		
	17	2009/053039	WO	A1	2009-04-30	Roche Diagnostics GmbH		
	18	2009/102878	WO	A2	2009-08-20	Nugen Technologies, Inc		
	19	2009/102896	WO	A2	2009-08-20	Nugen Technologies, Inc		
	20	2009/112844	WO	A1	2009-09-17	Stichting Het Nl Kanker I		
	21	2009/117698	WO	A2	2009-09-24	Nugen Technologies, Inc		
	22	2009/120372	WO	A2	2009-10-01	Pacific Biosciences Of California, Inc		
	23	2009/120374	WO	A2	2009-10-01	Pacific Biosciences Of California, Inc		
	24	2010/003153	WO	A2	2010-01-07	Life Technologies Corp		
	25	2010/030683	WO	A1	2010-03-18	Rosetta Inpharmatics LLC		
	26	2010/039991	WO	A2	2010-04-08	Texas A & M Univ Sys		

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	27	2010/063711	WO	A1	2010-06-10	Angeletti P Ist Richerche Bio		
	28	2010/064893	WO	A1	2010-06-10	Keygene NV		
	29	2010/085715	WO	A1	2010-07-29	Quanta Biosciences		
	30	2010/091246	WO	A2	2010-08-12	Univ Northwestern		
	31	2010/115154	WO	A1	2010-10-07	Fluidigm Corp		
	32	2010/129937	WO	A2	2010-11-11	Life Technologies Corp		
	33	2011/003630	WO	A1	2011-01-13	Max Planck Gesellschaft		
	34	2011/009941	WO	A1	2011-01-27	Olink Genomics AB		
	35	2011/019964	WO	A1	2011-02-17	Nugen Technologies, Inc		
	36	2011/032053	WO	A1	2011-03-17	Nugen Technologies, Inc.		
	37	2011/053987	WO	A1	2011-05-05	Nugen Technologies, Inc		

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	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
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	38	2011/151777	WO	A1	2011-12-08	Consiglio Nazionale Ricerche		
	39	2011/156529	WO	A2	2011-12-15	Nugen Technologies, Inc		
	40	2011032040	WO	A1	2011-03-17	Centrillion Technology Holding Corp		
	41	2012/013932	WO	A1	2012-02-02	Univ St Andrews		
	42	2012/054873	WO	A3	2012-08-23	Cold Spring Harbor Lab		
	43	2012/061832	WO	A1	2012-05-10	Illumina, Inc		
	44	2012/103154	WO	A1	2012-08-02	Nugen Technologies, Inc		
	45	2013/059740	WO	A1	2013-04-25	Foundation Medicine, Inc		
	46	2013/059746	WO	A1	2013-04-25	Nugen Technologies, Inc		
	47	2013/112923	WO	A1	2013-08-01	Nugen Technologies, Inc		
	48	2013/130512	WO	A3	2013-10-24	Univ North Carolina		

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	Attorney Docket Number	NUGN-001/04US 34079/550

	49	2013/138510	WO	A9	2014-07-10	Patel, Abhijit Ajit		
	50	2013/177220	WO	A1	2013-11-28	Scripps Research Inst		

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	1	Wolford, 2000, High-throughput SNP detection by using DNA pooling and denaturing high performance liquid chromatography (DHPLC), Hum Genet 107:483-487	
	2	Xi, 2011, Copy number variation detection in whole-genome sequencing data using the Bayesian information criterion, PNAS 108(46):e1128-e1136	
	3	Xu, 2012, FastUniq: A fast de novo duplicates removal tool for paired short reads, PLoSOne 7(12):e52249	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17381888
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First Named Inventor	Douglas A. Amorese
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	NUGN-001/04US 34079/550

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

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

OR

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See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

Signature	/Thomas C. Meyers/	Date (YYYY-MM-DD)	2022-08-04
Name/Print	Thomas C. Meyers	Registration Number	36,989

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Application Number	17381888
Filing Date	2021-07-21
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Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	NUGN-001/04US 34079/550

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	4362867		1982-12-07	Paddock	
	2	4458066		1984-07-03	Caruthers et al.	
	3	4469863		1984-09-04	Ts'o et al.	
	4	4582877		1986-04-15	Fairchok et al.	
	5	4876187		1989-10-24	Duck et al.	
	6	4925065		1990-05-15	Golias	
	7	4935357		1990-06-19	Szybalski	
	8	4942124		1990-07-17	Church	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	9	4988617		1991-01-29	Landegren et al.	
	10	4996143		1991-02-26	Heller et al.	
	11	5011769		1991-04-30	Duck et al.	
	12	5034506		1991-07-23	Summerton et al.	
	13	5035996		1991-07-30	Hartley	
	14	5043272		1991-08-27	Hartley	
	15	5082830		1992-01-21	Brakel et al.	
	16	5090591		1992-02-25	Long	
	17	5130238		1992-07-14	Malek et al.	
	18	5169766		1992-12-08	Schuster et al.	
	19	5171534		1992-12-15	Smith et al.	

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	20	5194370		1993-03-16	Berninger et al.	
	21	5216141		1993-06-01	Benner	
	22	5234809		1993-08-10	Boom et al.	
	23	5235033		1993-08-10	Summerton et al.	
	24	5242794		1993-09-07	Whiteley et al.	
	25	5384242		1995-01-24	Oakes	
	26	5386023		1995-01-31	Sanghvi et al.	
	27	5399491		1995-03-21	Kacian et al.	
	28	5409818		1995-04-25	Davey et al.	
	29	5418149		1995-05-23	Gelfand et al.	
	30	5422271		1995-06-06	Chen et al.	

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	31	5427929		1995-06-27	Richards et al.	
	32	5480784		1996-01-02	Kacian et al.	
	33	5494810		1996-02-27	Barany et al.	
	34	5508169		1996-04-16	Deugau et al.	
	35	5508178		1996-04-16	Rose et al.	
	36	5510270		1996-04-23	Fodor et al.	
	37	5525471		1996-06-11	Zeng	
	38	5545522		1996-08-13	Van Gelder et al.	
	39	5554516		1996-09-10	Kacian et al.	
	40	5554517		1996-09-10	Davey et al.	
	41	5556752		1996-09-17	Lockhart et al.	

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	42	5565340		1996-10-15	Chenchik et al.	
	43	5573913		1996-11-12	Rosemeyer et al.	
	44	5578832		1996-11-26	Trulson et al.	
	45	5589339		1996-12-31	Hampson et al.	
	46	5602240		1997-02-11	De Mesmaeker et al.	
	47	5637684		1997-06-10	Cook et al.	
	48	5641658		1997-06-24	Adams et al.	
	49	5644048		1997-07-01	Yau	
	50	5665549		1997-09-09	Pinkel et al.	
	51	5667976		1997-09-16	Van Ness et al.	
	52	5667979		1997-09-16	Berrens	

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	53	5679512		1997-10-21	Laney et al.	
	54	5681726		1997-10-28	Huse et al.	
	55	5683879		1997-11-04	Laney et al.	
	56	5688648		1997-11-18	Mathies et al.	
	57	5705628		1998-01-06	Hawkins	
	58	5708154		1998-01-13	Smith et al.	
	59	5710028		1998-01-20	Eyal et al.	
	60	5712126		1998-01-27	Weissman et al.	
	61	5716785		1998-02-10	Van Gelder et al.	
	62	5726329		1998-03-10	Jones et al.	
	63	5750341		1998-05-12	Macevicz	

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	64	5759822		1998-06-02	Chenchik et al.	
	65	5763178		1998-06-09	Chirikjian et al.	
	66	5789206		1998-08-04	Tavtigian et al.	
	67	5824517		1998-10-20	Cleuziat et al.	
	68	5824518		1998-10-20	Kacian et al.	
	69	5837832		1998-11-17	Chee et al.	
	70	5876976		1999-03-02	Richards et al.	
	71	5882867		1999-03-16	Ullman et al.	
	72	5888779		1999-03-30	Kacian et al.	
	73	5888819		1999-03-30	Goelet et al.	
	74	5945313		1999-08-31	Hartley et al.	

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	75	5952176		1999-09-14	McCarthy et al.	
	76	5958681		1999-09-28	Wetmur et al.	
	77	5965409		1999-10-12	Pardee et al.	
	78	5969119		1999-10-19	Macevicz	
	79	5972618		1999-10-26	Bloch	
	80	6004744		1999-12-21	Goelet et al.	
	81	6004745		1999-12-21	Arnold, Jr. et al.	
	82	6027889		2000-02-22	Barany et al.	
	83	6027923		2000-02-22	Wallace	
	84	6030774		2000-02-29	Laney et al.	
	85	6037152		2000-03-14	Richards et al.	

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	86	6056661		2000-05-02	Schmidt	
	87	6077674		2000-06-20	Schleifer et al.	
	88	6087103		2000-07-11	Burmer	
	89	6090553		2000-07-18	Matson	
	90	6090591		2000-07-18	Burg et al.	
	91	6107023		2000-08-22	Reyes et al.	
	92	6110709		2000-08-29	Ausubel et al.	
	93	6150112		2000-11-21	Weissman et al.	
	94	6159685		2000-12-12	Pinkel et al.	
	95	6160105		2000-12-12	Cunningham et al.	
	96	6169194		2001-01-02	Thompson et al.	

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	97	6172208		2001-01-09	Cook	
	98	6174680		2001-01-16	Makrigiorgos	
	99	6180338		2001-01-30	Adams	
	100	6190865		2001-02-20	Jendrisak et al.	
	101	6194211		2001-02-27	Richards et al.	
	102	6197501		2001-03-06	Cremer et al.	
	103	6197557		2001-03-06	Makarov et al.	
	104	6210891		2001-04-03	Nyren et al.	
	105	6225109		2001-05-01	Juncosa et al.	
	106	6225451		2001-05-01	Ballinger et al.	
	107	6232104		2001-05-15	Lishanski et al.	

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	108	6251639		2001-06-26	Kurn	
	109	6262490		2001-07-17	Hsu et al.	
	110	6270961		2001-08-07	Drmanac	
	111	6280935		2001-08-28	Macevicz	
	112	6287766		2001-09-11	Nolan et al.	
	113	6287825		2001-09-11	Weissman et al.	
	114	6291170		2001-09-18	Van Gelder et al.	
	115	6306365		2001-10-23	Ruoslahti et al.	
	116	6306597		2001-10-23	Macevicz	
	117	6309843		2001-10-30	Timms	
	118	6326142		2001-12-04	Royer	

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	119	6335167		2002-01-01	Pinkel et al.	
	120	6339147		2002-01-15	Lukhtanov et al.	
	121	6440705		2002-08-27	Stanton, Jr. et al.	
	122	6449562		2002-09-10	Chandler et al.	
	123	6582938		2003-06-24	Su et al.	
	124	6670461		2003-12-30	Wengel et al.	
	125	6686156		2004-02-03	Kurn	
	126	6692918		2004-02-17	Kurn	
	127	6770748		2004-08-03	Imanishi et al.	
	128	6777180		2004-08-17	Fisher et al.	
	129	6794499		2004-09-21	Wengel et al.	

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	130	6815164		2004-11-09	Kurn	
	131	6815167		2004-11-09	Crothers et al.	
	132	6825011		2004-11-30	Romantchikov	
	133	6833246		2004-12-21	Balasubramanian	
	134	6849404		2005-02-01	Park et al.	
	135	6858413		2005-02-22	Kurn	
	136	6913884		2005-07-05	Stuelpnagel et al.	
	137	6917726		2005-07-12	Levene et al.	
	138	6924104		2005-08-02	Weissman et al.	
	139	6946251		2005-09-20	Kurn	
	140	7001724		2006-02-21	Greenfield	

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	141	7033764		2006-04-25	Korlach et al.	
	142	7048481		2006-05-23	Sugata et al.	
	143	7052847		2006-05-30	Korlach et al.	
	144	7056676		2006-06-06	Korlach et al.	
	145	7056716		2006-06-06	Potter et al.	
	146	7060441		2006-06-13	Bourget et al.	
	147	7094536		2006-08-22	Kurn	
	148	7115400		2006-10-03	Adessi et al.	
	149	7170050		2007-01-30	Turner et al.	
	150	7175982		2007-02-13	McCarthy et al.	
	151	7176025		2007-02-13	Kurn et al.	

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	152	7189512		2007-03-13	Porat et al.	
	153	7211390		2007-05-01	Rothberg et al.	
	154	7232656		2007-06-19	Balasubramanian et al.	
	155	7244559		2007-07-17	Rothberg et al.	
	156	7244567		2007-07-17	Chen et al.	
	157	7264929		2007-09-04	Rothberg et al.	
	158	7273730		2007-09-25	Du Breuil Lastrucci	
	159	7276720		2007-10-02	Ulmer	
	160	7294461		2007-11-13	Kurn	
	161	7300755		2007-11-27	Petersdorf et al.	
	162	7302146		2007-11-27	Turner et al.	

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	163	7313308		2007-12-25	Turner et al.	
	164	7315019		2008-01-01	Turner et al.	
	165	7323305		2008-01-29	Leamon et al.	
	166	7335762		2008-02-26	Rothberg et al.	
	167	7351557		2008-04-01	Kurn	
	168	7354717		2008-04-08	Kurn	
	169	7361466		2008-04-22	Korlach et al.	
	170	7361468		2008-04-22	Liu et al.	
	171	7402386		2008-07-22	Kurn et al.	
	172	7405281		2008-07-29	Xu et al.	
	173	7414117		2008-08-19	Saito et al.	

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	174	7416844		2008-08-26	Korlach et al.	
	175	7462452		2008-12-09	Williams et al.	
	176	7462468		2008-12-09	Williams et al.	
	177	7476503		2009-01-13	Turner et al.	
	178	7476504		2009-01-13	Turner	
	179	7491498		2009-02-17	Lapidus et al.	
	180	7501245		2009-03-10	Quake et al.	
	181	7579153		2009-08-25	Brenner et al.	
	182	7704687		2010-04-27	Wang et al.	
	183	7741463		2010-06-22	Gormley et al.	
	184	7771934		2010-08-10	Kurn	

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	185	7771946		2010-08-10	Kurn	
	186	7803550		2010-09-28	Makarov et al.	
	187	7846666		2010-12-07	Kurn	
	188	7846733		2010-12-07	Kurn	
	189	7867703		2011-01-11	Sampson et al.	
	190	7939258		2011-05-10	Kurn et al.	
	191	7948015		2011-05-24	Rothberg et al.	
	192	7985565		2011-07-26	Mayer et al.	
	193	8017335		2011-09-13	Smith	
	194	8034568		2011-10-11	Kurn et al.	
	195	8053192		2011-11-08	Bignell et al.	

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	196	8071311		2011-12-06	Kurn	
	197	8143001		2012-03-27	Kurn et al.	
	198	8209130		2012-06-26	Kennedy et al.	
	199	8334116		2012-12-18	Kurn	
	200	8465950		2013-06-18	Kurn et al.	
	201	8492095		2013-07-23	Kurn	
	202	8512956		2013-08-20	Kurn	
	203	8551709		2013-10-08	Kurn et al.	
	204	8852867		2014-10-07	Kurn et al.	
	205	8999677		2015-04-07	Soldatov et al.	
	206	9175325		2015-11-03	Kurn et al.	

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	207	9175336		2015-11-03	Soldatov et al.	
	208	9181582		2015-11-10	Kurn	
	209	9206418		2015-12-08	Armour	
	210	9248076		2016-02-02	Sullivan et al.	
	211	9546399		2017-01-17	Amorese et al.	
	212	9702004		2017-07-11	Van Eijk et al.	
	213	9745627		2017-08-29	van Eijk et al.	
	214	9896721		2018-02-20	Van Eijk et al.	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20010000077	A1	2001-03-29	Engelhardt et al.	

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	2	20010031739	A1	2001-10-18	Dare	
	3	20010034048	A1	2001-10-25	Kurn	
	4	20010041334	A1	2001-11-15	Rashtchian et al.	
	5	20020028447	A1	2002-03-07	Li et al.	
	6	20020058270	A1	2002-05-16	Kurn	
	7	20020115088	A1	2002-08-22	Kurn	
	8	20020150919	A1	2002-10-17	Weismann et al.	
	9	20020155451	A1	2002-10-24	Makrigiorgos	
	10	20020164628	A1	2002-11-07	Kurn	
	11	20020164634	A1	2002-11-07	Patil et al.	
	12	20020197639	A1	2002-12-26	Shia et al.	

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	13	20030017591	A1	2003-01-23	Kurn	
	14	20030022207	A1	2003-01-30	Balasubramanian et al.	
	15	20030082543	A1	2003-05-01	Su et al.	
	16	20030087251	A1	2003-05-08	Kurn	
	17	20030119150	A1	2003-06-26	Ankenbauer et al.	
	18	20030143555	A1	2003-07-31	Bourget et al.	
	19	20030175780	A1	2003-09-18	Jones	
	20	20030180779	A1	2003-09-25	Lofton-Day et al.	
	21	20030186234	A1	2003-10-02	Kurn	
	22	20030207279	A1	2003-11-06	Crothers et al.	
	23	20030211616	A1	2003-11-13	Leong	

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	24	20030215926	A1	2003-11-20	Kurn et al.	
	25	20030224439	A1	2003-12-04	Lafferty et al.	
	26	20030232348	A1	2003-12-18	Jones et al.	
	27	20040002371	A1	2004-01-01	Paquin et al.	
	28	20040005614	A1	2004-01-08	Kurn et al.	
	29	20040023271	A1	2004-02-05	Kurn et al.	
	30	20040115815	A1	2004-06-17	Li et al.	
	31	20040137456	A1	2004-07-15	Yokota et al.	
	32	20040161742	A1	2004-08-19	Dean et al.	
	33	20040203019	A1	2004-10-14	Kurn	
	34	20040203025	A1	2004-10-14	Kurn	

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	35	20040248153	A1	2004-12-09	Dear et al.	
	36	20050003441	A1	2005-01-06	Kurn	
	37	20050014192	A1	2005-01-20	Kurn	
	38	20050019793	A1	2005-01-27	Kurn et al.	
	39	20050059048	A1	2005-03-17	Gunderson et al.	
	40	20050064456	A1	2005-03-24	Kurn	
	41	20050123956	A1	2005-06-09	Blume et al.	
	42	20050136417	A1	2005-06-23	Cole et al.	
	43	20050142577	A1	2005-06-30	Jones et al.	
	44	20050191656	A1	2005-09-01	Drmanac et al.	
	45	20050191682	A1	2005-09-01	Barone et al.	

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	46	20050208538	A1	2005-09-22	Kurn et al.	
	47	20060008824	A1	2006-01-12	Ronaghi et al.	
	48	20060014182	A1	2006-01-19	Kurn	
	49	20060024678	A1	2006-02-02	Buzby	
	50	20060024711	A1	2006-02-02	Lapidus et al.	
	51	20060035274	A1	2006-02-16	Dong	
	52	20060046251	A1	2006-03-02	Sampson et al.	
	53	20060051789	A1	2006-03-09	Kazakov et al.	
	54	20060068415	A1	2006-03-30	Jones et al.	
	55	20060134633	A1	2006-06-22	Chen et al.	
	56	20060216724	A1	2006-09-28	Christians et al.	

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	57	20060263789	A1	2006-11-23	Kincaid	
	58	20060281082	A1	2006-12-14	Zhu	
	59	20060286566	A1	2006-12-21	Lapidus et al.	
	60	20060292597	A1	2006-12-28	Shapero et al.	
	61	20070031857	A1	2007-02-08	Makarov et al.	
	62	20070134128	A1	2007-06-14	Korlach	
	63	20070141604	A1	2007-06-21	Gormley et al.	
	64	20070224607	A1	2007-09-27	Morgan et al.	
	65	20070224613	A1	2007-09-27	Strathmann	
	66	20070231823	A1	2007-10-04	McKernan et al.	
	67	20070238122	A1	2007-10-11	Allbritton et al.	

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	68	20070263045	A1	2007-11-15	Okazawa	
	69	20080038727	A1	2008-02-14	Spier	
	70	20080087826	A1	2008-04-17	Harris et al.	
	71	20080103058	A1	2008-05-01	Siddiqi	
	72	20080131937	A1	2008-06-05	Schroeder	
	73	20080160580	A1	2008-07-03	Adessi et al.	
	74	20080176311	A1	2008-07-24	Kurn	
	75	20080182300	A1	2008-07-31	Kurn	
	76	20080194413	A1	2008-08-14	Albert	
	77	20080194416	A1	2008-08-14	Chen	
	78	20080206764	A1	2008-08-28	Williams et al.	

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	79	20080213770	A1	2008-09-04	Williams et al.	
	80	20080217246	A1	2008-09-11	Benn et al.	
	81	20080241831	A1	2008-10-02	Fan et al.	
	82	20080242560	A1	2008-10-02	GUNDERSON et al.	
	83	20080286795	A1	2008-11-20	Kawashima et al.	
	84	20090011959	A1	2009-01-08	Costa et al.	
	85	20090024331	A1	2009-01-22	Tomaney et al.	
	86	20090026082	A1	2009-01-29	Rothberg et al.	

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	1	2444926	CA	A1	2002-11-14	Centre Nat Rech Scient		X

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	2	101565746	CN	A	2009-10-28	Univ Southeast		☒
	3	105890722	CN	A	2016-08-24	Univ Changzhou		☒
	4	1661102	CN	A	2005-08-31	Univ Shanghai Jiaotong		☒
	5	0329822	EP	B1	1994-06-08	Cangene Corp		☐
	6	0365627	EP	B1	1993-12-22	Univ Iowa Res Found		☐
	7	0667393	EP	A2	1995-08-16	Enzo Diagnostics, Inc		☐
	8	0843735	EP	B1	2002-07-17	Forfas Trading As Biores Irela		☐
	9	1071811	EP	B1	2002-03-06	Entpr Ie Trd As Bioresearch Ie		☐
	10	1929039	EP	B2	2013-11-20	Keygene NV		☐
	11	2272976	EP	A1	2011-01-12	Max Planck Gesellschaft		☐
	12	2322612	EP	A1	2011-05-18	Hitachi High Tech Corp		☐

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	13	2451973	EP	A1	2012-05-16	Max Planck Gesellschaft		☐
	14	2511381	EP	A1	2012-10-17	Monsanto Technology LLC		☐
	15	00/018957	WO	A1	2000-04-06	Applied Research Systems		☐
	16	00/08208	WO	A2	2000-02-17	Medical Res Council		☐
	17	00/39345	WO	A1	2000-07-06	Dana-farber Cancer Institute, Inc		☐
	18	00/52191	WO	A1	2000-09-08	Life Technologies, Inc		☐
	19	00/70039	WO	A1	2000-11-23	Univ Mcgill		☐
	20	01/20035	WO	A2	2001-03-22	Nugen Technologies, Inc		☐
	21	01/23613	WO	A1	2001-04-05	Isis Pharmaceuticals, Inc		☐
	22	01/46464	WO	A1	2001-06-28	Lion Bioscience AG		☐
	23	01/57248	WO	A2	2001-08-09	Solexa Ltd		☐

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	24	01/64952	WO	A2	2001-09-07	Molecular Staging, Inc		☐
	25	02/00938	WO	A2	2002-01-03	Nugen Tgechnologies, Inc		☐
	26	02/060318	WO	A2	2002-08-08	Univ Ohio State Res Found		☐
	27	02/072772	WO	A2	2002-09-19	Nugen Technologies, Inc		☐
	28	02/072773	WO	A2	2002-09-19	Nugen Technologies, Inc		☐
	29	02/081753	WO	A1	2002-10-17	Advanced Res & Tech Inst		☐
	30	02/090584	WO	A2	2002-11-14	Bio Merieux		☐
	31	02/28876	WO	A2	2002-04-11	Riken		☐
	32	02/29117	WO	A2	2002-04-11	Nugen Technologies, Inc		☐
	33	02/36821	WO	A2	2002-05-10	University College Cork - National University Of I		☐
	34	02/48402	WO	A2	2002-06-20	Nugen Technologies, Inc		☐

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	35	03/004690	WO	A2	2003-01-16	454 Corp		☐
	36	03/027259	WO	A2	2003-04-03	Epigenx Pharmaceuticals, Inc		☐
	37	03/078645	WO	A2	2003-09-25	Nugen Technologies, Inc		☐
	38	03/083435	WO	A2	2003-10-09	Nugen Technologies, Inc		☐
	39	03/106642	WO	A2	2003-12-24	Affymetrix, Inc		☐
	40	04/011665	WO	A2	2004-02-05	Nugen Technologies, Inc		☐
	41	2000/09756	WO	A1	2000-02-24	Perkin Elmer Corp		☐
	42	2000/55364	WO	A2	2000-09-21	Amersham Pharm Biotech UK Ltd		☐
	43	2003/002736	WO	A2	2003-01-09	Roche Diagnostics GmbH		☐
	44	2003/012118	WO	A1	2003-02-13	Affymetrix, Inc		☐
	45	2004/070007	WO	A2	2004-08-19	454 Corp		☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	46	2004/092418	WO	A2	2004-10-28	Nugen Technologies, Inc		☐
	47	2005/003375	WO	A2	2005-01-13	454 Corp		☐
	48	2005/038427	WO	A2	2005-04-28	Medical Gene Ct Ltd		☐
	49	2005/065321	WO	A2	2005-07-21	Nugen Technologies, Inc		☐
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	1	Amos, 2000, DNA pooling in mutation detection with reference to sequence analysis, Am J Hum Genet 66:1689-1692	
	2	Bellos, 2014, cnvCapSeq: detecting copy number variation in long-range targeted resequencing data, Nucleic Acids Res 42(20):e158	
	3	Benson, 2013, Genbank, Nucl Acids Res 41:D36-D42	
	4	Blomquist, 2013, Targeted RNA-Sequencing with Competitive Multiplex-PCR Amplicon Libraries, PLOS ONE 8(11): e79120	

IDT Exhibit 1020.10402

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	5	Bodi, 2013, Comparison of commercially available target enrichment methods for next-generation sequencing, J Biomolecular Tech 24:73-86
	6	Church, 1988, Multiplexed DNA sequencing, Science 240:185-188
	7	Colbert, 2001, High-throughput screening for induced point mutations, Plant Physiol 126:480-484
	8	Collard, 2005, An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvements: the basic concepts Euphytica 142:169-196
	9	Eminaga, 2013, Quantification of microRNA Expression with Next-Generation Sequencing, Unit 4.17 in Current Protocols in Molecular Biology, Wiley, New York, NY (14 pages)
	10	Fakhrai-Rad, 2002, Pyroseqeuncing: An accurate detection platform for single nucleotide polymorphisms, Human Mutation 19:479-485
	11	Grothues, 1993, PCR amplification of megabase DNA with tagged random primers (T-PCR), Nucl Acids Res 21:1321-1322
	12	Hajibabaei, 2005, Critical factors for assembling a high volume of DNA barcodes, Phil Trans R Soc B 360:1959-1967
	13	Illumina, 2008, Multiplexed Sequencing with the Illumina Genome Analyzer System, Illumina Sequencing, 4 pages
	14	Illumina, 2011, TruSeq RNA and DNA Sample Preparation Kits v2, 1-15 Illumina, dated 27 April 27, 2011 (4 pages)
	15	Ion Total RNA-Seq Kit v2, User Guide, 2012, Life Technologies (82 pages)

IDT Exhibit 1020.10403

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	16	Jiang, 2015, CODEX: a normalization and copy number variation detection method for whole exome sequencing, Nucleic Acids Res 43(6):e39
	17	Krumm, 2012, Copy number variation detection and genotyping from exome sequence data, Genome Res 22 (8):1525-1532
	18	Lai, 2004, Characterization of the maize endosperm transcriptome and its comparison to the rice genome, Genome Res14:1932-1937
	19	Langmead, 2009, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome, Genome Biol 10:R25
	20	Li, 2012, CONTRA: copy number analysis for targeted resequencing, Bioinformatics 28(10):1307-1313
	21	Lindstrom, 2004, Pyrosequencing for detection of Lamivudine-resistant Hepatitis B virus, J Clin Microb 42 (10):4788-4795
	22	Liu, 2008, Sequence space coverage, entropy of genomes and the potential to detect non-human DNA in human samples, BMC Genomics 9(509):1-17
	23	Ma, 2015, Quantitative Analysis of Copy Number Variants Based on Real-Time LightCycler PCR, Curr Protoc Hum Genet 80:7.21.1-7.23.8
	24	Machine translation generated on March 7, 2018, of CN 105890722 by website of European Patent Office (4 pages)
	25	Margulies, 2005, Genorne sequencing in open microfabricated high density picoliter reactors, Nature 437 (7057):376-380
	26	McCloskey, 2007, Encoding PCR products with batch-stamps and barcodes, Biochem Genet 45:761-767

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	27	Miner, 2004, Molecular barcodes detect redundancy and contamination in hairpin-bisulfite PCR, Nucl Acids Res 32 (17):e135, 4 pages
	28	Myers, 2013, Protocol for Creating Multiplexed miRNA Libraries for Use in Illumina Sequencing, Myers lab microRNA-seq Protocol, Hudson Alpha Institute for Biotechnology web site, dated May 2, 2013, (15 pages)
	29	NuGEN, 2014, User Guide Ovation Target Enrichment System, NuGEN Technologies Inc., San Carlos, CA (45 pages)
	30	Plagnol, 2012, A robust model for read count data in exome sequencing experiments and implications for copy number variant calling, Bioinformatics 28(21):2747-2754
	31	Qiu, 2003, DNA sequence-based "bar-codes" for tracking the origins of expressed sequence tags from a maize cDNA library constructed using multiple mRNA sources, Plant Physiol 133:475-481
	32	Querfurth, 2012, Creation and application of immortalized bait libraries for targeted enrichment and next-generation sequencing, Biotechniques 52(6):375-380
	33	Ronaghi, 2001, Pyrosequencing sheds light on DNA sequencing, Genome Res 11:3-11
	34	Sathirapongsasuti, 2011, Exome sequencing-based copy-number variation and loss of heterozygosity detection: ExomeCNV, Bioinformatics 27(19):2648-2654
	35	Schiemer, 2011, Illumina TruSeq Adapters Demystified,TUFTS UNIVERSITY CORE FACILITY XP055357867 (5 pages)
	36	Shapero, 2001, SNP Genotyping by multiplexed solid-phase amplification and fluorescent minisequencing, Genome Res 11:1926-1934
	37	Shendure, 2005, Accurate multiplex polony sequencing of an evolved bacterial genome, Science 309:1728

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	38	Soni, 2007, Progress toward ultrafast DNA sequencing using solid-state nanopores, Clin Chem 53(11):1996-2001
	39	Sood, 2006, Methods for reverse genetic screening in zebrafish by resequencing and TILLING, Methods 39:220-227
	40	Staroscik, 2004, Calculator for determining the number of copies of a template, URI Genomics, webpage archive dated April 6, 2017 (1 page), Retreived from the internet on March 7, 2018, from <https://web.archive.org/web/20170406174850/http://cels.uri.edu/gsc/cndna.html>
	41	Stratagene, 1998, Gene characterization kits, Stratagene Catalog, p. 39 (2 pages)
	42	Till, 2003, Large-scale discovery of induced point mutations with high-throughput TILLING, Genome Res 13:524-530
	43	Trapnell, 2010, Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation, Nat Biotech 28:511-515
	44	Trapnell, 2013, Differential analysis of gene regulation at transcript resolution with RNA-seq, Nat Biotech 31:46-53
	45	Unemo, 2004, Molecular typing of Neisseria gonorrhoeae isolates by pyrosequencing of highly polymorphic segments of the porB gene, J Clin Microb 42(7):2926-2934
	46	Vasil, 2012, Next generation sequencing for molecular diagnosis of neuromuscular diseases, Acta Neuropathol, 124:273-283, plus supplemental material pp. 1-10
	47	Vigal, 2002, A review on SNP and other types of molecular markers and their use in animal genetics, Genet Sel Evol 34:275-305
	48	Walker, 1992, Strand displacement amplification--an isothermal, in vitro DNA amplification technique, Nucl Acids Res 20(7):1691-1696

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	NUGN-001/04US 34079/550

	49	Westin 2000, Anchored multiplex amplification on a microelectronic chip array, Nat Biotech 18:199-204
	50	Wienholds, 2004, Target-selected gene inactivation in zebrafish, Meth Cell Biol 77:69-90

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Application Number	17381888
Filing Date	2021-07-21
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Art Unit	N/A
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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

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Signature	/Thomas C. Meyers/	Date (YYYY-MM-DD)	2022-08-04
Name/Print	Thomas C. Meyers	Registration Number	36,989

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by A Srivastava · 2019 · Cited by 136 — ... a unique barcode, which enables pooling of cells for sequencing ... The process of deduplication requires identifying duplicate reads ...

ResearchGate
https://www.researchgate.net › ... › De Novo Assembly

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Apr 10, 2019 — Using a combinatorial process up to 3.6 billion unique barcode sequences were generated on beads, ... This would eliminate duplicate reads.

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Degenerate adaptor sequences for detecting PCR duplicates in ...

Amplification introduces duplicate reads into the data, which arise from ... From this pool of possible adaptor sequences, a unique barcode is ligated to ...

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unique barcode attached to all cDNA molecules in each single cell ... still leaves plenty of non-duplicate reads (> 12 million).

bioRxiv
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Detection and removal of barcode swapping in single-cell ...

Jan 30, 2018 — ... molecular identifiers (UMIs) to collapse PCR duplicate reads. ... For transcripts labelled with this unique barcode, swapping will ...

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Alevin: An integrated method for dscRNA-seq quantification

by A Srivastava · 2018 · Cited by 3 — likely to be labeled by a unique barcode. ... reads in which to assess the UMIs, as true duplicate reads may contain sequences.

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Pipeline for scRNAseq analysis - RPubs

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ChIP-nexus (version 2019)

Aug 6, 2021 — makes use of a unique barcode to identify duplicate reads and a library ...
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Gale
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Haplotype phasing of whole human genomes using bead ...

by F Zhang · 2017 · Cited by 52 — Duplicate reads were removed and reads sharing the same barcode were arranged into ... Raw sequencing reads were demultiplexed by their unique...

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Nucleotide sequence generation by barcode bead ...

... the beads can be labeled with unique barcode sequences. ... Duplicate reads having the same barcodes can be removed so that only one read copy for a ...

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Sequencing-Based Measurable Residual Disease Testing in ...

by JM Yoest · 2020 · Cited by 20 — ... first ligated to unique barcode sequences (red, blue, and green). ... UMI-based error correction leverages duplicate reads to determine ...

GitHub
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Samples are assigned unique barcode tracking numbers and have a detailed sample ... Duplicate reads, QC-failed reads, supplementary reads, secondary reads ...

Journal of Thoracic Oncology
https://www.jto.org › article › fulltext

A Clinician's Guide to Bioinformatics for Next-Generation ...

by NB Larson · Cited by 1 — ... together for sequencing in a single run using unique barcode labels. ... First, duplicate reads (i.e., reads originated from the same ...

Rockefeller University Press
https://rupress.org › jcb › article › High-throughput-singl...

High-throughput single-cell epigenomic profiling by targeted ...

Nov 16, 2021 — ... plate received a unique barcode downstream of the T7 promoter. ... T7 duplicate reads were filtered based on read 1 start positions ...

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Barcoded bulk QTL mapping reveals highly polygenic and ...

by ANN Ba · 2022 · Cited by 14 — ... by looking for the unique barcode which is present in the set of ... of each error-corrected true barcode by removing duplicate reads, ...

Labome
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... ligation of "Y" adapters containing a unique barcode and primer binding sites. ... duplicate reads (typically a result of PCR over-amplification during ...

American Society for Microbiology
https://journals.asm.org › mSystems › Vol. 1, No. 3

A Universal Next-Generation Sequencing Protocol To ...

by LA Moser · 2016 · Cited by 32 — ... a primer with a random hexamer coupled to a unique barcode (BC-N6). ... Duplicate reads were removed, reads were demultiplexed based on ...

HackMD
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Reference-guided RAD analysis workflow - HackMD

During library prep we use a unique barcode to identify each sample in each well, ... percent of reads mapping, and proprotion of duplicate reads.

OpenReview
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Machine learning for discovery: deciphering RNA splicing logic

by SE Liao — Each reporter includes a unique barcode at the end of the third exon so that ... unique molecular identifiers (UMIs), the fraction of duplicate reads in ...

WordPress.com
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Notes on RNA-seq – Logan Thrasher Collins - WordPress.com

containing barcoded primers (each droplet possesses a unique barcode) with poly-dT sequences ... underrepresented), duplicate reads, and other artifacts.

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https://assets.thermofisher.com › LSG › manuals PDF

Torrent Suite™ Software 5.12 - USER GUIDE

Nov 5, 2019 — Assign a unique barcode to each sample in a Sample Set. ... Marking duplicate reads is not appropriate for Ion AmpliSeq™ data, because many.

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EMBO Press
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Using single-cell genomics to understand developmental ...

by JA Griffiths · 2018 · Cited by 177 — ... randomly generated nucleotide sequences that allow PCR duplicate reads to ... sequences of fluorescence across washes, building up a unique...

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by AR Vierstraete · 2022 · Cited by 1 — which contains the unique barcode- primer sequences, a fastq file with the sequences, and the ... duplicate reads in the comparison.

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by DA Cusanovich · 2015 · Cited by 949 — the plate contained a unique barcode combination) was added to each well as ... Finally, duplicate reads were removed with Picard. After.

rug.nl
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Single-cell whole genome sequencing reveals no evidence ...

by H van den Bos · 2016 · Cited by 143 — Illumina sequencing. Since each single-cell library received a unique barcode, ... (version 0.1.18) [25] and duplicate reads were marked.

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Advances in Transcriptomics - AHA Journals

by RC Wirka · 2018 · Cited by 30 — Furthermore, filtering out duplicate reads, originating from ... marked with a unique barcode, sheared and sequenced on the.

Washington University in St. Louis
https://digitalcommons.wustl.edu › viewcontent PDF

Sequencing-based measurable residual disease testing in ...

by JM Yoest · 2020 · Cited by 20 — distinguish duplicate reads originating from the same DNA ... are first ligated to unique barcode sequences (red, blue, and green).

EpiCypher
https://www.epicypher.com › content › manuals PDF

14-1001-2-cut-and-run-library-prep-kit-manual.pdf - EpiCypher

Each primer incorporates a unique barcode ... remove adapter dimers (using gel purification; FAQ 3), filter out duplicate reads, and/or increase sequencing ...

University of New Mexico
https://digitalrepository.unm.edu › viewcontent PDF

Comprehensive Sequencing with Surface Tagmentation ...

by K Feng · 2018 — With their unique barcode system, the genome is marked, and every piece is trackable. ... Remove the duplicate reads with Picard tools.

BMJ
https://jmg.bmj.com › content

Assessment of the incorporation of CNV surveillance into ...

by JM Ellingford · 2018 · Cited by 55 — After enrichment, samples were pooled using unique barcode identifiers, ... software.20 Duplicate reads were removed using SAMtools V.0.1.18...

More results

IDT Exhibit 1020.10414

IDT Exhibit 1020.10415

Bibliographic Data

Application No: 17/381,888

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /TERESA E STRZELECKA/ Examiner's Signature | Initials

Title: COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/21/2021	506	1637	NUGN-001/04US 34079/550
RULE			

APPLICANTS

Tecan Genomics, Inc., Redwood City, CA,

INVENTORS

Douglas A. Amorese, Los Altos, CA, UNITED STATES

Jonathan Scolnick, San Francisco, CA, UNITED STATES

Ben Schroeder, San Mateo, CA, UNITED STATES

CONTINUING DATA

This application is a CON of 16743604 01/15/2020 PAT 11098357

16743604 is a CON of 15406002 01/13/2017 PAT 10570448

15406002 is a DIV of 14540917 11/13/2014 PAT 9546399

14540917 has PRO of 61903826 11/13/2013

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

08/10/2021

STATE OR COUNTRY

UNITED STATES

ADDRESS

BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111
UNITED STATES

FILING FEE RECEIVED

$1,820

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/381,888	Amorese et al.
	Examiner	Art Unit
	TERESA E STRZELECKA	1637

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	03/17/2023								
	22	=								
	23	=								
	24	=								
	25	=								
	26	=								
	27	=								
	28	=								
	29	=								
	30	=								
	31	=								
	32	=								
	33	=								
	34	=								
	35	=								
	36	=								
	37	=								

IDT Exhibit 1020.10417

PALM INTRANET

Day : Tuesday
Date: 3/14/2023
Time: 17:49:47

Inventor Name Search

Enter the **first few letters** of the Inventor's Last Name.
Additionally, enter the **first few letters** of the Inventor's First name.

Last Name	First Name	
Amorese	Douglas	Search

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IDT Exhibit 1020.10418

PALM INTRANET

Day : Tuesday
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Time: 17:49:47

Inventor Name Search

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Scolnick	Jonathan	Search

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IDT Exhibit 1020.10419

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Time: 17:49:47

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Last Name	First Name	
Schroeder	Ben	Search

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IDT Exhibit 1020.10420

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/381,888	Amorese et al.
	Examiner	Art Unit
	TERESA E STRZELECKA	1637

CPC - Searched*		
Symbol	Date	Examiner
(C12Q1/6869 OR C12Q2525/191 OR C12Q2525/179) plus text search	03/15/2023	/TS/

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventor name search	03/14/2023	/TS/
Google	03/15/2023	/TS/
IDS (8/4/22)	03/15/2023	/TS/
PE2E search	03/15/2023	/TS/
PE2E search	03/16/2023	/TS/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	(C12Q1/6869 OR C12Q2525/191 OR C12Q2525/179) plus text search	03/15/2023	/TS/

IDT Exhibit 1020.10421

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/381,888	Amorese et al.
	Examiner	Art Unit
	TERESA E STRZELECKA	1637

IDT Exhibit 1020.10422

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-22)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17381888
Filing Date	2021-07-21
First Named Inventor	Douglas A. Amorese
Art Unit	N/A
Examiner Name	Not Yet Assigned Teresa Strzelecka
Attorney Docket Number	NUGN-001/04US 34079/550

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2013/190441	WO	A2	2013-12-27	Univ Hong Kong Chinese		
	2	2013/191775	WO	A2	2013-12-27	Nugen Technologies, Inc		
	3	2014/039556	WO	A1	2014-03-13	Guardant Health, Inc		

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	4	2014/082032	WO	A1	2014-05-30	Univ Toledo		
	5	2014/144092	WO	A1	2014-09-18	Nugen Technologies, Inc		
	6	2014/150931	WO	A1	2014-09-25	Nugen Technologies, Inc		
	7	2015/031691	WO	A1	2015-03-05	Cellular Research, Inc		
	8	2015/073711	WO	A1	2015-05-21	Nugen Technologies, Inc		
	9	2015/131107	WO	A1	2015-09-03	Nugen Technologies, Inc		
	10	89/09284	WO	A1	1989-10-05	Univ Iowa Res Found		
	11	92/07951	WO	A1	1992-05-14	Kow, Yoke		
	12	93/18052	WO	A1	1993-09-16	Isis Pharmaceuticals, Inc		
	13	94/16090	WO	A1	1994-07-21	Molecular Tool, Inc		
	14	96/40998	WO	A1	1996-12-19	Pioneer Hi-bred International, Inc		

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IDT Exhibit 1020.10424

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	15	97/12061	WO	A1	1997-04-03	Epicentre Technologies Corp		
	16	97/25416	WO	A2	1997-07-17	Nyfotek As		
	17	98/044151	WO	A1	1998-10-08	Glaxo Group Ltd		
	18	98/06736	WO	A1	1998-02-19	Life Technologies, Inc		
	19	98/38296	WO	A1	1998-09-03	Gesher Israel Advanced Biotecs		
	20	99/10540	WO	A1	1999-03-04	Lopez, Osvaldo		
	21	99/11819	WO	A1	1999-03-11	Perkin Elmer Corp		
	22	99/42618	WO	A1	1999-08-26	Dade Behring Inc		

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T5
	1		

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

EXAMINER SIGNATURE			
Examiner Signature	/TERESA E STRZELECKA/	Date Considered	03/15/2023

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

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

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

21710 7590 03/27/2023

BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111

EXAMINER
STRZELECKA, TERESA E

ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 03/27/2023

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/381,888	07/21/2021	Douglas A. Amorese	NUGN-001/04US 34079/550	6188

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	06/27/2023

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 40% the amount of undiscounted fees, and micro entity fees are 20% the amount of undiscounted fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PTOL-85 (Rev. 02/11)

IDT Exhibit 1020.10427

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

21710 7590 03/27/2023

BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/381,888	07/21/2021	Douglas A. Amorese	NUGN-001/04US 34079/550	6188

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	06/27/2023

EXAMINER	ART UNIT	CLASS-SUBCLASS
STRZELECKA, TERESA E	1637	506-004000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required)

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via Patent Center or EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

❑ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

❑ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

IDT Exhibit 1020.10428

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/381,888	07/21/2021	Douglas A. Amorese	NUGN-001/04US ~~34079/550~~	6188

21710 7590 03/27/2023

BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111

EXAMINER	
STRZELECKA, TERESA E	
ART UNIT	**PAPER NUMBER**
1637	

DATE MAILED: 03/27/2023

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

IDT Exhibit 1020.10429

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. 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 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

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

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

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 17/381,888	Applicant(s) Amorese et al.	
	Examiner TERESA E STRZELECKA	Art Unit 1637	AIA (FITF) Status Yes

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

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☐ This communication is responsive to _____.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ 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.

3. ☑ The allowed claim(s) is/are 22-37 . As a result of the allowed claim(s), 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.**

4. ☐ 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)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☑ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 8/4/22.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/TERESA E STRZELECKA/
Primary Examiner, Art Unit 1637

IDT Exhibit 1020.10431

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

2. Claims 22-37 are pending and under consideration.

Priority

3. The later-filed application must be an application for a patent for an invention which is also disclosed in the prior application (the parent or original nonprovisional application or provisional application). The disclosure of the invention in the parent application and in the later-filed application must be sufficient to comply with the requirements of 35 U.S.C. 112(a) or the first paragraph of pre-AIA 35 U.S.C. 112, except for the best mode requirement. See *Transco Products, Inc. v. Performance Contracting, Inc.*, 38 F.3d 551, 32 USPQ2d 1077 (Fed. Cir. 1994)

The disclosure of the prior-filed applications, Application No. 16/743,604 (filed January 15, 2020); Application No. 15/406,002 (filed January 13, 2017); Application No. 14/540,917 (filed November 13, 2014) and Application No. 61/903,826 (filed November 13, 2013), fail to provide adequate support or enablement in the manner provided by 35 U.S.C. 112(a) or pre-AIA 35 U.S.C. 112, first paragraph for one or more claims of this application.

Specifically, claim 22 of the instant application is drawn to a method for detecting duplicate sequencing reads, the method comprising:

obtaining amplicons each comprising an amplified fragment of a nucleic acid with an appended adaptor, wherein each adaptor comprises an identifier site comprising a plurality of nucleotides unique to the amplified fragment;

sequencing the amplicons to generate sequence reads that include identifier and target sequences; and

identifying sequence reads with identical identifier and target sequences as duplicates.

However, there is no support in the specification of the instant application and disclosures in the parent cases for the limitation of an adapter comprising an identifier site comprising a plurality of nucleotides unique to the amplified fragment. The claim requires that each fragment of the nucleic acid has a unique identifier specific to the fragment. Let us consider Example 1, in which 500 ng of fragments with about 100 bp were labeled with adaptors comprising a random 6 bp identifier. There are about 4.6 x 10^{12} double-stranded fragments in this sample, while the number of unique identifier is 4^6 or 4096. Therefore, there are not enough adapters to provide each of the fragments with a unique identifier. Even a 10 bp random identifier would have 4^{10} or 2.7 x 10^8 unique molecules, about 10,000-fold lower than necessary.

In Example 5 Applicant describes labeling 100 ng of 200 bp fragments with an adapter comprising a 6 bp random identifier. The number of double-stranded fragments would be 4.6 x 10^{11}, and the number of unique adapters 4096. In conclusion, Applicant did not describe any adapters which provide identifiers capable of uniquely labeling every fragment in a sample.

As a consequence, the priority date of the instant claims is the filing date of the instant application, July 21, 2021.

Information Disclosure Statement

4. The information disclosure statement (IDS) submitted on August 4, 2022 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement is being considered by the examiner.

5. No references were found teaching or suggesting claims 22-37. The closest prior art reference, Doan et al. (US 2022/0145380 A1; filed November 2019) teaches detection of mutations in sequenced DNA samples by appending adapters with unique identifiers to DNA fragments, grouping the fragments using the unique identifiers and removing duplicate reads, but does not teach or suggest using a combination of target sequence and identifier sequence for duplicate removal.

Further, as can be seen from Kinde et al. (PNAS, vol. 108, pp. 9530-9535, 2011), unique fragment identifiers wwere used to create consensus sequences from many sequence read duplicates, and the duplicates were not removed.

As a consequence, claims 22-37 are allowed.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to TERESA E STRZELECKA whose telephone number is (571)272-0789. The examiner can normally be reached M-F 9-7.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available

to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/TERESA E STRZELECKA/
Primary Examiner, Art Unit 1637
March 17, 2023

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/381,888	Amorese et al.
	Examiner	**Art Unit**
	TERESA E STRZELECKA	1637

CPC								
Symbol							**Type**	**Version**
C12Q	/	1	/	6874			F	2013-01-01
C12Q	/	1	/	6869			I	2013-01-01
C12Q	/	2525	/	179			A	2013-01-01
C12Q	/	2525	/	191			A	2013-01-01

CPC Combination Sets									
Symbol							**Type**	**Set**	**Ranking**	**Version**
C12Q	/	1	/	6869			I	1	1	2013-01-01
C12Q	/	2525	/	179			I	1	2	2013-01-01
C12Q	/	2525	/	191			I	1	3	2013-01-01
C12Q	/	2537	/	157			I	1	4	2013-01-01
C12Q	/	2563	/	179			I	1	5	2013-01-01
C12Q	/	2565	/	514			I	1	6	2013-01-01
C12Q	/	1	/	6869			I	2	1	2013-01-01
C12Q	/	2525	/	155			I	2	2	2013-01-01
C12Q	/	2525	/	179			I	2	3	2013-01-01
C12Q	/	2525	/	191			I	2	4	2013-01-01
C12Q	/	2535	/	122			I	2	5	2013-01-01
C12Q	/	2537	/	159			I	2	6	2013-01-01
C12Q	/	2563	/	179			I	2	7	2013-01-01

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 16	
/TERESA E STRZELECKA/ Primary Examiner, Art Unit 1637 (Primary Examiner)	17 March 2023 (Date)	O.G. Print Claim(s) 22	O.G. Print Figure None

U.S. Patent and Trademark Office

Part of Paper No.: 20230317

IDT Exhibit 1020.10436

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/381,888	Amorese et al.
	Examiner	**Art Unit**
	TERESA E STRZELECKA	1637

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C12Q1/6874	/	1	/	6874
C12Q1/6869	/	1	/	6869

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE (Assistant Examiner) (Date)	**Total Claims Allowed:** 16	
/TERESA E STRZELECKA/ Primary Examiner, Art Unit 1637 (Primary Examiner) 17 March 2023 (Date)	O.G. Print Claim(s) 22	O.G. Print Figure None

U.S. Patent and Trademark Office Part of Paper No.: 20230317

IDT Exhibit 1020.10437

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/381,888	Amorese et al.
	Examiner	**Art Unit**
	TERESA E STRZELECKA	1637

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 16	
/TERESA E STRZELECKA/ Primary Examiner, Art Unit 1637 (Primary Examiner)	17 March 2023 (Date)	O.G. Print Claim(s) 22	O.G. Print Figure None

IDT Exhibit 1020.10438

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L9	34	unique near3 (barcod$6 OR index$76 OR tag$7 OR identifier) AND duplicate near3 read AND read near3 (group$8 OR famil$8) near3 (barcod$6 OR index$76 OR tag$7 OR identifier) AND duplicate near3 (remov$7 OR collaps$7)	(US-PGPUB; USPAT)	ADJ	ON	ON	2023/03/16 05:14 PM
L10	42	unique near3 (barcod$6 OR index$76 OR tag$7 OR identifier) AND duplicate near3 read AND read near3 (group$8 OR famil$8) near3 (barcod$6 OR index$76 OR tag$7 OR identifier) AND duplicate near3 (remov$7 OR collaps$7)	(FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); EPO; JPO)	ADJ	ON	ON	2023/03/16 05:44 PM
L11	5	"63039174".fmid.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; JPO)	ADJ	ON	ON	2023/03/16 05:52 PM
L12	5	"61689730".fmid.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA,	ADJ	ON	ON	2023/03/16 05:56 PM

IDT Exhibit 1020.10439

			VN); FPRS; JPO)				
L13	5	"60412420".fmid.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; JPO)	ADJ	ON	ON	2023/03/16 06:01 PM

PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

IDT Exhibit 1020.10440

Notice of References Cited	Application/Control No. 17/381,888	Applicant(s)/Patent Under Reexamination Amorese et al.	
	Examiner TERESA E STRZELECKA	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20220145380-A1	05-2022	DOAN; Ryan N.	C12Q1/6851	1/1
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kinde, I. et al., Detection and quantification of rare mutations with massively parallel sequencing, PNAS, vol. 108, pp. 9530-9535, 2011) (Year: 2011)
	V	
	W	
	X	

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

IDT Exhibit 1020.10441

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-22)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17381888
Filing Date	2021-07-21
First Named Inventor	Douglas A. Amorese
Art Unit	N/A
Examiner Name	Not Yet Assigned Teresa Strzelecka
Attorney Docket Number	NUGN-001/04US 34079/550

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	4362867		1982-12-07	Paddock	
	2	4458066		1984-07-03	Caruthers et al.	
	3	4469863		1984-09-04	Ts'o et al.	
	4	4582877		1986-04-15	Fairchok et al.	
	5	4876187		1989-10-24	Duck et al.	
	6	4925065		1990-05-15	Golias	
	7	4935357		1990-06-19	Szybalski	
	8	4942124		1990-07-17	Church	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	9	4988617		1991-01-29	Landegren et al.	
	10	4996143		1991-02-26	Heller et al.	
	11	5011769		1991-04-30	Duck et al.	
	12	5034506		1991-07-23	Summerton et al.	
	13	5035996		1991-07-30	Hartley	
	14	5043272		1991-08-27	Hartley	
	15	5082830		1992-01-21	Brakel et al.	
	16	5090591		1992-02-25	Long	
	17	5130238		1992-07-14	Malek et al.	
	18	5169766		1992-12-08	Schuster et al.	
	19	5171534		1992-12-15	Smith et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

IDT Exhibit 1020.10443

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	20	5194370		1993-03-16	Berninger et al.	
	21	5216141		1993-06-01	Benner	
	22	5234809		1993-08-10	Boom et al.	
	23	5235033		1993-08-10	Summerton et al.	
	24	5242794		1993-09-07	Whiteley et al.	
	25	5384242		1995-01-24	Oakes	
	26	5386023		1995-01-31	Sanghvi et al.	
	27	5399491		1995-03-21	Kacian et al.	
	28	5409818		1995-04-25	Davey et al.	
	29	5418149		1995-05-23	Gelfand et al.	
	30	5422271		1995-06-06	Chen et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

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	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	31	5427929		1995-06-27	Richards et al.	
	32	5480784		1996-01-02	Kacian et al.	
	33	5494810		1996-02-27	Barany et al.	
	34	5508169		1996-04-16	Deugau et al.	
	35	5508178		1996-04-16	Rose et al.	
	36	5510270		1996-04-23	Fodor et al.	
	37	5525471		1996-06-11	Zeng	
	38	5545522		1996-08-13	Van Gelder et al.	
	39	5554516		1996-09-10	Kacian et al.	
	40	5554517		1996-09-10	Davey et al.	
	41	5556752		1996-09-17	Lockhart et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

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	Attorney Docket Number	NUGN-001/04US 34079/550

	42	5565340		1996-10-15	Chenchik et al.	
	43	5573913		1996-11-12	Rosemeyer et al.	
	44	5578832		1996-11-26	Trulson et al.	
	45	5589339		1996-12-31	Hampson et al.	
	46	5602240		1997-02-11	De Mesmaeker et al.	
	47	5637684		1997-06-10	Cook et al.	
	48	5641658		1997-06-24	Adams et al.	
	49	5644048		1997-07-01	Yau	
	50	5665549		1997-09-09	Pinkel et al.	
	51	5667976		1997-09-16	Van Ness et al.	
	52	5667979		1997-09-16	Berrens	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	53	5679512		1997-10-21	Laney et al.	
	54	5681726		1997-10-28	Huse et al.	
	55	5683879		1997-11-04	Laney et al.	
	56	5688648		1997-11-18	Mathies et al.	
	57	5705628		1998-01-06	Hawkins	
	58	5708154		1998-01-13	Smith et al.	
	59	5710028		1998-01-20	Eyal et al.	
	60	5712126		1998-01-27	Weissman et al.	
	61	5716785		1998-02-10	Van Gelder et al.	
	62	5726329		1998-03-10	Jones et al.	
	63	5750341		1998-05-12	Macevicz	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

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	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	64	5759822		1998-06-02	Chenchik et al.	
	65	5763178		1998-06-09	Chirikjian et al.	
	66	5789206		1998-08-04	Tavtigian et al.	
	67	5824517		1998-10-20	Cleuziat et al.	
	68	5824518		1998-10-20	Kacian et al.	
	69	5837832		1998-11-17	Chee et al.	
	70	5876976		1999-03-02	Richards et al.	
	71	5882867		1999-03-16	Ullman et al.	
	72	5888779		1999-03-30	Kacian et al.	
	73	5888819		1999-03-30	Goelet et al.	
	74	5945313		1999-08-31	Hartley et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

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	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	75	5952176		1999-09-14	McCarthy et al.	
	76	5958681		1999-09-28	Wetmur et al.	
	77	5965409		1999-10-12	Pardee et al.	
	78	5969119		1999-10-19	Macevicz	
	79	5972618		1999-10-26	Bloch	
	80	6004744		1999-12-21	Goelet et al.	
	81	6004745		1999-12-21	Arnold, Jr. et al.	
	82	6027889		2000-02-22	Barany et al.	
	83	6027923		2000-02-22	Wallace	
	84	6030774		2000-02-29	Laney et al.	
	85	6037152		2000-03-14	Richards et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	86	6056661		2000-05-02	Schmidt	
	87	6077674		2000-06-20	Schleifer et al.	
	88	6087103		2000-07-11	Burmer	
	89	6090553		2000-07-18	Matson	
	90	6090591		2000-07-18	Burg et al.	
	91	6107023		2000-08-22	Reyes et al.	
	92	6110709		2000-08-29	Ausubel et al.	
	93	6150112		2000-11-21	Weissman et al.	
	94	6159685		2000-12-12	Pinkel et al.	
	95	6160105		2000-12-12	Cunningham et al.	
	96	6169194		2001-01-02	Thompson et al.	

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	Attorney Docket Number	NUGN-001/04US 34079/550

	97	6172208		2001-01-09	Cook	
	98	6174680		2001-01-16	Makrigiorgos	
	99	6180338		2001-01-30	Adams	
	100	6190865		2001-02-20	Jendrisak et al.	
	101	6194211		2001-02-27	Richards et al.	
	102	6197501		2001-03-06	Cremer et al.	
	103	6197557		2001-03-06	Makarov et al.	
	104	6210891		2001-04-03	Nyren et al.	
	105	6225109		2001-05-01	Juncosa et al.	
	106	6225451		2001-05-01	Ballinger et al.	
	107	6232104		2001-05-15	Lishanski et al.	

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IDT Exhibit 1020.10451

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	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	108	6251639		2001-06-26	Kurn	
	109	6262490		2001-07-17	Hsu et al.	
	110	6270961		2001-08-07	Drmanac	
	111	6280935		2001-08-28	Macevicz	
	112	6287766		2001-09-11	Nolan et al.	
	113	6287825		2001-09-11	Weissman et al.	
	114	6291170		2001-09-18	Van Gelder et al.	
	115	6306365		2001-10-23	Ruoslahti et al.	
	116	6306597		2001-10-23	Macevicz	
	117	6309843		2001-10-30	Timms	
	118	6326142		2001-12-04	Royer	

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	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	119	6335167		2002-01-01	Pinkel et al.	
	120	6339147		2002-01-15	Lukhtanov et al.	
	121	6440705		2002-08-27	Stanton, Jr. et al.	
	122	6449562		2002-09-10	Chandler et al.	
	123	6582938		2003-06-24	Su et al.	
	124	6670461		2003-12-30	Wengel et al.	
	125	6686156		2004-02-03	Kurn	
	126	6692918		2004-02-17	Kurn	
	127	6770748		2004-08-03	Imanishi et al.	
	128	6777180		2004-08-17	Fisher et al.	
	129	6794499		2004-09-21	Wengel et al.	

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	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
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	130	6815164		2004-11-09	Kurn	
	131	6815167		2004-11-09	Crothers et al.	
	132	6825011		2004-11-30	Romantchikov	
	133	6833246		2004-12-21	Balasubramanian	
	134	6849404		2005-02-01	Park et al.	
	135	6858413		2005-02-22	Kurn	
	136	6913884		2005-07-05	Stuelpnagel et al.	
	137	6917726		2005-07-12	Levene et al.	
	138	6924104		2005-08-02	Weissman et al.	
	139	6946251		2005-09-20	Kurn	
	140	7001724		2006-02-21	Greenfield	

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	141	7033764		2006-04-25	Korlach et al.	
	142	7048481		2006-05-23	Sugata et al.	
	143	7052847		2006-05-30	Korlach et al.	
	144	7056676		2006-06-06	Korlach et al.	
	145	7056716		2006-06-06	Potter et al.	
	146	7060441		2006-06-13	Bourget et al.	
	147	7094536		2006-08-22	Kurn	
	148	7115400		2006-10-03	Adessi et al.	
	149	7170050		2007-01-30	Turner et al.	
	150	7175982		2007-02-13	McCarthy et al.	
	151	7176025		2007-02-13	Kurn et al.	

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	152	7189512		2007-03-13	Porat et al.	
	153	7211390		2007-05-01	Rothberg et al.	
	154	7232656		2007-06-19	Balasubramanian et al.	
	155	7244559		2007-07-17	Rothberg et al.	
	156	7244567		2007-07-17	Chen et al.	
	157	7264929		2007-09-04	Rothberg et al.	
	158	7273730		2007-09-25	Du Breuil Lastrucci	
	159	7276720		2007-10-02	Ulmer	
	160	7294461		2007-11-13	Kurn	
	161	7300755		2007-11-27	Petersdorf et al.	
	162	7302146		2007-11-27	Turner et al.	

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	163	7313308		2007-12-25	Turner et al.	
	164	7315019		2008-01-01	Turner et al.	
	165	7323305		2008-01-29	Leamon et al.	
	166	7335762		2008-02-26	Rothberg et al.	
	167	7351557		2008-04-01	Kurn	
	168	7354717		2008-04-08	Kurn	
	169	7361466		2008-04-22	Korlach et al.	
	170	7361468		2008-04-22	Liu et al.	
	171	7402386		2008-07-22	Kurn et al.	
	172	7405281		2008-07-29	Xu et al.	
	173	7414117		2008-08-19	Saito et al.	

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	174	7416844		2008-08-26	Korlach et al.	
	175	7462452		2008-12-09	Williams et al.	
	176	7462468		2008-12-09	Williams et al.	
	177	7476503		2009-01-13	Turner et al.	
	178	7476504		2009-01-13	Turner	
	179	7491498		2009-02-17	Lapidus et al.	
	180	7501245		2009-03-10	Quake et al.	
	181	7579153		2009-08-25	Brenner et al.	
	182	7704687		2010-04-27	Wang et al.	
	183	7741463		2010-06-22	Gormley et al.	
	184	7771934		2010-08-10	Kurn	

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	185	7771946		2010-08-10	Kurn	
	186	7803550		2010-09-28	Makarov et al.	
	187	7846666		2010-12-07	Kurn	
	188	7846733		2010-12-07	Kurn	
	189	7867703		2011-01-11	Sampson et al.	
	190	7939258		2011-05-10	Kurn et al.	
	191	7948015		2011-05-24	Rothberg et al.	
	192	7985565		2011-07-26	Mayer et al.	
	193	8017335		2011-09-13	Smith	
	194	8034568		2011-10-11	Kurn et al.	
	195	8053192		2011-11-08	Bignell et al.	

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	196	8071311		2011-12-06	Kum	
	197	8143001		2012-03-27	Kum et al.	
	198	8209130		2012-06-26	Kennedy et al.	
	199	8334116		2012-12-18	Kum	
	200	8465950		2013-06-18	Kum et al.	
	201	8492095		2013-07-23	Kum	
	202	8512956		2013-08-20	Kum	
	203	8551709		2013-10-08	Kum et al.	
	204	8852867		2014-10-07	Kum et al.	
	205	8999677		2015-04-07	Soldatov et al.	
	206	9175325		2015-11-03	Kum et al.	

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	207	9175336		2015-11-03	Soldatov et al.	
	208	9181582		2015-11-10	Kum	
	209	9206418		2015-12-08	Armour	
	210	9248076		2016-02-02	Sullivan et al.	
	211	9546399		2017-01-17	Amorese et al.	
	212	9702004		2017-07-11	Van Eijk et al.	
	213	9745627		2017-08-29	van Eijk et al.	
	214	9896721		2018-02-20	Van Eijk et al.	

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	1	20010000077	A1	2001-03-29	Engelhardt et al.	

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	2	20010031739	A1	2001-10-18	Dare	
	3	20010034048	A1	2001-10-25	Kurn	
	4	20010041334	A1	2001-11-15	Rashtchian et al.	
	5	20020028447	A1	2002-03-07	Li et al.	
	6	20020058270	A1	2002-05-16	Kurn	
	7	20020115088	A1	2002-08-22	Kurn	
	8	20020150919	A1	2002-10-17	Weismann et al.	
	9	20020155451	A1	2002-10-24	Makrigiorgos	
	10	20020164628	A1	2002-11-07	Kurn	
	11	20020164634	A1	2002-11-07	Patil et al.	
	12	20020197639	A1	2002-12-26	Shia et al.	

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	13	20030017591	A1	2003-01-23	Kurn	
	14	20030022207	A1	2003-01-30	Balasubramanian et al.	
	15	20030082543	A1	2003-05-01	Su et al.	
	16	20030087251	A1	2003-05-08	Kurn	
	17	20030119150	A1	2003-06-26	Ankenbauer et al.	
	18	20030143555	A1	2003-07-31	Bourget et al.	
	19	20030175780	A1	2003-09-18	Jones	
	20	20030180779	A1	2003-09-25	Lofton-Day et al.	
	21	20030186234	A1	2003-10-02	Kurn	
	22	20030207279	A1	2003-11-06	Crothers et al.	
	23	20030211616	A1	2003-11-13	Leong	

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	24	20030215926	A1	2003-11-20	Kurn et al.	
	25	20030224439	A1	2003-12-04	Lafferty et al.	
	26	20030232348	A1	2003-12-18	Jones et al.	
	27	20040002371	A1	2004-01-01	Paquin et al.	
	28	20040005614	A1	2004-01-08	Kurn et al.	
	29	20040023271	A1	2004-02-05	Kurn et al.	
	30	20040115815	A1	2004-06-17	Li et al.	
	31	20040137456	A1	2004-07-15	Yokota et al.	
	32	20040161742	A1	2004-08-19	Dean et al.	
	33	20040203019	A1	2004-10-14	Kurn	
	34	20040203025	A1	2004-10-14	Kurn	

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	35	20040248153	A1	2004-12-09	Dear et al.	
	36	20050003441	A1	2005-01-06	Kum	
	37	20050014192	A1	2005-01-20	Kum	
	38	20050019793	A1	2005-01-27	Kum et al.	
	39	20050059048	A1	2005-03-17	Gunderson et al.	
	40	20050064456	A1	2005-03-24	Kum	
	41	20050123956	A1	2005-06-09	Blume et al.	
	42	20050136417	A1	2005-06-23	Cole et al.	
	43	20050142577	A1	2005-06-30	Jones et al.	
	44	20050191656	A1	2005-09-01	Drmanac et al.	
	45	20050191682	A1	2005-09-01	Barone et al.	

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	46	20050208538	A1	2005-09-22	Kum et al.	
	47	20060008824	A1	2006-01-12	Ronaghi et al.	
	48	20060014182	A1	2006-01-19	Kum	
	49	20060024678	A1	2006-02-02	Buzby	
	50	20060024711	A1	2006-02-02	Lapidus et al.	
	51	20060035274	A1	2006-02-16	Dong	
	52	20060046251	A1	2006-03-02	Sampson et al.	
	53	20060051789	A1	2006-03-09	Kazakov et al.	
	54	20060068415	A1	2006-03-30	Jones et al.	
	55	20060134633	A1	2006-06-22	Chen et al.	
	56	20060216724	A1	2006-09-28	Christians et al.	

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	57	20060263789	A1	2006-11-23	Kincaid	
	58	20060281082	A1	2006-12-14	Zhu	
	59	20060286566	A1	2006-12-21	Lapidus et al.	
	60	20060292597	A1	2006-12-28	Shapero et al.	
	61	20070031857	A1	2007-02-08	Makarov et al.	
	62	20070134128	A1	2007-06-14	Korlach	
	63	20070141604	A1	2007-06-21	Gormley et al.	
	64	20070224607	A1	2007-09-27	Morgan et al.	
	65	20070224613	A1	2007-09-27	Strathmann	
	66	20070231823	A1	2007-10-04	McKernan et al.	
	67	20070238122	A1	2007-10-11	Allbritton et al.	

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	68	20070263045	A1	2007-11-15	Okazawa	
	69	20080038727	A1	2008-02-14	Spier	
	70	20080087826	A1	2008-04-17	Harris et al.	
	71	20080103058	A1	2008-05-01	Siddiqi	
	72	20080131937	A1	2008-06-05	Schroeder	
	73	20080160580	A1	2008-07-03	Adessi et al.	
	74	20080176311	A1	2008-07-24	Kum	
	75	20080182300	A1	2008-07-31	Kum	
	76	20080194413	A1	2008-08-14	Albert	
	77	20080194416	A1	2008-08-14	Chen	
	78	20080206764	A1	2008-08-28	Williams et al.	

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	Attorney Docket Number	NUGN-001/04US 34079/550

	79	20080213770	A1	2008-09-04	Williams et al.	
	80	20080217246	A1	2008-09-11	Benn et al.	
	81	20080241831	A1	2008-10-02	Fan et al.	
	82	20080242560	A1	2008-10-02	GUNDERSON et al.	
	83	20080286795	A1	2008-11-20	Kawashima et al.	
	84	20090011959	A1	2009-01-08	Costa et al.	
	85	20090024331	A1	2009-01-22	Tomaney et al.	
	86	20090026082	A1	2009-01-29	Rothberg et al.	

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	1	2444926	CA	A1	2002-11-14	Centre Nat Rech Scient		X

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
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	2	101565746	CN	A	2009-10-28	Univ Southeast		☒
	3	105890722	CN	A	2016-08-24	Univ Changzhou		☒
	4	1661102	CN	A	2005-08-31	Univ Shanghai Jiaotong		☒
	5	0329822	EP	B1	1994-06-08	Cangene Corp		☐
	6	0365627	EP	B1	1993-12-22	Univ Iowa Res Found		☐
	7	0667393	EP	A2	1995-08-16	Enzo Diagnostics, Inc		☐
	8	0843735	EP	B1	2002-07-17	Forfas Trading As Biores Irela		☐
	9	1071811	EP	B1	2002-03-06	Entpr Ie Trd As Bioresearch Ie		☐
	10	1929039	EP	B2	2013-11-20	Keygene NV		☐
	11	2272976	EP	A1	2011-01-12	Max Planck Gesellschaft		☐
	12	2322612	EP	A1	2011-05-18	Hitachi High Tech Corp		☐

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	13	2451973	EP	A1	2012-05-16	Max Planck Gesellschaft		☐
	14	2511381	EP	A1	2012-10-17	Monsanto Technology LLC		☐
	15	00/018957	WO	A1	2000-04-06	Applied Research Systems		☐
	16	00/08208	WO	A2	2000-02-17	Medical Res Council		☐
	17	00/39345	WO	A1	2000-07-06	Dana-farber Cancer Institute, Inc		☐
	18	00/52191	WO	A1	2000-09-08	Life Technologies, Inc		☐
	19	00/70039	WO	A1	2000-11-23	Univ Mcgill		☐
	20	01/20035	WO	A2	2001-03-22	Nugen Technologies, Inc		☐
	21	01/23613	WO	A1	2001-04-05	Isis Pharmaceuticals, Inc		☐
	22	01/46464	WO	A1	2001-06-28	Lion Bioscience AG		☐
	23	01/57248	WO	A2	2001-08-09	Solexa Ltd		☐

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	24	01/64952	WO	A2	2001-09-07	Molecular Staging, Inc		☐
	25	02/00938	WO	A2	2002-01-03	Nugen Tgechnologies, Inc		☐
	26	02/060318	WO	A2	2002-08-08	Univ Ohio State Res Found		☐
	27	02/072772	WO	A2	2002-09-19	Nugen Technologies, Inc		☐
	28	02/072773	WO	A2	2002-09-19	Nugen Technologies, Inc		☐
	29	02/081753	WO	A1	2002-10-17	Advanced Res & Tech Inst		☐
	30	02/090584	WO	A2	2002-11-14	Bio Merieux		☐
	31	02/28876	WO	A2	2002-04-11	Riken		☐
	32	02/29117	WO	A2	2002-04-11	Nugen Technologies, Inc		☐
	33	02/36821	WO	A2	2002-05-10	University College Cork - National University Of I		☐
	34	02/48402	WO	A2	2002-06-20	Nugen Technologies, Inc		☐

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	35	03/004690	WO	A2	2003-01-16	454 Corp		☐
	36	03/027259	WO	A2	2003-04-03	Epigenx Pharmaceuticals, Inc		☐
	37	03/078645	WO	A2	2003-09-25	Nugen Technologies, Inc		☐
	38	03/083435	WO	A2	2003-10-09	Nugen Technologies, Inc		☐
	39	03/106642	WO	A2	2003-12-24	Affymetrix, Inc		☐
	40	04/011665	WO	A2	2004-02-05	Nugen Technologies, Inc		☐
	41	2000/09756	WO	A1	2000-02-24	Perkin Elmer Corp		☐
	42	2000/55364	WO	A2	2000-09-21	Amersham Pharm Biotech UK Ltd		☐
	43	2003/002736	WO	A2	2003-01-09	Roche Diagnostics GmbH		☐
	44	2003/012118	WO	A1	2003-02-13	Affymetrix, Inc		☐
	45	2004/070007	WO	A2	2004-08-19	454 Corp		☐

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	46	2004/092418	WO	A2	2004-10-28	Nugen Technologies, Inc		☐
	47	2005/003375	WO	A2	2005-01-13	454 Corp		☐
	48	2005/038427	WO	A2	2005-04-28	Medical Gene Ct Ltd		☐
	49	2005/065321	WO	A2	2005-07-21	Nugen Technologies, Inc		☐
	50	2006/081222	WO	A2	2006-08-03	Compass Genetics, LLC		☐

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	1	Amos, 2000, DNA pooling in mutation detection with reference to sequence analysis, Am J Hum Genet 66:1689-1692	
	2	Bellos, 2014, cnvCapSeq: detecting copy number variation in long-range targeted resequencing data, Nucleic Acids Res 42(20):e158	
	3	Benson, 2013, Genbank, Nucl Acids Res 41:D36-D42	
	4	Blomquist, 2013, Targeted RNA-Sequencing with Competitive Multiplex-PCR Amplicon Libraries, PLOS ONE 8(11): e79120	

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IDT Exhibit 1020.10474

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	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	5	Bodi, 2013, Comparison of commercially available target enrichment methods for next-generation sequencing, J Biomolecular Tech 24:73-86
	6	Church, 1988, Multiplexed DNA sequencing, Science 240:185-188
	7	Colbert, 2001, High-throughput screening for induced point mutations, Plant Physiol 126:480-484
	8	Collard, 2005, An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvements: the basic concepts Euphytica 142:169-196
	9	Eminaga, 2013, Quantification of microRNA Expression with Next-Generation Sequencing, Unit 4.17 in Current Protocols in Molecular Biology, Wiley, New York, NY (14 pages)
	10	Fakhrai-Rad, 2002, Pyroseqeuncing: An accurate detection platform for single nucleotide polymorphisms, Human Mutation 19:479-485
	11	Grothues, 1993, PCR amplification of megabase DNA with tagged random primers (T-PCR), Nucl Acids Res 21:1321-1322
	12	Hajibabaei, 2005, Critical factors for assembling a high volume of DNA barcodes, Phil Trans R Soc B 360:1959-1967
	13	Illumina, 2008, Multiplexed Sequencing with the Illumina Genome Analyzer System, Illumina Sequencing, 4 pages
	14	Illumina, 2011, TruSeq RNA and DNA Sample Preparation Kits v2, 1-15 Illumina, dated 27 April 27, 2011 (4 pages)
	15	Ion Total RNA-Seq Kit v2, User Guide, 2012, Life Technologies (82 pages)

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

IDT Exhibit 1020.10475

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	16	Jiang, 2015, CODEX: a normalization and copy number variation detection method for whole exome sequencing, Nucleic Acids Res 43(6):e39
	17	Krumm, 2012, Copy number variation detection and genotyping from exome sequence data, Genome Res 22 (8):1525-1532
	18	Lai, 2004, Characterization of the maize endosperm transcriptome and its comparison to the rice genome, Genome Res14:1932-1937
	19	Langmead, 2009, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome, Genome Biol 10:R25
	20	Li, 2012, CONTRA: copy number analysis for targeted resequencing, Bioinformatics 28(10):1307-1313
	21	Lindstrom, 2004, Pyrosequencing for detection of Lamivudine-resistant Hepatitis B virus, J Clin Microb 42 (10):4788-4795
	22	Liu, 2008, Sequence space coverage, entropy of genomes and the potential to detect non-human DNA in human samples, BMC Genomics 9(509):1-17
	23	Ma, 2015, Quantitative Analysis of Copy Number Variants Based on Real-Time LightCycler PCR, Curr Protoc Hum Genet 80:7.21.1-7.23.8
	24	Machine translation generated on March 7, 2018, of CN 105890722 by website of European Patent Office (4 pages)
	25	Margulies, 2005, Genorne sequencing in open microfabricated high density picoliter reactors, Nature 437 (7057):376-380
	26	McCloskey, 2007, Encoding PCR products with batch-stamps and barcodes, Biochem Genet 45:761-767

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2021-07-21
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	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	27	Miner, 2004, Molecular barcodes detect redundancy and contamination in hairpin-bisulfite PCR, Nucl Acids Res 32 (17):e135, 4 pages
	28	Myers, 2013, Protocol for Creating Multiplexed miRNA Libraries for Use in Illumina Sequencing, Myers lab microRNA-seq Protocol, Hudson Alpha Institute for Biotechnology web site, dated May 2, 2013, (15 pages)
	29	NuGEN, 2014, User Guide Ovation Target Enrichment System, NuGEN Technologies Inc., San Carlos, CA (45 pages)
	30	Plagnol, 2012, A robust model for read count data in exome sequencing experiments and implications for copy number variant calling, Bioinformatics 28(21):2747-2754
	31	Qiu, 2003, DNA sequence-based "bar-codes" for tracking the origins of expressed sequence tags from a maize cDNA library constructed using multiple mRNA sources, Plant Physiol 133:475-481
	32	Querfurth, 2012, Creation and application of immortalized bait libraries for targeted enrichment and next-generation sequencing, Biotechniques 52(6):375-380
	33	Ronaghi, 2001, Pyrosequencing sheds light on DNA sequencing, Genome Res 11:3-11
	34	Sathirapongsasuti, 2011, Exome sequencing-based copy-number variation and loss of heterozygosity detection: ExomeCNV, Bioinformatics 27(19):2648-2654
	35	Schiemer, 2011, Illumina TruSeq Adapters Demystified,TUFTS UNIVERSITY CORE FACILITY XP055357867 (5 pages)
	36	Shapero, 2001, SNP Genotyping by multiplexed solid-phase amplification and fluorescent minisequencing, Genome Res 11:1926-1934
	37	Shendure, 2005, Accurate multiplex polony sequencing of an evolved bacterial genome, Science 309:1728

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

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	38	Soni, 2007, Progress toward ultrafast DNA sequencing using solid-state nanopores, Clin Chem 53(11):1996-2001
	39	Sood, 2006, Methods for reverse genetic screening in zebrafish by resequencing and TILLING, Methods 39:220-227
	40	Staroscik, 2004, Calculator for determining the number of copies of a template, URI Genomics, webpage archive dated April 6, 2017 (1 page), Retreived from the internet on March 7, 2018, from <https://web.archive.org/web/20170406174850/http://cels.uri.edu/gsc/cndna.html>
	41	Stratagene, 1998, Gene characterization kits, Stratagene Catalog, p. 39 (2 pages)
	42	Till, 2003, Large-scale discovery of induced point mutations with high-throughput TILLING, Genome Res 13:524-530
	43	Trapnell, 2010, Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation, Nat Biotech 28:511-515
	44	Trapnell, 2013, Differential analysis of gene regulation at transcript resolution with RNA-seq, Nat Biotech 31:46-53
	45	Unemo, 2004, Molecular typing of Neisseria gonorrhoeae isolates by pyrosequencing of highly polymorphic segments of the porB gene, J Clin Microb 42(7):2926-2934
	46	Vasil, 2012, Next generation sequencing for molecular diagnosis of neuromuscular diseases, Acta Neuropathol, 124:273-283, plus supplemental material pp. 1-10
	47	Vigal, 2002, A review on SNP and other types of molecular markers and their use in animal genetics, Genet Sel Evol 34:275-305
	48	Walker, 1992, Strand displacement amplification--an isothermal, in vitro DNA amplification technique, Nucl Acids Res 20(7):1691-1696

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IDT Exhibit 1020.10478

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	Art Unit	N/A
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	49	Westin 2000, Anchored multiplex amplification on a microelectronic chip array, Nat Biotech 18:199-204
	50	Wienholds, 2004, Target-selected gene inactivation in zebrafish, Meth Cell Biol 77:69-90

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	1	20090029385	A1	2009-01-29	Christians et al.	
	2	20090036663	A1	2009-02-05	Kurn	
	3	20090061425	A1	2009-03-05	Lo et al.	
	4	20090061439	A1	2009-03-05	Buzby	
	5	20090068645	A1	2009-03-12	Sibson	

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	6	20090068655	A1	2009-03-12	Williams	
	7	20090068709	A1	2009-03-12	Kurn et al.	
	8	20090105081	A1	2009-04-23	Rodesch et al.	
	9	20090117573	A1	2009-05-07	Fu et al.	
	10	20090117621	A1	2009-05-07	Boutell et al.	
	11	20090124514	A1	2009-05-14	Fu et al.	
	12	20090127589	A1	2009-05-21	Rothberg et al.	
	13	20090130721	A1	2009-05-21	Kurn et al.	
	14	20090203085	A1	2009-08-13	KURN et al.	
	15	20090203531	A1	2009-08-13	Kurn	
	16	20090233802	A1	2009-09-17	Bignell et al.	

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	17	20090233804	A1	2009-09-17	Kurn et al.	
	18	20090239232	A1	2009-09-24	Kurn	
	19	20090275486	A1	2009-11-05	Kurn et al.	
	20	20090280538	A1	2009-11-12	Patel et al.	
	21	20090298075	A1	2009-12-03	Travers et al.	
	22	20100015666	A1	2010-01-21	Brenner et al.	
	23	20100021973	A1	2010-01-28	Makarov et al.	
	24	20100022403	A1	2010-01-28	Kurn et al.	
	25	20100029511	A1	2010-02-04	Raymond et al.	
	26	20100105052	A1	2010-04-29	DRMANAC et al.	
	27	20100113296	A1	2010-05-06	Myerson	

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	28	20100129879	A1	2010-05-27	Ach et al.	
	29	20100137143	A1	2010-06-03	Rothberg et al.	
	30	20100159559	A1	2010-06-24	Kurn et al.	
	31	20100167954	A1	2010-07-01	Earnshaw et al.	
	32	20100173394	A1	2010-07-08	Colston, JR. et al.	
	33	20100203597	A1	2010-08-12	Chen et al.	
	34	20100267043	A1	2010-10-21	Braverman et al.	
	35	20100273219	A1	2010-10-28	May et al.	
	36	20100311066	A1	2010-12-09	Kurn	
	37	20100323348	A1	2010-12-23	Hamady et al.	
	38	20110015096	A1	2011-01-20	CHIU	

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	39	20110039732	A1	2011-02-17	Raymond et al.	
	40	20110104785	A1	2011-05-05	Vaidyanathan et al.	
	41	20110105364	A1	2011-05-05	Kum	
	42	20110129827	A1	2011-06-02	Causey et al.	
	43	20110189679	A1	2011-08-04	Kum et al.	
	44	20110224105	A1	2011-09-15	Kum et al.	
	45	20110269647	A1	2011-11-03	ULE et al.	
	46	20110288780	A1	2011-11-24	Rabinowitz et al.	
	47	20110294132	A1	2011-12-01	Kum	
	48	20110319290	A1	2011-12-29	Raymond et al.	
	49	20120003657	A1	2012-01-05	Myllykangas et al.	

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	50	20120028310	A1	2012-02-02	Kurn et al.	
	51	20120045797	A1	2012-02-23	Kurn et al.	
	52	20120071331	A1	2012-03-22	Casbon et al.	
	53	20120074925	A1	2012-03-29	Oliver	
	54	20120102054	A1	2012-04-26	POPESCU et al.	
	55	20120107811	A1	2012-05-03	Kelso et al.	
	56	20120122701	A1	2012-05-17	Ryan et al.	
	57	20120149068	A1	2012-06-14	Kurn	
	58	20120156728	A1	2012-06-21	Li et al.	
	59	20120157322	A1	2012-06-21	Myllykangas et al.	
	60	20120190587	A1	2012-07-26	Kurn et al.	

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	61	20120208705	A1	2012-08-16	Steemers et al.	
	62	20120220483	A1	2012-08-30	KURN et al.	
	63	20120220494	A1	2012-08-30	Samuels et al.	
	64	20120237943	A1	2012-09-20	Soldatov et al.	
	65	20120238738	A1	2012-09-20	Hendrickson	
	66	20120245041	A1	2012-09-27	Brenner et al.	
	67	20120270212	A1	2012-10-25	RABINOWITZ et al.	
	68	20120283145	A1	2012-11-08	Wang	
	69	20120289426	A1	2012-11-15	Roos et al.	
	70	20120309002	A1	2012-12-06	Link	
	71	20130005585	A1	2013-01-03	Anderson et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

IDT Exhibit 1020.10486

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	72	20130059738	A1	2013-03-07	LEAMON et al.	
	73	20130231253	A1	2013-09-05	Amorese et al.	
	74	20140038188	A1	2014-02-06	Kurn	
	75	20140038236	A1	2014-02-06	Kurn et al.	
	76	20140065692	A1	2014-03-06	KURN et al.	
	77	20140274729	A1	2014-09-18	Kurn et al.	
	78	20140274731	A1	2014-09-18	RAYMOND et al.	
	79	20140274738	A1	2014-09-18	Amorese et al.	
	80	20140303000	A1	2014-10-09	Armour	
	81	20140378345	A1	2014-12-25	Hindson et al.	
	82	20150011396	A1	2015-01-08	Schroeder et al.	

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IDT Exhibit 1020.10487

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	83	20150017635	A1	2015-01-15	Myllykangas et al.	
	84	20150101595	A1	2015-04-16	Hancock et al.	
	85	20150133319	A1	2015-05-14	Fu et al.	
	86	20150284769	A1	2015-10-08	Schroeder	
	87	20150299767	A1	2015-10-22	Armour et al.	
	88	20150299784	A1	2015-10-22	Fan et al.	
	89	20150299812	A1	2015-10-22	Talasaz	
	90	20160122756	A1	2016-05-05	Armour	
	91	20160130576	A1	2016-05-12	Armour	
	92	20160153039	A1	2016-06-02	Amorese et al.	
	93	20160203259	A1	2016-07-14	SCOLNICK et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /T.E.S/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	94	20160220994	A1	2016-08-04	Wright	
	95	20160251711	A1	2016-09-01	AMORESE et al.	
	96	20160265042	A1	2016-09-15	Schroeder et al.	
	97	20160275240	A1	2016-09-22	HUELGA et al.	
	98	20160296930	A1	2016-10-13	Matear et al.	

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2006/086668	WO	A2	2006-08-17	Epict Technologies		
	2	2006/137733	WO	A1	2006-12-28	Keygene NV		
	3	2007/018601	WO	A1	2007-02-15	Rubicon Genomics, Inc		
	4	2007/019444	WO	A2	2007-02-15	Euclid Diagnostics LLC		

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	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	5	2007/030759	WO	A2	2007-03-15	Nugen Technologies, Inc		
	6	2007/037678	WO	A2	2007-04-05	Keygene NV		
	7	2007/052006	WO	A1	2007-05-10	Solexa Ltd		
	8	2007/057652	WO	A1	2007-05-24	Solexa Ltd		
	9	2007/073165	WO	A1	2007-06-28	Keygene NV		
	10	2007/136717	WO	A1	2007-11-29	Nugen Technologies, Inc		
	11	2008/005459	WO	A2	2008-01-10	Nugen Technologies, Inc		
	12	2008/015396	WO	A2	2008-02-07	Solexa Ltd		
	13	2008/033442	WO	A2	2008-03-20	Univ Leland Stanford Junior		
	14	2008/093098	WO	A2	2008-08-07	Solexa Ltd		
	15	2008/115185	WO	A2	2008-09-25	Nimblegen Systems, Inc		

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	16	2008150432	WO	A1	2008-12-11	454 Life Sciences Corp		
	17	2009/053039	WO	A1	2009-04-30	Roche Diagnostics GmbH		
	18	2009/102878	WO	A2	2009-08-20	Nugen Technologies, Inc		
	19	2009/102896	WO	A2	2009-08-20	Nugen Technologies, Inc		
	20	2009/112844	WO	A1	2009-09-17	Stichting Het Nl Kanker I		
	21	2009/117698	WO	A2	2009-09-24	Nugen Technologies, Inc		
	22	2009/120372	WO	A2	2009-10-01	Pacific Biosciences Of California, Inc		
	23	2009/120374	WO	A2	2009-10-01	Pacific Biosciences Of California, Inc		
	24	2010/003153	WO	A2	2010-01-07	Life Technologies Corp		
	25	2010/030683	WO	A1	2010-03-18	Rosetta Inpharmatics LLC		
	26	2010/039991	WO	A2	2010-04-08	Texas A & M Univ Sys		

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	27	2010/063711	WO	A1	2010-06-10	Angeletti P Ist Richerche Bio		
	28	2010/064893	WO	A1	2010-06-10	Keygene NV		
	29	2010/085715	WO	A1	2010-07-29	Quanta Biosciences		
	30	2010/091246	WO	A2	2010-08-12	Univ Northwestern		
	31	2010/115154	WO	A1	2010-10-07	Fluidigm Corp		
	32	2010/129937	WO	A2	2010-11-11	Life Technologies Corp		
	33	2011/003630	WO	A1	2011-01-13	Max Planck Gesellschaft		
	34	2011/009941	WO	A1	2011-01-27	Olink Genomics AB		
	35	2011/019964	WO	A1	2011-02-17	Nugen Technologies, Inc		
	36	2011/032053	WO	A1	2011-03-17	Nugen Technologies, Inc.		
	37	2011/053987	WO	A1	2011-05-05	Nugen Technologies, Inc		

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	38	2011/151777	WO	A1	2011-12-08	Consiglio Nazionale Ricerche		
	39	2011/156529	WO	A2	2011-12-15	Nugen Technologies, Inc		
	40	2011032040	WO	A1	2011-03-17	Centrillion Technology Holding Corp		
	41	2012/013932	WO	A1	2012-02-02	Univ St Andrews		
	42	2012/054873	WO	A3	2012-08-23	Cold Spring Harbor Lab		
	43	2012/061832	WO	A1	2012-05-10	Illumina, Inc		
	44	2012/103154	WO	A1	2012-08-02	Nugen Technologies, Inc		
	45	2013/059740	WO	A1	2013-04-25	Foundation Medicine, Inc		
	46	2013/059746	WO	A1	2013-04-25	Nugen Technologies, Inc		
	47	2013/112923	WO	A1	2013-08-01	Nugen Technologies, Inc		
	48	2013/130512	WO	A3	2013-10-24	Univ North Carolina		

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IDT Exhibit 1020.10493

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17381888
	Filing Date	2021-07-21
	First Named Inventor	Douglas A. Amorese
	Art Unit	N/A
	Examiner Name	Not Yet Assigned Teresa Strzelecka
	Attorney Docket Number	NUGN-001/04US 34079/550

	49	2013/138510	WO	A9	2014-07-10	Patel, Abhijit Ajit		
	50	2013/177220	WO	A1	2013-11-28	Scripps Research Inst		

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	Wolford, 2000, High-throughput SNP detection by using DNA pooling and denaturing high performance liquid chromatography (DHPLC), Hum Genet 107:483-487	
	2	Xi, 2011, Copy number variation detection in whole-genome sequencing data using the Bayesian information criterion, PNAS 108(46):e1128-e1136	
	3	Xu, 2012, FastUniq: A fast de novo duplicates removal tool for paired short reads, PLoSOne 7(12):e52249	

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EXAMINER SIGNATURE

Examiner Signature	/TERESA E STRZELECKA/	Date Considered	03/15/2023

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

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

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Electronic Patent Application Fee Transmittal

Application Number:	17381888
Filing Date:	21-Jul-2021
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas A. Amorese
Filer:	Adam M Schoen/Michelle Aiello
Attorney Docket Number:	NUGN-001/04US 34079/550

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1200	1200

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1200**

Electronic Acknowledgement Receipt

EFS ID:	48205348
Application Number:	17381888
International Application Number:	
Confirmation Number:	6188
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas A. Amorese
Customer Number:	21710
Filer:	Adam M Schoen/Michelle Aiello
Filer Authorized By:	Adam M Schoen
Attorney Docket Number:	NUGN-001/04US 34079/550
Receipt Date:	26-JUN-2023
Filing Date:	21-JUL-2021
Time Stamp:	16:55:27
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1200
RAM confirmation Number	E20236PG55405110
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1	Issue Fee Payment (PTO-85B)	NUGN-001-04US-Issue-Fee-Transmittal.pdf	37011 6d4dbfdea9637f06397af172d2012606de961617	no	1
Warnings:					
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2	Fee Worksheet (SB06)	fee-info.pdf	37953 bf4f5736a7a62042cd7eb7f4213a0f023ed62188	no	2
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		Total Files Size (in bytes):	74964		

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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

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PART B–FEE(S) TRANSMITTAL

Complete and send this form, together with the applicable fee(s), by mail or fax, or via EFS-Web.

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Alexandria, VA 22313-1450

By fax, send to: (571) 273-2885

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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

Michelle Aiello	(Typed or printed name)
/Michelle Aiello/	(Signature)
June 26, 2023	(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
17/381,888	07/21/2021	Douglas A. Amorese	NUGN-001/04US 34079/550	6188

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ

APPLN: TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1,200.00	$0.00	$0.00	$1,200.00	06/27/2023

EXAMINER	ART UNIT	CLASS-SUBCLASS
Strzelecka, Teresa E.	1637	506-004000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363)

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1. Brown Rudnick LLP
2. Adam M. Schoen
3.

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Tecan Genomics, Inc.

(B) RESIDENCE: (CITY and STATE or COUNTRY): Redwood City, California

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required)

4b. Method of Payment (*Please first reapply any previously paid fee shown above*):

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☐ Applicant certifying micro entity status. See 37 CFR 1.29. NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27. NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

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Authorized Signature: /Adam M. Schoen/ Date: June 26, 2023

Typed or printed name: Adam M. Schoen Registration No. 58,576

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/381,888	08/15/2023	11725241	NUGN-001/04US 34079/550	6188

21710 7590 07/26/2023

BROWN RUDNICK LLP
ONE FINANCIAL CENTER
BOSTON, MA 02111

ISSUE NOTIFICATION

The projected patent number and issue date are specified above. The patent will issue electronically. The electronically issued patent is the official patent grant pursuant to 35 U.S.C. § 153. The patent may be accessed on or after the issue date through Patent Center at https://patentcenter.uspto.gov/. The patent will be available in both the public and the private sides of Patent Center. Further assistance in electronically accessing the patent, or about Patent Center, is available by calling the Patent Electronic Business Center at 1-888-217-9197.

The USPTO is implementing electronic patent issuance with a transition period, during which period the USPTO will mail a ceremonial paper copy of the electronic patent grant to the correspondence address of record. Additional copies of the patent (i.e., certified and presentation copies) may be ordered for a fee from the USPTO's Certified Copy Center at https://certifiedcopycenter.uspto.gov/index.html. The Certified Copy Center may be reached at (800)972-6382.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 187 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PAIR WEB site http://pair.uspto.gov for additional inventors):

Douglas A. Amorese, Los Altos, CA;
Jonathan Scolnick, San Francisco, CA;
Ben Schroeder, San Mateo, CA;

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Tecan Genomics, Inc., Redwood City, CA;

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/381,888	07/21/2021	Douglas A. Amorese	NUGN-001/04US 34079/550	6188

21710 7590 08/15/2023

BROWN RUDNICK LLP
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BOSTON, MA 02111

EXAMINER	
STRZELECKA, TERESA E	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
08/15/2023	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.

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IDT Exhibit 1020.10502

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Alexandria, Virginia 22313-1450
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APPLICATION NO.	ISSUE DATE	PATENT NO.
17/381,888	15-Aug-2023	11725241

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BOSTON, MA 02111

EGRANT NOTIFICATION

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