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(54) Title: MULTI-PRIMER AMPLIFICATION METHOD FOR BARCODING OF TARGET NUCLEIC ACIDS

(57) Abstract: In certain embodiments, the present invention provides amplification methods in which nucleotide tag(s) and, optionally, a barcode nucleotide sequence are added to target nucleotide sequences. In other embodiments, the present invention provides a microfluidic device that includes a plurality of first input lines and a plurality of second input lines. The microfluidic device also includes a plurality of sets of first chambers and a plurality of sets of second chambers. Each set of first chambers is in fluid communication with one of the plurality of first input lines. Each set of second chambers is in fluid communication with one of the plurality of second input lines. The microfluidic device further includes a plurality of first pump elements in fluid communication with a first portion of the plurality of second input lines and a plurality of second pump elements in fluid communication with a second portion of the plurality of second input lines.

MULTI-PRIMER AMPLIFICATION METHOD FOR BARCODING OF TARGET NUCLEIC ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of prior U.S. provisional application no. 61/166,181, filed April 2, 2009; prior U.S. provisional application no. 61/166,105, filed April 2, 2009; prior U.S. provisional application no. 61/186,327, filed June 11, 2009; and prior U.S. provisional application no. 61/305,907, filed February 18, 2010, which are all hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the area of high-throughput assays for detection and/ or sequencing of particular target nucleic acids. In certain embodiments, the present invention provides amplification methods in which nucleotide tag(s) and a barcode nucleotide sequence are added to target nucleotide sequences.

BACKGROUND OF THE INVENTION

[0003] The ability to detect specific nucleic acid sequences in a sample has resulted in new approaches in diagnostic and predictive medicine, environmental, food and agricultural monitoring, molecular biology research, and many other fields. In addition, new sequencing methodologies provide the means for rapid high-throughput nucleic acid sequencing.

[0004] Additional methods, especially methods that facilitate analysis of many targets and/or the analysis of many samples simultaneously across a broad range of concentrations in a sample would be of great benefit.

[0005] Microfluidic devices can be used for analytical, preparative, metering, and other manipulative functions on a scale not imagined until recently. The advantages of microfluidic devices include conservation of precious reagents and samples, high density and throughput of sample analysis or synthesis, fluidic precision and accuracy at a level scarcely visible to the unaided eye, and a space reduction accompanying the replacement of counterpart equipment operating at the

macrofluidic scale. Associated with the reduction in size and the increased density of microfluidic devices is increased complexity and higher engineering and fabrication costs associated with increasingly intricate device architecture.

[0006] Recently, there have been concerted efforts to develop and manufacture microfluidic systems to perform various chemical and biochemical analyses and syntheses. Additionally, microfluidic devices have the potential to be adapted for use with automated systems, thereby providing the additional benefits of further cost reductions and decreased operator errors because of the reduction in human involvement. Microfluidic devices have been proposed for use in a variety of applications, including, for instance, capillary electrophoresis, gas chromatography, and cell separations.

[0007] However, realization of these benefits has often been thwarted because of various complications associated with the microfluidic devices that have thus far been manufactured. For instance, many of the current microfluidic devices are manufactured from silica-based substrates, which are difficult and complicated to machine. As a result, many devices made from such materials are fragile. Furthermore, transport of fluid through many existing microfluidic devices requires regulation of complicated electrical fields to transport fluids in a controlled fashion through the device.

[0008] Thus, in view of the foregoing benefits that can be achieved with microfluidic devices but the current limitations of existing devices, there remains a need for microfluidic devices designed for use in conducting a variety of chemical and biochemical analyses. Because of its importance in modern biochemistry, there is a particular need for devices that can be utilized to conduct a variety of nucleic acid amplification reactions, while having sufficient versatility for use in other types of analyses as well.

[0009] Devices with the ability to conduct nucleic acid amplifications would have diverse utilities. For example, such devices could be used as an analytical tool to determine whether a particular target nucleic acid of interest is present or absent in a sample. Thus, the devices could be utilized to test for the presence of particular pathogens (e.g., viruses, bacteria, or fungi), and for identification purposes (e.g., paternity and forensic applications). Such devices could also be utilized to detect or

characterize specific nucleic acids previously correlated with particular diseases or genetic disorders. When used as analytical tools, the devices could also be utilized to conduct genotyping analyses and gene expression analyses (e.g., differential gene expression studies). Alternatively, the devices can be used in a preparative fashion to amplify sufficient nucleic acid for further analysis such as sequencing of amplified product, cell-typing, DNA fingerprinting, and the like. Amplified products can also be used in various genetic engineering applications, such as insertion into a vector that can then be used to transform cells for the production of a desired protein product.

[0010] Despite these advances in microfluidic design and use, it would be useful to reduce the complexity of microfluidic chips and simplify their operation. Additionally, a need exists for an increased ability to recover reaction products from microfluidic devices. Thus, there is a need in the art for improved methods and systems related to microfluidic devices.

SUMMARY OF THE INVENTION

[0011] In certain embodiments, the invention provides a method for amplifying, tagging, and barcoding a plurality of target nucleic acids in a plurality of samples. The method entails preparing an amplification mixture for each target nucleic acid. Each amplification mixture includes:

a forward primer comprising a target-specific portion;

a reverse primer comprising a target-specific portion, wherein the forward primer additionally comprises a first nucleotide tag and/or the reverse primer additionally comprises a second nucleotide tag; and

at least one barcode primer including a barcode nucleotide sequence and a first and/or second nucleotide tag-specific portion, wherein the barcode primer is in excess of the forward and/or reverse primer(s).

Each amplification mixture is subjected to amplification to produce a plurality of target amplicons, wherein each target amplicon includes a tagged target nucleotide sequence, with first and/or second nucleotide tags flanking the target nucleotide sequence, and at least one barcode nucleotide sequence at the 5' or 3' end of the target amplicon.

[0012] In specific embodiments, the forward primer additionally includes a first nucleotide tag. If desired, the reverse primer can additionally include a second nucleotide tag.

[0013] In certain embodiments of the tagging/barcoding method, the concentration of the barcode primer in the amplification mixtures is at least 4-fold the concentration of the forward and/or reverse primer(s). In variations of such embodiments, the concentration of the barcode primer in the amplification mixtures is at least 50-fold the concentration of the forward and/or reverse primer(s).

[0014] In particular embodiments of the barcoding/tagging method, the first and/or second nucleotide tags and/or the barcode nucleotide sequence are selected so as to avoid substantial annealing to the target nucleic acids. In illustrative embodiments, the barcode nucleotide sequence identifies a particular sample. Where the barcode primer includes a barcode nucleotide sequence and a first nucleotide tag-specific portion, in certain embodiments, a plurality of forward primers include the same first nucleotide tag. For, example, where multiple targets are to be amplified in different samples, the set of forward primers corresponding to the set of targets can all have the same first nucleotide tag.

[0015] In particular embodiments of the barcoding/tagging method, the forward and reverse primers for each target are initially combined separately from the sample, and each barcode primer is initially combined with its corresponding sample. For example, where T targets are to be amplified in S samples, T and S being integers greater than one, the method can additionally include preparing S×T amplification mixtures wherein the initially combined forward and reverse primers are added to the initially combined samples and barcode primers.

[0016] In certain embodiments of the barcoding/tagging method, the amplification is carried out for at least 3 cycles to introduce the first and second nucleotide tags and the barcode nucleotide sequence. In variations of these embodiments, the amplification is carried out for between 5 and 50 cycles. In particular embodiments, the amplification is carried out for a sufficient number of cycles to normalize target amplicon copy number across targets and across samples.

[0017] In certain embodiments of the barcoding/tagging method, at least 50 percent of the target amplicons produced upon amplification are present at greater

than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

[0018] In other embodiments, the invention provides a method in which barcoding is, optionally, omitted and the target nucleotide sequences are tagged after amplification. This method entails amplifying a plurality of target nucleic acids, typically, in a plurality of samples. An amplification mixture is prepared for each target nucleic acid, wherein each amplification mixture includes:

a forward primer including a target-specific sequence; and

a reverse primer including a target-specific sequence;

Each amplification mixture is subjected to amplification to produce a plurality of target nucleotide sequences. The target nucleotide sequences are then tagged (e.g., by ligation of nucleotide tags unto one or both ends of the target nucleotide sequences) to produce a plurality of target amplicons. Each target amplicon includes first and/or second nucleotide tags flanking the target nucleotide sequence. In particular embodiments, at least 50 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

[0019] In certain embodiments of the amplification methods described herein, at least 70 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons. In illustrative embodiments, at least 90 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

[0020] In various embodiments, the average length of the target amplicons is at least 25 bases, 50 bases, 100 bases, 200 bases, 500 bases, and 750 bases. Longer average lengths, such as 1 kilobase or more are also possible, as, for example, when amplification is carried out by long-range PCR. In such embodiments, amplification may yield target amplicons wherein at least 70 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

[0021] An advantage of the methods described herein is that amplification can (but need not) be carried out in small reaction volumes. In particular embodiments, the volume of the amplification mixtures is in the range of about 1 picoliter to about 50 nanoliters. In certain embodiments, the volume of the amplification mixtures is in the range of about 5 picoliters to about 25 nanoliters.

[0022] The methods described herein can, optionally, include recovering the target amplicons from the amplification mixtures. In certain embodiments, the target amplicons are recovered in a volume and/or copy number that varies less than about 50% among the recovered target amplicons. The recovered amplicons can be employed for further amplification and/or analysis (e.g., DNA sequencing). In some embodiments, at least one target amplicon can be subjected to amplification using primers specific for the first and second nucleotide tags to produce a target amplicon lacking the barcode nucleotide sequence, if such is desired.

[0023] In particular embodiments of the methods described herein, the target nucleic acids include genomic DNA. In variations of these embodiments, the genomic DNA can be DNA intended for DNA sequencing, e.g., automated DNA sequencing

[0024] In certain embodiments, one or more of the forward primer, reverse primer, and barcode primer can include at least one additional primer binding site. For example, if a barcode primer is employed, the barcode primer can include at least a first additional primer binding site upstream of the barcode nucleotide sequence, which is upstream of the first nucleotide tag. In such an embodiment, the reverse primer can include at least a second additional primer binding site downstream of the second nucleotide tag. In particular embodiments, where the target nucleotide sequences are to be sequenced by automated DNA sequencing, the first and second additional primer binding sites are capable of being bound by DNA sequencing primers.

[0025] If a barcode primer is not employed, and the target nucleotide sequences are tagged after amplification, the first and second nucleotide tags can be capable of being bound by DNA sequencing primers.

[0026] Thus, the methods described herein can, optionally, include subjecting at least one target amplicon to DNA sequencing.

[0027] In certain embodiments, the method can, optionally, include quantifying the amount of target amplicons in the amplification mixtures. This step may be carried out, for example, prior to automated DNA sequencing. In particular embodiments, quantification includes recovering the target amplicons and subjecting them to digital amplification. Digital amplification includes, in particular embodiments,

distributing the preamplified target amplicons into discrete reaction mixtures, wherein each reaction mixture, on average, includes no more than one amplicon per reaction mixture; and

subjecting the reaction mixtures to amplification.

Quantification in digital amplification may be carried out by real-time PCR and/or endpoint PCR.

[0028] The amplification methods described herein can, optionally, include determining the amount of each target nucleic acid present in each sample. In certain embodiments, the methods can be performed in determining the copy numbers of the target nucleic acids in each sample. In particular embodiments, the methods can be performed in determining the genotypes at loci corresponding to the target nucleic acids. In other embodiments, the methods can be performed in determining the expression levels of the target nucleic acids.

[0029] In particular embodiments, the present invention relates to microfluidic devices. More particularly, the present invention relates to a microfluidic device that provides for recovery of reaction products. Merely by way of example, the method and apparatus has been applied to a PCR sample preparation system used to prepare libraries for next generation sequencing. However, it would be recognized that the invention has a much broader range of applicability.

[0030] According to an embodiment of the present invention, a microfluidic device is provided. The microfluidic device includes a plurality of first input lines and a plurality of second input lines. The microfluidic device also includes a plurality of sets of first chambers and a plurality of sets of second chambers. Each set of first chambers is in fluid communication with one of the plurality of first input lines and each set of second chambers is in fluid communication with one of the plurality of second input lines. The microfluidic device further includes a plurality of first pump

elements in fluid communication with a first portion of the plurality of second input lines and a plurality of second pump elements in fluid communication with a second portion of the plurality of second input lines.

[0031] According to another embodiment of the present invention, a method of operating a microfluidic device having an assay chamber, a sample chamber, and a harvesting port is provided. The method includes closing a fluid line between the assay chamber and the sample chamber, flowing a sample into the sample chamber via a sample input line, and flowing an assay into the assay chamber via an assay input line. The method also includes opening the fluid line between the assay chamber and the sample chamber, combining at least a portion of the sample and at least a portion of the assay to form a mixture, and reacting the mixture to form a reaction product. The method further includes closing the fluid line between the assay chamber and the sample chamber, flowing a harvesting reagent from the harvesting port to the sample chamber, and removing the reaction product from the microfluidic device.

[0032] According to a particular embodiment of the present invention, a method of preparing reaction products is provided. The method includes providing M samples and providing N assays. The method also includes mixing the M samples and N assays to form MxN pairwise combinations. Each of the MxN pairwise combinations are contained in a closed volume. The method further includes forming MxN reaction products from the MxN pairwise combinations and recovering the MxN reaction products.

[0033] Many benefits are achieved by way of the present invention over conventional techniques. For example, embodiments of the present invention provide for mixing and reaction of MxN samples and assays followed by recovery of the reaction products in sample-by-sample pools. Additionally, dilation pumping is utilized to remove substantially all of the reaction products from the microfluidic device, providing uniformity between the various reaction product pools. Utilizing the systems and methods described herein, the time and labor required to prepare libraries is reduced in comparison with conventional techniques. These and other embodiments of the invention along with many of its advantages and features are described in more detail in conjunction with the text below and attached figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] The invention may be understood by reference to the following description taken in conjunction with the accompanying drawings that illustrate certain specific embodiments of the present invention.

[0035] Figure 1 depicts an illustrative matrix-type microfluidic device in plan view.

[0036] Figure 2 is a simplified perspective illustration of a carrier and a microfluidic device that permits recovery of reaction products.

[0037] Figure 3 is a simplified schematic diagram of a microfluidic device that permits recovery of reaction products.

[0038] Figure 4 is a simplified schematic diagram of several unit cells of the microfluidic device illustrated in Fig. 3.

[0039] Figure 5A is simplified schematic diagram of a microfluidic device that permits recovery of reaction products.

[0040] Figure 5B is a simplified schematic diagram of portions of the microfluidic device illustrated in Fig. 5A.

[0041] Figure 6 is a simplified schematic diagram of several unit cells of the microfluidic device illustrated in Fig. 5A.

[0042] Figure 7 is a simplified flowchart of a method of operating a microfluidic device that permits recovery of reaction products.

[0043] Figures 8A-8D are simplified schematic diagrams illustrating fluid flow through unit cells of a microfluidic device that permits recovery of reaction products during operation.

[0044] Figures 9A-9D are simplified schematic diagrams illustrating fluid flow through a microfluidic device that permits recovery of reaction products during operation.

[0045] Figure 10 illustrates an embodiment of a 3-primer amplification method for barcoding target nucleic acids prior to sequencing.

[0046] Figure 11 shows a photograph of the gel described in Example 1. The lanes are as follows: (2) molecular markers, (4) sample amplified with 454 tails; (5)

sample (NTC) amplified with 454 tails; (7) sample amplified with A5 primer pair; (8) sample (NTC) amplified with A5 primer pair; (10) sample amplified with 3 primers; and (11) sample (NTC) amplified with 3 primers.

[0047] Figure 12 shows gel-view electropherograms obtained from 4 Agilent 1K Biolanalyzer chips for each of the individual samples run on the Access Array IFC (Integrated Fluidic Circuit) in Example 4. Each column in the figure shows the size distribution of DNA products in each sample. All samples produce similar distributions of products

[0048] Figure 13A-13B shows results from Example 4. A) Predicted sizes of all PCR products for this set of target specific primers. B) Electropherogram of one of the sample pools obtained from the Access Array IFC. Distribution of product size within a single product pool. All products fall within the predicted size range shown in (B).

[0049] Figure 14A-14C shows results from Example 4. A) Number of sequences counted per barcode on the 454 sequence run. Upper horizontal line represents 2x average number of counts per barcode. Lower horizontal line represents 50% of average number of counts per barcode. B) Number of sequences counted per amplicon. Each point on the plot represents the number of times the sequence for an individual chamber on the Access Array IFC were measured on the sequencer. Triangular points represent PCR reactions with greater than 2x the average representation. Dark grey points represent PCR reactions with less than 0.5x the average representation. C) Frequency distribution of amplicon representation. The dark grey line represents the number of amplicons present at a given representation. The light grey line represents the number of reads that would be measured at a given coverage (e.g. 98% at 20x coverage). Percentage of amplicons within 2-fold of average: 95.8%; percentage of amplicons within 5-fold of average: 99.7%.

[0050] Figure 15A-15B shows an example of a multi-primer reaction set-up using 4 outer primers with different combinations of primer binding site and nucleotide tags. (Example 5.) A) Two forward barcode primers (454B-BC-Tag8, 454A-BC-Tag8 and two reverse barcode primers (454A-BC-Tag5, 454B-BC-Tag8) are combined with one inner primer pair (Tag8-TSF and Tag5-TSR). B) The two major PCR products formed from this PCR reaction. PCR products containing 454-A

Tag8 and 454A-Tag5 at each end or 454B-Tag8 and 454B-Tag5 at each end do not produce significant PCR products due to PCR suppression

[0051] Figure 16A-16B shows the representation of each of the primer sequences in each of the samples for each of the amplicons in Figure 15B. The number of sequences counted per amplicon were normalized to the average number of counts per amplicon within a sample. The normalized counts for an individual amplicon were summed between the A and B emulsions (A) for Tag5 amplicons in Emulsion A plus Tag 8 amplicons in Emulsion B and (B) for Tag5 amplicons in Emulsion B plus Tag 8 amplicons in Emulsion A. The middle dark grey line represents the average representation of each amplicon. The upper light grey line represents 2x average coverage. The lower light grey line represents 50% of average representation.

[0052] Figure 17 shows the results from Example 6: Successful amplication of a PCR product using the 4-primer strategy designed for use on the Illumina GA II sequencer. The barcode primers listed in Table 14 are labelled as Outer Short.

[0053] Figure 18 shows results from Example 8: PCR reactions of three pools of 10 sets of PCR primers (A, B, C) when the PCR reactions were run for template-specific primers only and in 4-primer mode. The presence of higher molecular weight products in the 4-primer strategy demonstrates successful 4-primer assembly.

[0054] Figure 19 shows results from Example 8: Changing the ratio of inner and outer primers impacts yield in multiplex 4-primer PCR using inner and outer primers.

DETAILED DESCRIPTION

[0055] In certain embodiments, the present invention provides amplification methods in which nucleotide tag(s) and a barcode nucleotide sequence are added to target nucleotide sequences. The added sequences can then serve as primer and/or probe-binding sites. The barcode nucleotide sequence can encode information, such as, e.g., sample origin, about the target nucleotide sequence to which it is attached. Tagging and/or barcoding target nucleotide sequences can increase the number of samples that can be analyzed for one or multiple targets in a single assay, while

minimizing increases in assay cost. The methods are particularly well-suited for increasing the efficiency of assays performed on microfluidic devices.

[0056] In particular embodiments, the methods are used to prepare nucleic acids for DNA sequencing by, e.g., adding binding sites for DNA sequencing primers, optionally followed by sample calibration for DNA sequencing. In specific, illustrative embodiments, the method can be employed to add binding sites for DNA sequencing primers in a microfluidic device that permits recovery of reaction products. In illustrative devices of this type, dilation pumping can utilized to remove substantially all of the reaction products from the microfluidic device, providing uniformity between the various reaction product pools. Thus, it is possible to produce pools of barcoded reaction products that are uniform with respect to volume and copy number. In various embodiments, the volume and/or copy number uniformity is such that the variability, with respect to volume and/or copy number, of each pool recovered from the device is less than about 100 percent, less than about 90 percent, less than about 80 percent, less than about 70 percent, less than about 60 percent, less than about 50 percent, less than about 40 percent, less than about 30 percent, less than about 20 percent, less than about 17 percent, or less than about 15, 12, 10, 9, 8, 7, 6, 5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, or 0.5 percent. Those of skill in the art appreciate that the volume and/or copy number variability may fall within any range bounded by any of these values (e.g., about 2 to about 7 percent). In an illustrative embodiment, the volume samples recovered from a microfluidic device vary by no more than approximately 10%. Standard pipetting error is on the order of between 5 and 10%. Thus, the observed variability in volumes is largely attributable to pipetting error. Utilizing the systems and methods described herein, the time and labor required to prepare sequencing libraries is reduced in comparison with conventional techniques.

[0057] It is understood that the invention is not limited to the particular methodology, protocols, and reagents, etc., described herein, as these can be varied by the skilled artisan. It is also understood that the terminology used herein is used for the purpose of describing particular illustrative embodiments only, and is not intended to limit the scope of the invention. It also noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a

cell" is a reference to one or more cells and equivalents thereof known to those skilled in the art.

[0058] The embodiments of the invention and the various features and advantageous details thereof are explained more fully with reference to the non-limiting embodiments and examples that are described and/or illustrated in the accompanying drawings and detailed in the following description. It should be noted that the features illustrated in the drawings are not necessarily drawn to scale, and features of one embodiment may be employed with other embodiments as the skilled artisan would recognize, even if not explicitly stated herein. Descriptions of well-known components and processing techniques may be omitted so as to not unnecessarily obscure the embodiments of the invention.

Definitions

[0059] Terms used in the claims and specification are defined as set forth below unless otherwise specified. These terms are defined specifically for clarity, but all of the definitions are consistent with how a skilled artisan would understand these terms.

[0060] The term "adjacent," when used herein to refer two nucleotide sequences in a nucleic acid, can refer to nucleotide sequences separated by 0 to about 20 nucleotides, more specifically, in a range of about 1 to about 10 nucleotides, or sequences that directly abut one another.

[0061] The term "nucleic acid" refers to a nucleotide polymer, and unless otherwise limited, includes known analogs of natural nucleotides that can function in a similar manner (e.g., hybridize) to naturally occurring nucleotides.

[0062] The term nucleic acid includes any form of DNA or RNA, including, for example, genomic DNA; complementary DNA (cDNA), which is a DNA representation of mRNA, usually obtained by reverse transcription of messenger RNA (mRNA) or by amplification; DNA molecules produced synthetically or by amplification; and mRNA.

[0063] The term nucleic acid encompasses double- or triple-stranded nucleic acids, as well as single-stranded molecules. In double- or triple-stranded nucleic

acids, the nucleic acid strands need not be coextensive (i.e, a double-stranded nucleic acid need not be double-stranded along the entire length of both strands).

[0064] The term nucleic acid also encompasses any chemical modification thereof, such as by methylation and/or by capping. Nucleic acid modifications can include addition of chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and functionality to the individual nucleic acid bases or to the nucleic acid as a whole. Such modifications may include base modifications such as 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitutions of 5-bromo-uracil, backbone modifications, unusual base pairing combinations such as the isobases isocytidine and isoguanidine, and the like.

[0065] More particularly, in certain embodiments, nucleic acids, can include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and any other type of nucleic acid that is an N- or C-glycoside of a purine or pyrimidine base, as well as other polymers containing nonnucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids (PNAs)) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, Oregon, as Neugene) polymers, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. The term nucleic acid also encompasses linked nucleic acids (LNAs), which are described in U.S. Patent Nos. 6,794,499, 6,670,461, 6,262,490, and 6,770,748, which are incorporated herein by reference in their entirety for their disclosure of LNAs.

[0066] The nucleic acid(s) can be derived from a completely chemical synthesis process, such as a solid phase-mediated chemical synthesis, from a biological source, such as through isolation from any species that produces nucleic acid, or from processes that involve the manipulation of nucleic acids by molecular biology tools, such as DNA replication, PCR amplification, reverse transcription, or from a combination of those processes.

[0067] The term "target nucleic acids" is used herein to refer to particular nucleic acids to be detected in the methods of the invention.

[0068] As used herein the term "target nucleotide sequence" refers to a molecule that includes the nucleotide sequence of a target nucleic acid, such as, for example, the amplification product obtained by amplifying a target nucleic acid or the cDNA produced upon reverse transcription of an RNA target nucleic acid.

[0069] As used herein, the term "complementary" refers to the capacity for precise pairing between two nucleotides. I.e., if a nucleotide at a given position of a nucleic acid is capable of hydrogen bonding with a nucleotide of another nucleic acid, then the two nucleic acids are considered to be complementary to one another at that position. Complementarity between two single-stranded nucleic acid molecules may be "partial," in which only some of the nucleotides bind, or it may be complete when total complementarity exists between the single-stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands.

[0070] "Specific hybridization" refers to the binding of a nucleic acid to a target nucleotide sequence in the absence of substantial binding to other nucleotide sequences present in the hybridization mixture under defined stringency conditions. Those of skill in the art recognize that relaxing the stringency of the hybridization conditions allows sequence mismatches to be tolerated.

[0071] In particular embodiments, hybridizations are carried out under stringent hybridization conditions. The phrase "stringent hybridization conditions" generally refers to a temperature in a range from about 5°C to about 20°C or 25°C below than the melting temperature (T_m) for a specific sequence at a defined ionic strength and pH. As used herein, the T_m is the temperature at which a population of double-stranded nucleic acid molecules becomes half-dissociated into single strands. Methods for calculating the T_m of nucleic acids are well known in the art (see, e.g., Berger and Kimmel (1987) METHODS IN ENZYMOLOGY, VOL.152: GUIDE TO MOLECULAR CLONING TECHNIQUES, San Diego: Academic Press, Inc. and Sambrook et al. (1989) MOLECULAR CLONING: A LABORATORY MANUAL, 2ND ED., VOLS. 1-3, Cold Spring Harbor Laboratory), both incorporated herein by reference). As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\%\ G+C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (see, e.g., Anderson and Young, Quantitative Filter Hybridization in NUCLEIC ACID HYBRIDIZATION (1985)). The melting

temperature of a hybrid (and thus the conditions for stringent hybridization) is affected by various factors such as the length and nature (DNA, RNA, base composition) of the primer or probe and nature of the target nucleic acid (DNA, RNA, base composition, present in solution or immobilized, and the like), as well as the concentration of salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol). The effects of these factors are well known and are discussed in standard references in the art. Illustrative stringent conditions suitable for achieving specific hybridization of most sequences are: a temperature of at least about 60°C and a salt concentration of about 0.2 molar at pH7.

[0072] The term "oligonucleotide" is used to refer to a nucleic acid that is relatively short, generally shorter than 200 nucleotides, more particularly, shorter than 100 nucleotides, most particularly, shorter than 50 nucleotides. Typically, oligonucleotides are single-stranded DNA molecules.

[0073] The term "primer" refers to an oligonucleotide that is capable of hybridizing (also termed "annealing") with a nucleic acid and serving as an initiation site for nucleotide (RNA or DNA) polymerization under appropriate conditions (i.e., in the presence of four different nucleoside triphosphates and an agent for polymerization, such as DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer, but primers are typically at least 7 nucleotides long and, more typically range from 10 to 30 nucleotides, or even more typically from 15 to 30 nucleotides, in length. Other primers can be somewhat longer, e.g., 30 to 50 nucleotides long. In this context, "primer length" refers to the portion of an oligonucleotide or nucleic acid that hybridizes to a complementary "target" sequence and primes nucleotide synthesis. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. The term "primer site" or "primer binding site" refers to the segment of the target nucleic acid to which a primer hybridizes.

[0074] A primer is said to anneal to another nucleic acid if the primer, or a portion thereof, hybridizes to a nucleotide sequence within the nucleic acid. The statement that a primer hybridizes to a particular nucleotide sequence is not intended

to imply that the primer hybridizes either completely or exclusively to that nucleotide sequence. For example, in certain embodiments, amplification primers used herein are said to "anneal to a nucleotide tag." This description encompasses primers that anneal wholly to the nucleotide tag, as well as primers that anneal partially to the nucleotide tag and partially to an adjacent nucleotide sequence, e.g., a target nucleotide sequence. Such hybrid primers can increase the specificity of the amplification reaction.

[0075] As used herein, the selection of primers "so as to avoid substantial annealing to the target nucleic acids" means that primers are selected so that the majority of the amplicons detected after amplification are "full-length" in the sense that they result from priming at the expected sites at each end of the target nucleic acid, as opposed to amplicons resulting from priming within the target nucleic acid, which produces shorter-than-expected amplicons. In various embodiments, primers are selected to that at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% are full-length.

[0076] The term "primer pair" refers to a set of primers including a 5' "upstream primer" or "forward primer" that hybridizes with the complement of the 5' end of the DNA sequence to be amplified and a 3' "downstream primer" or "reverse primer" that hybridizes with the 3' end of the sequence to be amplified. As will be recognized by those of skill in the art, the terms "upstream" and "downstream" or "forward" and "reverse" are not intended to be limiting, but rather provide illustrative orientation in particular embodiments.

[0077] A "probe" is a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, generally through complementary base pairing, usually through hydrogen bond formation, thus forming a duplex structure. The probe binds or hybridizes to a "probe binding site." The probe can be labeled with a detectable label to permit facile detection of the probe, particularly once the probe has hybridized to its complementary target. Alternatively, however, the probe may be unlabeled, but may be detectable by specific binding with a ligand that is labeled, either directly or indirectly. Probes can vary significantly in size. Generally, probes are at least 7 to 15 nucleotides in length. Other probes are at least 20, 30, or 40 nucleotides long. Still other probes are

somewhat longer, being at least 50, 60, 70, 80, or 90 nucleotides long. Yet other probes are longer still, and are at least 100, 150, 200 or more nucleotides long. Probes can also be of any length that is within any range bounded by any of the above values (e.g., 15-20 nucleotides in length).

[0078] The primer or probe can be perfectly complementary to the target nucleic acid sequence or can be less than perfectly complementary. In certain embodiments, the primer has at least 65% identity to the complement of the target nucleic acid sequence over a sequence of at least 7 nucleotides, more typically over a sequence in the range of 10-30 nucleotides, and often over a sequence of at least 14-25 nucleotides, and more often has at least 75% identity, at least 85% identity, at least 90% identity, or at least 95%, 96%, 97%. 98%, or 99% identity. It will be understood that certain bases (e.g., the 3' base of a primer) are generally desirably perfectly complementary to corresponding bases of the target nucleic acid sequence. Primer and probes typically anneal to the target sequence under stringent hybridization conditions.

[0079] The term "nucleotide tag" is used herein to refer to a predetermined nucleotide sequence that is added to a target nucleotide sequence. The nucleotide tag can encode an item of information about the target nucleotide sequence, such the identity of the target nucleotide sequence or the identity of the sample from which the target nucleotide sequence was derived. In certain embodiments, such information may be encoded in one or more nucleotide tags, e.g., a combination of two nucleotide tags, one on either end of a target nucleotide sequence, can encode the identity of the target nucleotide sequence.

[0080] As used herein the term "barcode primer" refers to a primer that includes a specific barcode nucleotide sequence that encodes information about the amplicon produced when the barcode primer is employed in an amplification reaction. For example, a different barcode primer can be employed to amplify one or more target sequences from each of a number of different samples, such that the barcode nucleotide sequence indicates the sample origin of the resulting amplicons.

[0081] As used herein, the term "encoding reaction" refers to reaction in which at least one nucleotide tag is added to a target nucleotide sequence. Nucleotide tags can be added, for example, by an "encoding PCR" in which the at least one

primer comprises a target-specific portion and a nucleotide tag located on the 5' end of the target-specific portion, and a second primer that comprises only a target-specific portion or a target-specific portion and a nucleotide tag located on the 5' end of the target-specific portion. For illustrative examples of PCR protocols applicable to encoding PCR, see pending WO Application US03/37808 as well as U.S. Pat. No.6,605,451. Nucleotide tags can also be added by an "encoding ligation" reaction that can comprise a ligation reaction in which at least one primer comprises a target-specific portion and nucleotide tag located on the 5' end of the target-specific portion, and a second primer that comprises a target-specific portion only or a target-specific portion and a nucleotide tag located on the 5' end of the target specific portion. Illustrative encoding ligation reactions are described, for example, in U.S. Patent Publication No. 2005/0260640, which is hereby incorporated by reference in its entirety, and in particular for ligation reactions.

[0082] As used herein an "encoding reaction" produces a "tagged target nucleotide sequence," which includes a nucleotide tag linked to a target nucleotide sequence.

[0083] As used herein with reference to a portion of a primer, the term "target-specific" nucleotide sequence refers to a sequence that can specifically anneal to a target nucleic acid or a target nucleotide sequence under suitable annealing conditions.

[0084] As used herein with reference to a portion of a primer, the term "nucleotide tag-specific nucleotide sequence" refers to a sequence that can specifically anneal to a nucleotide tag under suitable annealing conditions.

[0085] Amplification according to the present teachings encompasses any means by which at least a part of at least one target nucleic acid is reproduced, typically in a template-dependent manner, including without limitation, a broad range of techniques for amplifying nucleic acid sequences, either linearly or exponentially. Illustrative means for performing an amplifying step include ligase chain reaction (LCR), ligase detection reaction (LDR), ligation followed by Q-replicase amplification, PCR, primer extension, strand displacement amplification (SDA), hyperbranched strand displacement amplification, multiple displacement amplification (MDA), nucleic acid strand-based amplification (NASBA), two-step

multiplexed amplifications, rolling circle amplification (RCA), and the like, including multiplex versions and combinations thereof, for example but not limited to, OLA/PCR, PCR/OLA, LDR/PCR, PCR/PCR/LDR, PCR/LDR, LCR/PCR, PCR/LCR (also known as combined chain reaction--CCR), and the like. Descriptions of such techniques can be found in, among other sources, Ausbel et al.; PCR Primer: A Laboratory Manual, Diffenbach, Ed., Cold Spring Harbor Press (1995); The Electronic Protocol Book, Chang Bioscience (2002); Msuih et al., J. Clin. Micro. 34:501-07 (1996); The Nucleic Acid Protocols Handbook, R. Rapley, ed., Humana Press, Totowa, N.J. (2002); Abramson et al., Curr Opin Biotechnol. 1993 Feb.;4(1):41-7, U.S. Pat. No. 6,027,998; U.S. Pat. No. 6,605,451, Barany et al., PCT Publication No. WO 97/31256; Wenz et al., PCT Publication No. WO 01/92579; Day et al., Genomics, 29(1): 152-162 (1995), Ehrlich et al., Science 252:1643-50 (1991); Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press (1990); Favis et al., Nature Biotechnology 18:561-64 (2000); and Rabenau et al., Infection 28:97-102 (2000); Belgrader, Barany, and Lubin, Development of a Multiplex Ligation Detection Reaction DNA Typing Assay, Sixth International Symposium on Human Identification, 1995 (available on the world wide web at: promega.com/geneticidproc/ussymp6proc/blegrad.html-); LCR Kit Instruction Manual, Cat. #200520, Rev. #050002, Stratagene, 2002; Barany, Proc. Natl. Acad. Sci. USA 88:188-93 (1991); Bi and Sambrook, Nucl. Acids Res. 25:2924-2951 (1997); Zirvi et al., Nucl. Acid Res. 27:e40i-viii (1999); Dean et al., Proc Natl Acad Sci USA 99:5261-66 (2002); Barany and Gelfand, Gene 109:1-11 (1991); Walker et al., Nucl. Acid Res. 20:1691-96 (1992); Polstra et al., BMC Inf. Dis. 2:18- (2002); Lage et al., Genome Res. 2003 Feb.;13(2):294-307, and Landegren et al., Science 241:1077-80 (1988), Demidov, V., Expert Rev Mol Diagn. 2002 Nov.;2(6):542-8., Cook et al., J Microbiol Methods. 2003 May;53(2):165-74, Schweitzer et al., Curr Opin Biotechnol. 2001 Feb.;12(1):21-7, U.S. Pat. No. 5,830,711, U.S. Pat. No. 6,027,889, U.S. Pat. No. 5,686,243, PCT Publication No. WO0056927A3, and PCT Publication No. WO9803673A1.

[0086] In some embodiments, amplification comprises at least one cycle of the sequential procedures of: annealing at least one primer with complementary or substantially complementary sequences in at least one target nucleic acid; synthesizing at least one strand of nucleotides in a template-dependent manner using a

polymerase; and denaturing the newly-formed nucleic acid duplex to separate the strands. The cycle may or may not be repeated. Amplification can comprise thermocycling or can be performed isothermally.

[0087] The term "qPCR" is used herein to refer to quantitative real-time polymerase chain reaction (PCR), which is also known as "real-time PCR" or "kinetic polymerase chain reaction."

[0088] A "reagent" refers broadly to any agent used in a reaction, other than the analyte (e.g., nucleic acid being analyzed). Illustrative reagents for a nucleic acid amplification reaction include, but are not limited to, buffer, metal ions, polymerase, reverse transcriptase, primers, template nucleic acid, nucleotides, labels, dyes, nucleases, and the like. Reagents for enzyme reactions include, for example, substrates, cofactors, buffer, metal ions, inhibitors, and activators.

[0089] The term "universal detection probe" is used herein to refer to any probe that identifies the presence of an amplification product, regardless of the identity of the target nucleotide sequence present in the product.

[0090] The term "universal qPCR probe" is used herein to refer to any such probe that identifies the presence of an amplification product during qPCR. In particular embodiments, nucleotide tags according to the invention can comprise a nucleotide sequence to which a detection probe, such as a universal qPCR probe binds. Where a tag is added to both ends of a target nucleotide sequence, each tag can, if desired, include a sequence recognized by a detection probe. The combination of such sequences can encode information about the identity or sample source of the tagged target nucleotide sequence. In other embodiments, one or more amplification primers can comprise a nucleotide sequence to which a detection probe, such as a universal qPCR probe binds. In this manner, one, two, or more probe binding sites can be added to an amplification product during the amplification step of the methods of the invention. Those of skill in the art recognize that the possibility of introducing multiple probe binding sites during preamplification (if carried out) and amplification facilitates multiplex detection, wherein two or more different amplification products can be detected in a given amplification mixture or aliquot thereof.

[0091] The term "universal detection probe" is also intended to encompass primers labeled with a detectable label (e.g., a fluorescent label), as well as non-

sequence-specific probes, such as DNA binding dyes, including double-stranded DNA (dsDNA) dyes, such as SYBR Green.

[0092] The term "target-specific qPCR probe" is used herein to refer to a qPCR probe that identifies the presence of an amplification product during qPCR, based on hybridization of the qPCR probe to a target nucleotide sequence present in the product.

[0093] "Hydrolysis probes" are generally described in U.S. Patent No. 5,210,015, which is incorporated herein by reference in its entirety for its description of hydrolysis probes. Hydrolysis probes take advantage of the 5'-nuclease activity present in the thermostable Taq polymerase enzyme typically used in the PCR reaction (TaqMan® probe technology, Applied Biosystems, Foster City CA). The hydrolysis probe is labeled with a fluorescent detector dye such as fluorescin, and an acceptor dye or quencher. In general, the fluorescent dye is covalently attached to the 5' end of the probe and the quencher is attached to the 3' end of the probe, and when the probe is intact, the fluorescence of the detector dye is quenched by fluorescence resonance energy transfer (FRET). The probe anneals downstream of one of the primers that defines one end of the target nucleic acid in a PCR reaction. Using the polymerase activity of the Taq enzyme, amplification of the target nucleic acid is directed by one primer that is upstream of the probe and a second primer that is downstream of the probe but anneals to the opposite strand of the target nucleic acid. As the upstream primer is extended, the Taq polymerase reaches the region where the labeled probe is annealed, recognizes the probe-template hybrid as a substrate, and hydrolyzes phosphodiester bonds of the probe. The hydrolysis reaction irrevocably releases the quenching effect of the quencher dye on the reporter dye, thus resulting in increasing detector fluorescence with each successive PCR cycle. In particular, hydrolysis probes suitable for use in the invention can be capable of detecting 8-mer or 9-mer motifs that are common in the human and other genomes and/or transcriptomes and can have a high T_m of about 70°C enabled by the use of linked nucleic acid (LNA) analogs.

[0094] The term "label," as used herein, refers to any atom or molecule that can be used to provide a detectable and/or quantifiable signal. In particular, the label can be attached, directly or indirectly, to a nucleic acid or protein. Suitable labels that can be attached to probes include, but are not limited to, radioisotopes, fluorophores,

chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates.

[0095] The term "dye," as used herein, generally refers to any organic or inorganic molecule that absorbs electromagnetic radiation at a wavelength greater than or equal 340 nm.

[0096] The term "fluorescent dye," as used herein, generally refers to any dye that emits electromagnetic radiation of longer wavelength by a fluorescent mechanism upon irradiation by a source of electromagnetic radiation, such as a lamp, a photodiode, or a laser.

[0097] The term "elastomer" has the general meaning used in the art. Thus, for example, Allcock et al. (Contemporary Polymer Chemistry, 2nd Ed.) describes elastomers in general as polymers existing at a temperature between their glass transition temperature and liquefaction temperature. Elastomeric materials exhibit elastic properties because the polymer chains readily undergo torsional motion to permit uncoiling of the backbone chains in response to a force, with the backbone chains recoiling to assume the prior shape in the absence of the force. In general, elastomers deform when force is applied, but then return to their original shape when the force is removed.

[0098] A "polymorphic marker" or "polymorphic site" is a locus at which nucleotide sequence divergence occurs. Illustrative markers have at least two alleles, each occurring at frequency of greater than 1%, and more typically greater than 10% or 20% of a selected population. A polymorphic site may be as small as one base pair. Polymorphic markers include restriction fragment length polymorphism (RFLPs), variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, deletions, and insertion elements such as Alu. The first identified allelic form is arbitrarily designated as the reference form and other allelic forms are designated as alternative or variant alleles. The allelic form occurring most frequently in a selected population is sometimes referred to as the wildtype form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms.

[0099] A "single nucleotide polymorphism" (SNP) occurs at a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (e.g., sequences that vary in less than 1/100 or 1/1000 members of the populations). A SNP usually arises due to substitution of one nucleotide for another at the polymorphic site. A transition is the replacement of one purine by another purine or one pyrimidine by another pyrimidine. A transversion is the replacement of a purine by a pyrimidine or vice versa. SNPs can also arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele.

Amplification Methods

In General

[0100] In particular embodiments, the invention provides an amplification method for introducing a plurality (e.g., at least three) of selected nucleotide sequences into one or more target nucleic acid(s). The method entails amplifying a plurality of target nucleic acids, typically, in a plurality of samples. In illustrative embodiments, the same set of target nucleic acids can be amplified in each of two or more different samples. The samples can differ from one another in any way, e.g., the samples can be from different tissues, subjects, environmental sources, etc. At least three primers can be used to amplify each target nucleic acid, namely: forward and reverse amplification primers, each primer including a target-specific portion and one or both primers including a nucleotide tag. The target-specific portions can specifically anneal to a target under suitable annealing conditions. The nucleotide tag for the forward primer can have a sequence that is the same as, or different from, the nucleotide tag for the reverse primer. Generally, the nucleotide tags are 5' of the target-specific portions. The third primer is a barcode primer comprising a barcode nucleotide sequence and a first and/or second nucleotide tag-specific portion. The barcode nucleotide sequence is a sequence selected to encode information about the amplicon produced when the barcode primer is employed in an amplification reaction. The tag-specific portion can specifically anneal to the one or both nucleotide tags in the forward and reverse primers. The barcode primer is generally 5' of the tag-specific portion.

[0101] The barcode primer is typically present in the amplification mixture in excess of the forward and/or reverse primer(s). More specifically, if the barcode primer anneals to the nucleotide tag in the forward primer, the barcode primer is generally present in excess of the forward primer. If the barcode primer anneals to the nucleotide tag in the reverse primer, the barcode primer is generally present in excess of the reverse primer. In each instance the third primer in the amplification mixture, i.e., the reverse primer or the forward primer, respectively, can be present, in illustrative embodiments, at a concentration approximately similar to that of the barcode primer. Generally the barcode primer is present in substantial excess. For example, the concentration of the barcode primer in the amplification mixtures can be at least 2-fold, at least 4-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 30-fold, at least 35-fold, at least 40-fold, at least 45-fold, at least 50-fold, at least 100-fold, at least 500-fold, at least 10^3-fold, at least 5 $\times 10^3$-fold, at least 10^4-fold, at least 5 $\times 10^4$-fold, at least 10^5-fold, at least 5 $\times 10^5$-fold, at least 10^6-fold, or higher, relative to the concentration of the forward and/or reverse primer(s). In addition, the concentration excess of the barcode primer can fall within any range having any of the above values as endpoints (e.g., 2-fold to 10^5-fold). In illustrative embodiments, where the barcode primer has a tag-specific portion that is specific for the nucleotide tag on the forward primer, the forward primer can be present in picomolar to nanomolar concentrations, e.g., about 5 pM to 500 nM, about 5 pM to 100 nM, about 5 pM to 50 nM, about 5 pM to 10 nM, about 5 pM to 5 nM, about 10 pM to 1 nM, about 50 pM to about 500 pM, about 100 pM or any other range having any of these values as endpoints (e.g., 10 pM to 50 pM). Suitable, illustrative concentrations of barcode primer that could be used on combination with any of these concentrations of forward primer include about 10 nM to about 10 μM, about 25 nM to about 7.5 μM, about 50 nM to about 5 μM, about 75 nM to about 2.5 μM, about 100 nM to about 1 μM, about 250 nM to about 750 nM, about 500 nM or any other range having any of these values as endpoints (e.g., 100 nM to 500 nM). In amplification reactions using such concentrations of forward and barcode primers, the reverse primer have a concentration on the same order as the barcode primer (e.g. within about 10-fold, within about 5-fold, or equal).

[0102] Each amplification mixture can be subjected to amplification to produce target amplicons comprising tagged target nucleotide sequences, each

comprising first and second nucleotide tags flanking the target nucleotide sequence, and at least one barcode nucleotide sequence at the 5' or 3' end of the target amplicon (relative to one strand of the target amplicon). In certain embodiments, the first and second nucleotide tags and/or the barcode nucleotide sequence are selected so as to avoid substantial annealing to the target nucleic acids. In such embodiments, the tagged target nucleotide sequences can include molecules having the following elements: 5'-(barcode nucleotide sequence)-(first nucleotide tag from the forward primer)-(target nucleotide sequence)-(second nucleotide tag sequence from the reverse primer)-3' or 5'-(first nucleotide tag from the forward primer)-(target nucleotide sequence)-(second nucleotide tag sequence from the reverse primer) -(barcode nucleotide sequence)-3'.

[0103] In illustrative embodiments, the barcode nucleotide sequence identifies a particular sample. Thus, for example, a set of T target nucleic acids can be amplified in each of S samples, where S and T are integers, typically greater than one. In such embodiments, amplification can be performed separately for each sample, wherein the same set of forward and reverse primers is used for each sample and the set of forward and reverse primers has at least one nucleotide tag that is common to all primers in the set. A different barcode primer can be used for each sample, wherein the bar code primers have different barcode nucleotide sequences, but the same tag-specific portion that can anneal to the common nucleotide tag. This embodiment has the advantage of reducing the number of different primers that would need to be synthesized to encode sample origin in amplicons produced for a plurality of target sequences. Alternatively, different sets of forward and reverse primers can be employed for each sample, wherein each set has a nucleotide tag that is different from the primers in the other set, and different barcode primers are used for each sample, wherein the barcode primers have different barcode nucleotide sequences and different tag-specific portions. In either case, the amplification produces a set of T amplicons from each sample that bear sample-specific barcodes.

[0104] In embodiments, wherein the same set of forward and reverse primers is used for each sample, the forward and reverse primers for each target can be initially combined separately from the sample, and each barcode primer can be initially combined with its corresponding sample. Aliquots of the initially combined forward and reverse primers can then be added to aliquots of the initially combined

sample and barcode primer to produce S×T amplification mixtures. These amplification mixtures can be formed in any article that can be subjected to conditions suitable for amplification. For example, the amplification mixtures can be formed in, or distributed into, separate compartments of a microfluidic device prior to amplification. Suitable microfluidic devices include, in illustrative embodiments, matrix-type microfluidic devices, such as those described below.

[0105] Any amplification method can be employed to produce amplicons from the amplification mixtures. In illustrative embodiments, PCR is employed. The amplification is generally carried out for at least three cycles to introduce the first and second nucleotide tags and the barcode nucleotide sequence. In various embodiments, amplification is carried out for 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 cycles, or for any number of cycles falling within a range having any of these values as endpoints (e.g. 5-10 cycles). In particular embodiments, amplification is carried out for a sufficient number of cycles to normalize target amplicon copy number across targets and across samples (e.g., 15, 20, 25, 30, 35, 40, 45, or 50 cycles, or for any number of cycles falling within a range having any of these values as endpoints).

[0106] Particular embodiments of the above-described method provide substantially uniform amplification, yielding a plurality of target amplicons wherein the majority of amplicons are present at a level relatively close to the average copy number calculated for the plurality of target amplicons. Thus, in various embodiments, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, or at least 99 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

[0107] The invention also provides, in certain embodiments, a method for amplifying a plurality of target nucleotides in which barcoding is, optionally, omitted and the target nucleotide sequences are tagged after amplification. More specifically, the invention provides a method for amplifying a plurality of target nucleic acids, typically, in a plurality of samples, that entails preparing an amplification mixture for each target nucleic acid. Each amplification mixture includes a forward primer including a target-specific sequence and a reverse primer including a target-specific sequence. The amplification mixtures are subjected to amplification to produce a

plurality of target nucleotide sequences. The target nucleotide sequences are then tagged to produce a plurality of target amplicons, each including first and/or second nucleotide tags flanking the target nucleotide sequence. This method produces a plurality of target amplicons, wherein at least 50 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons. In various embodiments of this method at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, or at least 99 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

[0108] In various embodiments, the target nucleotide sequence amplified can be, e.g., 25 bases, 50 bases, 100 bases, 200 bases, 500 bases, or 750 bases. In certain embodiments of the above-described methods, a long-range amplification method, such as long-range PCR can be employed to produce amplicons from the amplification mixtures. Long-range PCR permits the amplification of target nucleotide sequences ranging from one or a few kilobases (kb) to over 50 kb. In various embodiments, the target nucleotide sequences that are amplified by long-range PCR are at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, or 50 kb in length. Target nucleotide sequences can also fall within any range having any of these values as endpoints (e.g., 25 bases to 100 bases or 5-15 kb). The use of long-range PCR in the above-described methods can, in some embodiments, yield a plurality of target amplicons wherein at least 50, at least 55, at least 60, at least 65, or at least 70 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

[0109] Long-range PCR is well known in the art. See, e.g., Cheng S, Fockler C, Barnes WM, Higuchi R (June 1994). "Effective amplification of long targets from cloned inserts and human genomic DNA". Proc. Natl. Acad. Sci. U.S.A. 91 (12): 5695–9. Enzymes, protocols, and kits for long-range PCR that are suitable for use in the methods described here are commercially available; examples include: SequalPrep™ Long PCR Kit (Invitrogen, USA), PfuUltra® II Fusion HS DNA

polymerase (Stratagene), Phusion® DNA polymerases, Phusion® Flash High Fidelity PCR Master Mix (Finnzymes).

[0110] In certain embodiments, the target amplicons can be recovered from the amplification mixtures. For example, a matrix-type microfluidic device that is adapted to permit recovery of the contents of each reaction chamber (see below) can be employed for the amplification to generate the target amplicons. In variations of these embodiments, the target amplicons can be subjected to further amplification and/or analysis. For example, one or more target amplicon(s) can be subjected to amplification using primers specific for the first and second nucleotide tags to produce a target amplicon lacking the barcode nucleotide sequence. In certain embodiments, the amount of target amplicons produced in the amplification mixtures can be quantified during amplication, e.g., by quantitative real-time PCR, or after.

[0111] In particular embodiments, the above-described amplification methods are employed to produce amplicons suitable for automated DNA sequencing. In particular, the ability of the methods to provide substantially uniform amplification, as described above, of target nucleotide sequences is helpful in preparing DNA sequencing libraries having good coverage. In the context of automated DNA sequencing, the term "coverage" refers to the number of times the sequence is measured upon sequencing. A DNA sequencing library that has substantially uniform coverage can yield sequence data where the coverage is also substantially uniform. Thus, in various embodiments, upon performing automated sequencing of a plurality of target amplicons prepared as described herein, the sequences of at least 50 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicon sequences and less than 2-fold the average number of copies of target amplicon sequences. In various embodiments of this method at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, or at least 99 percent of the target amplicon sequences are present at greater than 50 percent of the average number of copies of target amplicon sequences and less than 2-fold the average number of copies of target amplicon sequences.

Preparation of Nucleic Acids for DNA Sequencing

[0112] Many current DNA sequencing techniques rely on "sequencing by synthesis." These techniques entail library creation, massively parallel PCR amplification of library molecules, and sequencing. Library creation starts with conversion of sample nucleic acids to appropriately sized fragments, ligation of adaptor sequences onto the ends of the fragments, and selection for molecules properly appended with adaptors. The presence of the adaptor sequences on the ends of the library molecules enables amplification of random-sequence inserts. The above-described methods for tagging nucleotide sequences can be substituted for ligation, to introduce adaptor sequences, as described in greater detail below.

[0113] In particular embodiments, the number of library DNA molecules produced in the massively parallel PCR step is low enough that the chance of two molecules associating with the same substrate, e.g. the same bead (in 454 DNA sequencing) or the same surface patch (in Solexa DNA sequencing) is low, but high enough so that the yield of amplified sequences is sufficient to provide a high throughput. As discussed further below, after suitable adaptor sequences are introduced, digital PCR can be employed to calibrate the number of library DNA molecules prior to sequencing by synthesis.

Addition of DNA Sequencing Primers to Nucleic Acids

[0114] The DNA to be sequenced can be any type of DNA. In particular embodiments, the DNA is genomic DNA from an organism. In variations of such embodiments, total genomic DNA obtained from a sample taken from an organism or from a DNA library is prepared for sequencing.

[0115] As described above, at least three primers are employed to prepare the DNA for sequencing: forward, reverse, and barcode primers. However, one or more of the forward primer, reverse primer, and barcode primer includes at least one additional primer binding site. In specific embodiments, the barcode primer includes at least a first additional primer binding site upstream of the barcode nucleotide sequence, which is upstream of the first nucleotide tag-specific portion. In certain embodiments, two of the forward primer, reverse primer, and barcode primer include at least one additional primer binding site (i.e, such that the amplicon produced upon amplification includes the nucleotide tag sequences, the barcode nucleotide sequence,

and the two additional binding sites). For example, if the barcode primer includes a first additional primer binding site upstream of the barcode nucleotide sequence, in specific embodiments, the reverse primer can include at least a second additional primer binding site downstream of the second nucleotide tag. Amplification then yields a molecule having the following elements: 5'-(first additional primer binding site)-(barcode nucleotide sequence)-(first nucleotide tag from the forward primer)-(target nucleotide sequence)-(second nucleotide tag from the reverse primer)-(second additional primer binding site)-3'. In specific embodiments, the first and second additional primer binding sites are capable of being bound by DNA sequencing primers, to facilitate sequencing of the entire amplicon, including the barcode, which can, as discussed above, indicate sample origin.

[0116] In some embodiments, more than three primers can be employed to add desired elements to a target nucleotide sequence. For example, four primers can be employed to produce molecules having the same five elements discussed above, plus an optional additional barcode e.g., 5'-(first additional primer binding site)-(barcode nucleotide sequence)-(first nucleotide tag from the forward primer)-(target nucleotide sequence)-(second nucleotide tag from the reverse primer)-(additional barcode nucleotide sequence)-(second additional primer binding site)-3'. In an illustrative four-primer embodiment, the forward primer includes a target-specific portion and first nucleotide tag, and the reverse primer includes a target-specific portion and a second nucleotide tag. Together, these two primers constitute the "inner primers." The remaining two primers are the "outer primers," which anneal to the first and second nucleotide tags present in the inner primers. One outer primer is the barcode primer, which can contain at least a first additional primer binding site upstream of the barcode nucleotide sequence, which is upstream of the first nucleotide tag-specific portion (i.e., the same barcode primer discussed in the previous paragraph). The second outer primer can include a second tag-specific portion, an additional barcode nucleotide sequence and, downstream of this, a second additional primer binding site.

[0117] Amplification to incorporate elements from more than three primers can be carried out in one or multiple amplification reactions. For example, a four-primer amplification can be carried out in one amplification reaction, in which all four primers are present. Alternatively, a four-primer amplification can be carried out,

e.g., in two amplification reactions: one to incorporate the inner primers and a separate amplification reaction to incorporate the outer primers. Where all four primers are present in one amplification reaction, the outer primers are generally present in the reaction mixture in excess. The relative concentration values give above for the barcode primer relative to the forward and/or reverse primers also apply to the relative concentrations of the outer primers relative to inner primers in a one-step, four-primer amplification reaction.

[0118] In an illustrative embodiment of the four-primer amplification reaction, each of the outer primers contains a unique barcode. For example, one barcode primer would be constructed of the elements 5'-(first additional primer binding site)-(first barcode nucleotide sequence)-(first nucleotide tag)-3', and the second barcode primer would be constructed of the elements 5'-(second additional primer binding site)-(second barcode nucleotide sequence)-(second nucleotide tag)-3'. In this embodiment, a number (J) of first barcode primers can be combined with a number (K) of second barcode primers to create JxK unique amplification products.

[0119] In a further illustrative embodiment of the invention, more than four primers can be combined in a single reaction to append different combinations of additional primer binding sites, barcode sequences, and nucleotide tags. For example, outer barcode primers containing the following elements: 5'-(first additional primer binding site)-(first barcode nucleotide sequence)-(first nucleotide tag)-3', 5'-(first additional primer binding site)-(first barcode nucleotide sequence)-(second nucleotide tag)-3', 5'-(second additional primer binding site)-(first barcode nucleotide sequence)-(first nucleotide tag)-3', 5'-(second additional primer binding site)-(first barcode nucleotide sequence)-(second nucleotide tag)-3', can be combined with inner target-specific primers as described above to produce amplification product pools containing all combinations of the barcode primers with the desired amplicon sequence.

[0120] In other illustrative embodiments of the invention, outer barcode primers in any of the combinations described above, or other combinations that would be obvious to one of skill in the art, can be combined with more than one pair of target primer sequences bearing the same first and second nucleotide tag sequences. For example, inner primers containing up to ten different target-specific forward primer sequences combined with the same first nucleotide tag and up to ten different

target-specific reverse primer sequences combined with the same second nucleotide tag can be combined with the up to 2 or up to 4 outer barcode primers to generate multiple amplification products as described above. In various embodiments, at least 10, at least 20, at least 50, at least 100, at least 200, at least 500, at least 1000, at least 2000, at least 5000 or at least 10000 different target-specific primer pairs bearing the same first nucleotide tag and second nucleotide tag would be combined with the up to 2 or up to 4 outer barcode primers to generate multiple amplification products.

[0121] The methods of the invention can include subjecting at least one target amplicon to DNA sequencing using any available DNA sequencing method. In particular embodiments, a plurality of target amplicons is sequenced using a high throughput sequencing method. Such methods typically use an in vitro cloning step to amplify individual DNA molecules. Emulsion PCR (emPCR) isolates individual DNA molecules along with primer-coated beads in aqueous droplets within an oil phase. PCR produces copies of the DNA molecule, which bind to primers on the bead, followed by immobilization for later sequencing. emPCR is used in the methods by Marguilis et al. (commercialized by 454 Life Sciences, Branford, CT), Shendure and Porreca et al. (also known as "polony sequencing") and SOLiD sequencing, (Applied Biosystems Inc., Foster City, CA). See M. Margulies, et al. (2005) "Genome sequencing in microfabricated high-density picolitre reactors" Nature 437: 376–380; J. Shendure, et al. (2005) "Accurate Multiplex Polony Sequencing of an Evolved Bacterial Genome" Science 309 (5741): 1728–1732. In vitro clonal amplification can also be carried out by "bridge PCR," where fragments are amplified upon primers attached to a solid surface. Braslavsky et al. developed a single-molecule method (commercialized by Helicos Biosciences Corp., Cambridge, MA) that omits this amplification step, directly fixing DNA molecules to a surface. I. Braslavsky, et al. (2003) "Sequence information can be obtained from single DNA molecules" Proceedings of the National Academy of Sciences of the United States of America 100: 3960–3964.

[0122] DNA molecules that are physically bound to a surface can be sequenced in parallel. "Sequencing by synthesis," like dye-termination electrophoretic sequencing, uses a DNA polymerase to determine the base sequence. Reversible terminator methods (commercialized by Illumina, Inc., San Diego, CA and Helicos Biosciences Corp., Cambridge, MA) use reversible versions of dye-

terminators, adding one nucleotide at a time, and detect fluorescence at each position in real time, by repeated removal of the blocking group to allow polymerization of another nucleotide. "Pyrosequencing" also uses DNA polymerization, adding one nucleotide at a time and detecting and quantifying the number of nucleotides added to a given location through the light emitted by the release of attached pyrophosphates (commercialized by 454 Life Sciences, Branford, CT). See M. Ronaghi, et al. (1996). "Real-time DNA sequencing using detection of pyrophosphate release" Analytical Biochemistry 242: 84-89.

Sample Preparation by Digital PCR

[0123] In some embodiments, samples are loaded into an amplification device, for example, a PCR plate or a microfluidic device, at sample concentrations containing on average less than one amplification template per well or chamber. Each well or chamber in the device is prepared such that it contains suitable tagged target-specific primers and a unique combination of forward and reverse barcode primers. For example, one well can contain barcode primers containing the elements 5'-(first additional primer binding site)-(first barcode sequence)-(first nucleotide tag)-3', 5'-(second additional primer binding site)-(second barcode sequence)-(second nucleotide tag)-3'. A second well or chamber can contain barcode primers containing the elements 5'-(first additional primer binding site)-(third barcode sequence)-(first nucleotide tag)-3', 5'-(second additional primer binding site)-(fourth barcode sequence)-(second nucleotide tag)-3'. Amplification products produced in each well would be labeled uniquely with the combinations of barcode sequences loaded into these wells.

Sample Calibration by Digital PCR

[0124] In particular embodiments, the number of target amplicons produced, e.g. from a DNA library, using the above-described methods can be calibrated using a digital amplification method. The step is finds particular application in preparing DNA for sequencing by synthesis. For discussions of "digital PCR" see, for example, Vogelstein and Kinzler, 1999, *Proc Natl Acad Sci USA* 96:9236-41; McBride et al., U.S Patent Application Publication No. 20050252773, especially Example 5 (each of these publications are hereby incorporated by reference in their entirety, and in particular for their disclosures of digital amplification). Digital amplification methods

can make use of certain-high-throughput devices suitable for digital PCR, such as microfluidic devices typically including a large number and/or high density of small-volume reaction sites (e.g., nano-volume reaction sites or reaction chambers). In illustrative embodiments, digital amplification is performed using a microfluidic device, such as the Digital Array microfluidic devices described below. Digital amplification can entail distributing or partitioning a sample among hundreds to thousands of reaction mixtures disposed in a reaction/assay platform or microfluidic device. In such embodiments, a limiting dilution of the sample is made across a large number of separate amplification reactions such that most of the reactions have no template molecules and give a negative amplification result. In counting the number of positive amplification results, e.g, at the reaction endpoint, one is counting the individual template molecules present in the input sample one-by-one. A major advantage of digital amplification is that the quantification is independent of variations in the amplification efficiency – successful amplifications are counted as one molecule, independent of the actual amount of product.

[0125] In certain embodiments, digital amplification can be carried out after preamplification of sample nucleic acids. Typically, preamplification prior to digital amplification is performed for a limited number of thermal cycles (e.g., 5 cycles, or 10 cycles). In certain embodiments, the number of thermal cycles during preamplification can range from about 4 to 15 thermal cycles, or about 4-10 thermal cycles. In certain embodiments the number of thermal cycles can be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more than 15. The above-described amplification to produce adaptor sequence-containing amplicons for DNA sequencing can be substituted for the typical preamplification step.

[0126] Digital amplication methods are described in U.S. Publication No. 20090239308, published September 24, 2009, which is hereby incorporated by reference in its entirety and, in particular, for its disclosure of digital amplification methods and devices. Generally, in digital amplification, identical (or substantially similar) amplification reactions are run on a nucleic acid sample, such as genomic DNA. The number of individual reactions for a given nucleic acid sample may vary from about 2 to over 1,000,000. Typically, the number of reactions performed on a sample is about 100 or greater, more typically about 200 or greater, and even more typically about 300 or greater. Larger scale digital amplification can also be

performed in which the number of reactions performed on a sample is about 500 or greater, about 700 or greater, about 765 or greater, about 1,000 or greater, about 2,500 or greater, about 5,000 or greater, about 7,500 or greater, or about 10,000or greater. The number of reactions performed may also be significantly higher, such up to about 25,000, up to about 50,000, up to about 75,000, up to about 100,000, up to about 250,000, up to about 500,000, up to about 750,000, up to about 1,000,000, or even greater than 1,000,000 assays per genomic sample.

[0127] In particular embodiments, the quantity of nucleic acid subjected to digital amplification is generally selected such that, when distributed into discrete reaction mixtures, each individual amplification reaction is expected to include one or fewer amplifiable nucleic acids. One of skill in the art can determine the concentration of target amplicon(s) produced as described above and calculate an appropriate amount for use in digital amplification. More conveniently, a set of serial dilutions of the target amplicon(s) can be tested. For example, a device that is commercially available from Fluidigm Corp. as the 12.765 Digital Array microfluidic device allows 12 different dilutions to be tested simultaneously. Optionally, a suitable dilution can be determined by generating a linear regression plot. For the optimal dilution, the line should be straight and pass through the origin. Subsequently the concentration of the original samples can be calculated from the plot.

[0128] The appropriate quantity of target amplicon(s) can be distributed into discrete locations or reaction wells or chambers such that each reaction includes, for example, an average of no more than about one amplicon per volume. The target amplicon(s) can be combined with reagents selected for quantitative or nonquantitative amplification, prior to distribution or after.

[0129] Following distribution, the reaction mixtures are subjected to amplification to identify those reaction mixtures that contained a target amplicon. Any amplification method can be employed, but conveniently, PCR is used, e.g., real-time PCR or endpoint PCR. This amplification can employ any primers capable of amplifying the target amplicon(s). Thus, in particular embodiments, the primers can be DNA sequencing primers that anneal to the primer binding sites introduced in the previous amplification step.

[0130] The concentration of any target amplicon (copies/μL) is correlated with the number of positive (i.e., amplification product-containing) reaction mixtures. See copending U.S. Application No. 12/170,414, entitled "Method and Apparatus for Determining Copy Number Variation Using Digital PCR," which is incorporated by reference for all purposes, and, in particular, for analysis of digital PCR results. Also see Dube et al., 2008, "Mathematical Analysis of Copy Number Variation in a DNA Sample Using Digital PCR on a Nanofluidic Device" PLoS ONE 3(8): e2876. doi:10.1371/journal.pone.0002876, which is incorporated by reference for all purposes and, in particular, for analysis of digital PCR results.

[0131] In an illustrative embodiment of sample calibration for DNA sequencing by digital PCR, a PCR reaction mix containing roughly 100-360 amplicons per μl can be loaded onto a Digital Array microfluidic device, such as Fluidigm Corporation's (South San Francisco, CA) 12.765 Digital Array microfluidic device, described below. The microfluidic chip has 12 panels and each panel contains 765 chambers. Replicate panels on the digital chip can be assayed in order to obtain absolute quantification of the initial concentration of library. The diluted samples having typical relative coefficients of variation (between replicates) within 9-12% (or lower) can be used for sequencing. See. e.g., White III RA, Blainey PC, Fan CH, Quake SR. "Digital PCR provides sensitive and absolute calibration for high throughput sequencing" BMC Genomics 10:116 doi:10.1186/1471-2164-10-116.

Sample Nucleic Acids

[0132] Preparations of nucleic acids ("samples") can be obtained from biological sources and prepared using conventional methods known in the art. In particular, DNA or RNA useful in the methods described herein can be extracted and/or amplified from any source, including bacteria, protozoa, fungi, viruses, organelles, as well higher organisms such as plants or animals, particularly mammals, and more particularly humans. Suitable nucleic acids can also be obtained from environmental sources (e.g., pond water), from man-made products (e.g., food), from forensic samples, and the like. Nucleic acids can be extracted or amplified from cells, bodily fluids (e.g., blood, a blood fraction, urine, etc.), or tissue samples by any of a variety of standard techniques. Illustrative samples include samples of plasma, serum, spinal fluid, lymph fluid, peritoneal fluid, pleural fluid, oral fluid, and external

sections of the skin; samples from the respiratory, intestinal genital, and urinary tracts; samples of tears, saliva, blood cells, stem cells, or tumors. For example, samples of fetal DNA can be obtained from an embryo or from maternal blood. Samples can be obtained from live or dead organisms or from in vitro cultures. Illustrative samples can include single cells, paraffin-embedded tissue samples, and needle biopsies. Nucleic acids useful in the invention can also be derived from one or more nucleic acid libraries, including cDNA, cosmid, YAC, BAC, P1, PAC libraries, and the like.

[0133] Nucleic acids of interest can be isolated using methods well known in the art, with the choice of a specific method depending on the source, the nature of nucleic acid, and similar factors. The sample nucleic acids need not be in pure form, but are typically sufficiently pure to allow the amplification steps of the methods of the invention to be performed. Where the target nucleic acids are RNA, the RNA can be reversed transcribed into cDNA by standard methods known in the art and as described in Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989), for example. The cDNA can then be analyzed according to the methods of the invention.

Target Nucleic Acids

[0134] Any target nucleic acid that can be tagged in an encoding reaction of the invention (described herein) can be detected using the methods of the invention. In typical embodiments, at least some nucleotide sequence information will be known for the target nucleic acids. For example, if the encoding reaction employed is PCR, sufficient sequence information is generally available for each end of a given target nucleic acid to permit design of suitable amplification primers. In an alternative embodiment, the target-specific sequences in primers could be replaced by random or degenerate nucleotide sequences.

[0135] The targets can include, for example, nucleic acids associated with pathogens, such as viruses, bacteria, protozoa, or fungi; RNAs, e.g., those for which over- or under-expression is indicative of disease, those that are expressed in a tissue- or developmental-specific manner; or those that are induced by particular stimuli; genomic DNA, which can be analyzed for specific polymorphisms (such as SNPs), alleles, or haplotypes, e.g., in genotyping. Of particular interest are genomic DNAs

that are altered (e.g., amplified, deleted, and/or mutated) in genetic diseases or other pathologies; sequences that are associated with desirable or undesirable traits; and/or sequences that uniquely identify an individual (e.g., in forensic or paternity determinations).

Primer Design

[0136] Primers suitable for nucleic acid amplification are sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact length and composition of the primer will depend on many factors, including, for example, temperature of the annealing reaction, source and composition of the primer, and where a probe is employed, proximity of the probe annealing site to the primer annealing site and ratio of primer:probe concentration. For example, depending on the complexity of the target nucleic acid sequence, an oligonucleotide primer typically contains in the range of about 15 to about 30 nucleotides, although it may contain more or fewer nucleotides. The primers should be sufficiently complementary to selectively anneal to their respective strands and form stable duplexes. One skilled in the art knows how to select appropriate primer pairs to amplify the target nucleic acid of interest.

[0137] For example, PCR primers can be designed by using any commercially available software or open source software, such as Primer3 (*see, e.g.,* Rozen and Skaletsky (2000) *Meth. Mol. Biol.,* 132: 365-386; www.broad.mit.edu/node/1060, and the like) or by accessing the Roche UPL website. The amplicon sequences are input into the Primer3 program with the UPL probe sequences in brackets to ensure that the Primer3 program will design primers on either side of the bracketed probe sequence.

[0138] Primers may be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.* (1979) *Meth. Enzymol.* 68: 90-99; the phosphodiester method of Brown *et al.* (1979) *Meth. Enzymol.* 68: 109-151; the diethylphosphoramidite method of Beaucage *et al.* (1981) *Tetra. Lett.*, 22: 1859-1862; the solid support method of U.S. Patent No. 4,458,066 and the like, or can be provided from a commercial source.

[0139] Primers may be purified by using a Sephadex column (Amersham Biosciences, Inc., Piscataway, NJ) or other methods known to those skilled in the art. Primer purification may improve the sensitivity of the methods of the invention.

Microfluidic Devices

[0140] In certain embodiments, any of the methods of the invention can be carried out using a microfluidic device. In illustrative embodiments, the device is a matrix-type microfluidic device is one that allows the simultaneous combination of a plurality of substrate solutions with reagent solutions in separate isolated reaction chambers. It will be recognized, that a substrate solution can comprise one or a plurality of substrates and a reagent solution can comprise one or a plurality of reagents. For example, the microfluidic device can allow the simultaneous pair-wise combination of a plurality of different amplification primers and samples. In certain embodiments, the device is configured to contain a different combination of primers and samples in each of the different chambers. In various embodiments, the number of separate reaction chambers can be greater than 50, usually greater than 100, more often greater than 500, even more often greater than 1000, and sometimes greater than 5000, or greater than 10,000.

[0141] In particular embodiments, the matrix-type microfluidic device is a Dynamic Array ("DA") microfluidic device, an example of which is shown in Fig. 1. A DA microfluidic device is a matrix-type microfluidic device designed to isolate pair-wise combinations of samples and reagents (*e.g.*, amplification primers, detection probes, *etc.*) and suited for carrying out qualitative and quantitative PCR reactions including real-time quantitative PCR analysis. In some embodiments, the DA microfluidic device is fabricated, at least in part, from an elastomer. DA microfluidic devices are described in PCT publication WO05107938A2 (Thermal Reaction Device and Method For Using The Same) and US Pat. Publication US20050252773A1, both incorporated herein by reference in their entireties for their descriptions of DA microfluidic devices. DA microfluidic devices may incorporate high-density matrix designs that utilize fluid communication vias between layers of the microfluidic device to weave control lines and fluid lines through the device and between layers. By virtue of fluid lines in multiple layers of an elastomeric block, high density reaction cell arrangements are possible. Alternatively DA microfluidic devices may

be designed so that all of the reagent and sample channels are in the same elastomeric layer, with control channels in a different layer.

[0142] U.S. Patent Publication No. 2008/0223721 and PCT Publication No. WO 05/107938A2 describe illustrative matrix-type devices that can be used to practice the methods described herein. Figure 21 of the latter is reproduced as Fig. 1 and shows an illustrative matrix design having a first elastomeric layer 2110 (1st layer) and a second elastomeric layer 2120 (2d layer) each having fluid channels formed therein. For example, a reagent fluid channel in the first layer 2110 is connected to a reagent fluid channel in the second layer 2120 through a via 2130, while the second layer 2120 also has sample channels therein, the sample channels and the reagent channels terminating in sample and reagent chambers 2180, respectively. The sample and reagent chambers 2180 are in fluid communication with each other through an interface channel 2150 that has an interface valve 2140 associated therewith to control fluid communication between each of the chambers 2180 of a reaction cell 2160. In use, the interface is first closed, then reagent is introduced into the reagent channel from the reagent inlet and sample is introduced into the sample channel through the sample inlet; containment valves 2170 are then closed to isolate each reaction cell 2160 from other reaction cells 2160. Once the reaction cells 2160 are isolated, the interface valve 2140 is opened to cause the sample chamber and the reagent chamber to be in fluid communication with each other so that a desired reaction may take place. It will be apparent from this (and the description in WO 05/107938A2) that the DA microfluidic device may be used for reacting M number of different samples with N number of different reagents.

[0143] Although the DA microfluidic devices described above in WO 05/107938 are well suited for conducting the methods described herein, the invention is not limited to any particular device or design. Any device that partitions a sample and/or allows independent pair-wise combinations of reagents and sample may be used. U.S. Patent Publication No. 20080108063 (which is hereby incorporated by reference it its entirety) includes a diagram illustrating the 48.48 Dynamic Array IFC (Integrated Fluidic Circuit), a commercially available device available from Fluidigm Corp. (South San Francisco Calif.). It will be understood that other configurations are possible and contemplated such as, for example, 48×96; 96×96; 30×120; *etc.*

[0144] In specific embodiments, the microfluidic device can be a Digital Array microfluidic device, which is adapted to perform digital amplification. Such devices can have integrated channels and valves that partition mixtures of sample and reagents into nanolitre volume reaction chambers. In some embodiments, the Digital Array microfluidic device is fabricated, at least in part, from an elastomer. Illustrative Digital Array microfluidic devices are described in copending U.S. Applications owned by Fluidigm, Inc., such as U.S. Application No. 12/170,414, entitled "Method and Apparatus for Determining Copy Number Variation Using Digital PCR." One illustrative embodiment has 12 input ports corresponding to 12 separate sample inputs to the device. The device can have 12 panels, and each of the 12 panels can contain 765 6 nL reaction chambers with a total volume of 4.59 μL per panel. Microfluidic channels can connect the various reaction chambers on the panels to fluid sources. Pressure can be applied to an accumulator in order to open and close valves connecting the reaction chambers to fluid sources. In illustrative embodiments, 12 inlets can be provided for loading of the sample reagent mixture. 48 inlets can be used to provide a source for reagents, which are supplied to the biochip when pressure is applied to accumulator. Additionally, two or more inlets can be provided to provide hydration to the biochip. Hydration inlets are in fluid communication with the device to facilitate the control of humidity associated with the reaction chambers. As will be understood to one of skill in the art, some elastomeric materials that can utilized in the fabrication of the device are gas permeable, allowing evaporated gases or vapor from the reaction chambers to pass through the elastomeric material into the surrounding atmosphere. In a particular embodiment, fluid lines located at peripheral portions of the device provide a shield of hydration liquid, for example, a buffer or master mix, at peripheral portions of the biochip surrounding the panels of reaction chambers, thus reducing or preventing evaporation of liquids present in the reaction chambers. Thus, humidity at peripheral portions of the device can be increased by adding a volatile liquid, for example water, to hydration inlets. In a specific embodiment, a first inlet is in fluid communication with the hydration fluid lines surrounding the panels on a first side of the biochip and the second inlet is in fluid communication with the hydration fluid lines surrounding the panels on the other side of the biochip.

[0145] While the Digital Array microfluidic devices are well-suited for carrying out the digital amplification methods described herein, one of ordinary skill in the art would recognize many variations and alternatives to these devices. The microfluidic device which is the 12.765 Dynamic Array commercially available from Fluidigm Corp. (South San Francisco, CA), includes 12 panels, each having 765 reaction chambers with a volume of 6 nL per reaction chamber. However, this geometry is not required for the digital amplification methods described herein. The geometry of a given Digital Array microfluidic device will depend on the particular application. Additional description related to devices suitable for use in the methods described herein is provided in U.S. Patent Application Publication No. 2005/0252773, incorporated herein by reference for its disclosure of Digital Array microfluidic devices.

[0146] In certain embodiments, the methods described herein can be performed using a microfluidic device that provides for recovery of reaction products. Such devices are described in detail in copending U.S. Application No. 61/166,105, filed April 2, 2009, which is hereby incorporated by reference in its entirety and specifically for its description of microfluidic devices that permit reaction product recovery and related methods. For example, the digital PCR method for calibrating DNA samples prior to sequencing can be preformed on such devices, permitting recovery of amplification products, which can then serve as templates for DNA sequencing.

[0147] Fig. 2 is a simplified perspective illustration of a carrier and a microfluidic device according to an embodiment of the present invention. As illustrated in Fig. 2, the carrier 100 supports a microfluidic device 110, which may also be referred to as a Digital Array microfluidic device. The carrier 100 may be made from materials providing suitable mechanical support for the various elements of the carrier. As an example, the carrier is made using a plastic or other suitable material. The outer portion of the carrier has the same footprint as a standard 384-well microplate and enables stand-alone valve operation. Additionally, the carrier 100 is compatible with conventional stand-alone thermal cyclers. As described below, there are 48 sample input ports 120 located on a first side of the carrier 100 and 48 assay input ports 122 located on an opposing side of the carrier. The banks of sample input ports 120 and assay input ports 122 are recessed with respect to the top

of the carrier. Utilizing these recessed features, pressure can be applied concurrently to all of the sample input ports or the assay input ports, driving fluids present in the respective ports through fluid lines 140 connecting the input ports and either vias, fluid input lines, or combinations thereof, present on the microfluidic device 110. The samples may include encoded primers and the assays may also be referred to as amplicon-specific (AS) primers.

[0148] The carrier 100 also includes four sources 130, 132, 134, and 136, which may be used to actuate control lines present in the microfluidic device. In an embodiment, sources 130, 132, and 134 are used to pressurize control lines operable to open and close valves present in the microfluidic device. For example, application of pressure greater than atmospheric pressure to source 132 will result in the liquid present in source 132 flowing into control lines present on the microfluidic device, thereby actuating valves operable to obstruct flow through one or more fluid input lines also present on the microfluidic device. In an embodiment, source 130 is used as a fluid well containing harvesting reagent. Pressure can be applied to source 130, forcing the harvesting reagent to flow through fluid lines provided on the carrier to fluid lines provided on the microfluidic device. Thus, application of pressure to source 130 can result in the flow of a harvesting reagent or other suitable fluid through the microfluidic device. The control lines that are in fluid communication with the sources 130-136 can include control lines for interface valves, containment valves, valves used in dilation pumping, fluid lines for the flow of harvesting reagent, or the like. In a particular embodiment, valve 1 is controlled by source 132, valve 2 is controlled by source 134, harvesting reagent is provided in source 130, and hydration reagent is provided in source 136. In this particular embodiment, the interface valves are controlled by source 150 and containment valves are controlled by source 152. This particular embodiment is not intended to limit the present invention, but merely to provide an example of one configuration. Other configurations can be utilized as appropriate to the particular application.

[0149] As described more fully in relation to Fig. 3, fluid lines 140 present on the carrier 100 are in fluid communication with one or more fluid lines present on the microfluidic device 110. These fluid lines can serve to carry fluids into and out of the microfluidic device or may be used as control lines to actuate valves present on the microfluidic device. Thus, fluids provided in sample input ports 120 or assay input

ports 122 can be loaded into appropriate fluid input lines and chambers of the microfluidic device. Other fluids (e.g., liquids) provided in sources 130-136 can also be loaded into either fluid input lines or control lines of the microfluidic device. Reaction products from the chambers of the microfluidic device can be recovered as they are pumped through fluid lines on the microfluidic device, back into the fluid lines 140 present on the carrier and into the sample or assay input ports 120 or 122.

[0150] Pressure accumulators 150 and 152 may be utilized to pressurize other control lines, provide for hydration of the microfluidic device, or they may not be used in some embodiments. Although 48 sample input ports and 48 assay input ports are shown in the embodiment of the present invention illustrated in Fig. 2, this is not required by the present invention. Other embodiments utilize a different number of samples and assays depending on the particular application. One of ordinary skill in the art would recognize many variations, modifications, and alternatives.

[0151] Fig. 3 is a simplified schematic diagram of a microfluidic device according to an embodiment of the present invention. The microfluidic device 200 includes vias 210 connected to fluid input lines 212 that are used to provide fluid flow paths for 24 different samples. The 24 samples, which can be loaded into a subset of the sample input ports 120 illustrated in Fig. 1, flow through vias 210 and fluid input lines 212 to sample input lines 312 and eventually to sample chambers 310 as illustrated in Fig. 4. The microfluidic device 200 also includes vias 220 connected to fluid input lines 222 that are used to provide fluid flow paths for 21 different assays. The via 250 on the side of the microfluidic device opposing the assay input fluid lines provides for hydration, actuation of a control line, or other suitable operations. The array portion 230 of the microfluidic device is illustrated (in part) in Fig. 4. In the array portion 230, the samples and assays are loaded into sample and assay chambers and then can be mixed to form pairwise combinations.

[0152] As described more fully throughout the present specification, after samples and assays are mixed and reacted, the reaction products can be recovered from the microfluidic device by flowing a recovery fluid through the fluid input lines 212, through the array portion 230 of the microfluidic device as illustrated in Fig. 4, and through the output fluid lines 240 and vias 242. These output fluid lines are in fluid communication with output ports provided on a carrier. Thus, reaction products

pooled from the combination of a sample with each of the various assays are separately provided through each of the independent output fluid lines 240.

[0153] The particular number of sample and assay input lines illustrated in Fig. 3 are provided merely by way of example and particular implementations are not limited to these particular numbers. In other embodiments, additional sample and assay input lines are provided in order to facilitate additional pairwise combinations of samples and assays in the microfluidic device.

[0154] Fig. 4 is a simplified schematic diagram of several unit cells of the microfluidic device illustrated in Fig. 3. In Fig. 4, four unit cells from the array portion 230 are illustrated for purposes of clarity. The sample input lines 316 are in fluid communication with the fluid input lines 212 and the assay input lines 318 are in fluid communication with the assay input lines 222 as illustrated in Fig. 3. The unit cell section of the microfluidic device includes sample chambers 310 and assay chambers 312. Fluid lines 314 provide for fluid communication between the sample chambers and the assay chambers when the interface valves 330 are in the open position. In a specific embodiment, sample input lines 316 are provided in a layer of the microfluidic device underlying the layer containing the sample chambers. In a similar manner, assay input lines 318 are provided in a layer of the microfluidic device underlying the layer containing the assay chambers. Samples flow from the sample input lines 212 illustrated in Fig. 3 to sample input lines 316 and up through one or more vias (not shown) passing from the sample input lines 316 to the sample chambers 310. Although the sample input lines 316 are illustrated as branching into three input lines as the fluid passes to the sample chambers, this particular number is not required by the present invention and other numbers of sample input lines, for example, 1 input line, 2, 4, or more than 4 sample input lines may be utilized. Similar design criteria are applicable to the three fluid lines 314 connecting the sample chambers and corresponding assay chambers. The sample input lines 316 provide a continuous flow path in the row direction of the figure, enabling a single sample to be distributed evenly among multiple sample chambers, for example, the top row of sample chambers or the bottom row of sample chambers.

[0155] Utilizing interface valves 330 and containment valves 340, each of the sample chambers can be isolated from each of the other sample chambers as well as the assay chambers. The assay chambers can be isolated from the other assay

chambers using the containment valves. Both the isolation and containment valves are actuated by application of pressure to a corresponding control line present on the carrier or by other means, for example, electrostatic actuation.

[0156] Fig. 4 illustrates assay input lines 318, which provide for assay flow from assay input lines 222 illustrated in Fig. 3 to the assay chambers 312. When the containment valves are in the open position, assays are able to flow from the assay input lines to the assay chambers 312. In a specific embodiment, the assays flow through vias connecting the input lines and the assay chambers in a manner similar to the filling of the sample chambers. The loading of assays along the columns of the microfluidic device provide a different assay for each row of samples, resulting in M x N pairwise combinations.

[0157] Opening of the interface valves 330 enables the samples and the assays to mix in pairwise combinations via free interface diffusion. After the samples and assays are mixed, thermocycling can be performed to form reaction products. Reaction products are recovered from the microfluidic device by opening harvest valves 350, which enable the reaction products to flow into portions 360 of the sample input lines adjacent the sample chambers. Using sample input lines 316 and on-chip pumps (not shown), reaction products flow through the sample input lines toward recovery ports on the carrier.

[0158] In the embodiment illustrated in Fig. 4, samples load from a first side of the microfluidic device. The assays load from an adjacent side of the microfluidic device. After processing, a harvesting reagent is input from the first side of the device using the sample input lines and reaction products exit the microfluidic device out fluid lines running toward the side of the microfluidic device opposing the first side. In this embodiment, the remaining side of the microfluidic device is not used for sample or assay loading or reaction product unloading. Other configurations are included within the scope of the present invention and the example configuration illustrated in Fig. 4 is merely provided by way of example. One of ordinary skill in the art would recognize many variations, modifications, and alternatives.

[0159] A benefit provided by the systems described herein is that the volume of samples and assays used in the reactions are fixed, regardless of the pipetting volume dispensed into the sample input and the assay input ports. If the volume in

the sample and/or assay input ports is above a predetermined threshold sufficient to fill the sample/assay input lines and the sample/assay chambers, then application of pressure to the sample/assay input ports will result in complete filling of the sample/assay chambers. The completely filled chambers thus provide a fixed reaction volume not available with conventional microtiter plate techniques.

[0160] Although systems have been developed by the present assignee to perform many simultaneous binding assays, including, but not limited to immunological experiments such as ELISA assays, embodiments of the present invention provide for dilation pumping "on-chip" as well as separate sample and assay chambers. Thus, pairwise combinations of samples and assays are possible using embodiments described herein that are not possible with previously developed techniques. Additional description of binding assays is provided in U.S. Patent Application Publication No. 2007/0074972, filed on September 13, 2006, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

[0161] Embodiments of the present invention provide a system suitable for PCR sample preparation that features reduced cost, time, and labor in the preparation of amplicon libraries from an input DNA template. In a typical use case, the first amplification will be used to generate libraries for next-generation sequencing. Utilizing embodiments of the present invention, samples and encoded primers are combined with amplicon-specific (AS) primers to create a mixture that is suitable for desired reactions. Based on an MxN architecture of the microfluidic device, each of the M samples is combined with each of the N AS primers (i.e., assays) to form MxN pairwise combinations. That is, one reaction site is provided for each sample and assay pair. After the completion of the reaction (e.g., PCR), the reaction products are recovered from the system, typically using a harvest reagent that flows through the microfluidic device. In a specific embodiment, reaction products associated with each sample are recovered in a separate reaction pool, enabling further processing or study of the pool containing a given sample reacted with each of the various assays.

[0162] Thus, in embodiments described herein, a microfluidic device is provided in which independent sample inputs are combined with primer inputs in an MxN array configuration. Thus, each reaction is a unique combination of a particular sample and a particular primer. As described more fully throughout the present specification, samples are loaded into sample chambers in the microfluidic device

through sample input lines arranged as columns in one implementation. AS primers or assays are loaded into assay chambers in the microfluidic device through assay input lines arranged as rows crossing the columns. The sample chambers and the assay chambers are in fluidic isolation during loading. After the loading process is completed, an interface valve operable to obstruct a fluid line passing between pairs of sample and assay chambers is opened to enable free interface diffusion of the pairwise combinations of samples and assays. Precise mixture of the samples and assays enables reactions to occur between the various pairwise combinations, producing a reaction product including a set of specific PCR reactions for which each sample has been effectively coded with a unique barcode. The reaction products are harvested and can then be used for subsequent sequencing processes. The terms "assay" and "sample" as used herein are descriptive of particular uses of the devices in some embodiments. However, the uses of the devices are not limited to the use of "sample(s)" and "assay(s)" in all embodiments. For example, in other embodiments, "sample(s)" may refer to "a first reagent" or a plurality of "first reagents" and "assay(s)" may refer to "a second reagent" or a plurality of "second reagents." The MxN character of the devices enable the combination of any set of first reagents to be combined with any set of second reagents.

[0163] According to one particular process implemented using an embodiment of the present invention, after 25 cycles of PCR, the reaction products from the MxN pairwise combinations will be recovered from the microfluidic device in discrete pools, one for each of the M samples. Typically, the discrete pools are contained in a sample input port provided on the carrier. In some processes, the reaction products may be harvested on a "per amplicon" basis for purposes of normalization. Utilizing embodiments of the present invention, it is possible to achieve results (for replicate experiments assembled from the same input solutions of samples and assays) for which the copy number of amplification products varies by no more than ± 25% within a sample and no more than ± 25% between samples. Thus, the amplification products recovered from the microfluidic device will be representative of the input samples as measured by the distribution of specific known genotypes. Preferably, output sample concentration will be greater than 2,000 copies/amplicon/microliter and recovery of reaction products will be performed in less than two hours.

[0164] Applications in which embodiments of the present invention can be used include sequencer-ready amplicon preparation and long-range PCR amplicon library production. For the sequencer-ready amplicon preparation, multiple-forward primer and 3-primer combination protocols can be utilized.

[0165] Fig. 5A is simplified schematic diagram of a microfluidic device according to another embodiment of the present invention. The microfluidic device illustrated in Fig.5A shares common features as well as differences with the microfluidic device illustrated in Fig. 2. Samples are loaded into the microfluidic device through 48 vias and corresponding sample input lines provided at one edge of the microfluidic device (i.e., the bottom edge in Fig. 5A). Samples flow through the sample input lines into the array portion 430 of the microfluidic device. The assays are loaded from vias and assay input lines on two sides of the microfluidic device (i.e., the left and right sides in Fig. 5A). Additional discussion of the unit cells present in the array portion 430 is provided in relation to Fig. 6. Reaction products are removed through the sample input lines and are recovered in the sample input ports 120 provided on the carrier 100. Thus, in Fig. 5A, loading of samples and recovery of reaction products are illustrated as flowing through the bottom side of the microfluidic device.

[0166] Fig. 5B is a simplified schematic diagram of portions of the microfluidic device illustrated in Fig. 5A. The portions illustrated in Fig. 5B include sample input lines 410, assay input lines for even assays (assay input lines 420), assay input lines for odd assays (assay input lines 422), and a harvesting reagent input lines 430. In an embodiment, the sample input lines 410 are in fluid communication with vias 412 that are aligned with sample input lines 140, which are in fluid communication with sample input ports 120 as illustrated in Fig. 2. In other embodiments, additional sample input lines (not shown) are provided to enable fluid communication between the sample input ports 120 and the sample input lines 410. Thus, pressurization of the bank of sample input ports will result in flow of the various samples into the illustrated sample input lines 410.

[0167] As discussed in relation to Fig. 6 below, sample input lines 410 are in fluid communication with sample input lines 516 and sample chambers 510 present in the microfluidic device. For an array with 48 sample chambers and 48 assay chambers, the 48 sample input lines 410 illustrated in Fig. 5B will provide samples to

48 separate columns of sample chambers, two of which are illustrated in Fig. 6. It should be noted that the various fluid lines illustrated in Fig. 5B can be integrated in carrier 100, integrated in the microfluidic device 110, or present in one or more other structures, depending on the particular implementation. Thus, the illustration of the sample input lines 410 in Fig. 5B is not intended to limit the scope of the present invention but merely to illustrate fluid lines suitable for providing controlled fluid flow to the various chambers of the microfluidic device.

[0168] In an embodiment, even assay input lines 420 and odd assay input lines 422 are in fluid communication with vias 424 that are aligned with assay input lines 140, which are in fluid communication with assay input ports 122 as illustrated in Fig. 2. In other embodiments, additional assay input lines (not shown) are provided to enable fluid communication between the assay input ports 122 and the assay input lines 420 and 422. Thus, pressurization of the bank of assay input ports 122 will result in flow of the various assays into the illustrated assay input lines. After flowing through the input lines illustrated in Fig. 5B, the various fluids will eventually enter into the unit cells illustrated in Fig. 6.

[0169] As discussed above, the various fluid lines can be integrated into the carrier, the microfluidic device, or other suitable structure. In a 48 sample x 48 assay array configuration, the 24 even assay input lines 420 will provide inputs to half of the rows of assay input lines 518 shown in Fig. 6. The 24 odd assay input lines 422 will provide inputs to half of the rows of assay inputs lines 518 shown in Fig. 6. Thus, although loading of assays is only illustrated from the right side of the array in Fig. 6, actual implementation will typically load assays from both sides in an even/odd configuration. In some embodiments, additional vias are provide for loading of hydration fluids or the like. Moreover, in some embodiments, in order to provide compatibility with existing carriers, some fluid lines are unused or modified to provide for such compatibility.

[0170] The harvesting reagent input line 430 provides for harvesting reagent used in recovering reaction products from the microfluidic device. The harvesting reagent input line 430 illustrated in Fig. 5B is in fluid communication with the harvesting reagent input port 136 illustrated in Fig. 2 and passes along the microfluidic device adjacent to the even assay input lines to the top portion of the device and then branches off into a plurality of harvesting reagent input lines. The

harvesting reagent multiplexor has a substantially equal volume for every sample input line to provide uniform pumping during the reaction product recovery operation. It should be noted that the particular branching system illustrated in Fig. 5B is merely provided as an example and other branching systems are included within the scope of the present invention. The harvesting reagent input lines 430 are in fluid communication with sample input lines 516 discussed in relation to Fig. 6. As discussed in relation to Figs. 9A-D, embodiments utilize a separate harvesting reagent input line for each column of sample chambers, for example, 48 harvesting reagent input lines for an microfluidic device with a 48 x 48 array configuration. Additionally, although the harvesting reagent input line enters the microfluidic device at a location adjacent the even assay input lines 420, this is not required by embodiments of the present invention and other configurations are within the scope of the present invention. One of ordinary skill in the art would recognize many variations, modifications, and alternatives.

[0171] As described in relation to Fig. 8C, harvesting reagent flows from a harvesting reagent input port on the carrier, through the harvesting reagent input lines 430 and into one end of the sample input lines 516. As discussed more fully throughout the present specification, the sample input lines function both as input lines and reaction product recovery lines. For loading, the sample flow path is from the sample input ports 120, through input lines 140, through vias 412, through sample input lines 410, through sample input lines 516, to the reaction chambers 510 and to the loading bowls 830. For reaction product recovery, the product flow path is from the harvesting reagent input port 136,through the harvesting reagent input lines 430, through the sample input lines 516, through the sample input lines 410, through the vias 412, and to the sample input ports 120, which serve during harvesting, as a fluid recovery well. Thus, the use of the term "input" lines should be considered in the context of the particular process being performed, since the "input" lines can serve to both load samples and recover or remove reaction products from the microfluidic device and the carrier.

[0172] By applying pressure to the bank of sample input ports 120 and the bank of assay input ports 122, samples and reagents can be loaded through the illustrated sample and assay input lines into sample and assay chambers present in the microfluidic device. By applying pressure to the harvesting reagent input port 136,

the reaction products can be recovered from the sample chambers and delivered to the sample input ports. Valves present in the microfluidic device are utilized to control the flow of samples, assays, and reaction products, as described more fully throughout the present specification. Fig. 5B only illustrates a portion of the sample input lines, assay input lines, and harvesting reagent input lines and additional portions of these input lines are illustrated in Fig. 5A and Fig. 6.

[0173] Fig. 6 is a simplified schematic diagram of several unit cells of the microfluidic device illustrated in Fig. 5A. In Fig. 6, four unit cells from the array portion 430 shown in Fig. 5A are illustrated for purposes of clarity. The unit cell section of the microfluidic device includes sample chambers 510 and assay chambers 512. Fluid lines 514 provide for fluid communication between the sample chambers and the assay chambers when the interface valves 530 are in the open position. In a specific embodiment, sample input lines 516 are provided in a layer of the microfluidic device underlying the layer containing the sample chambers. In a similar manner, assay input lines 518 are provided in a layer of the microfluidic device underlying the layer containing the assay chambers. Samples flow from the sample input lines 410 illustrated in Fig. 5B to sample input lines 516 and up through one or more vias passing from the sample input lines to the sample chambers. Although two sample lines are illustrated for each sample chambers, this particular number is not required by the present invention and other numbers of sample input lines, for example, 1 input line, 3, 4, or more than 4 sample input lines may be utilized. The sample input lines 516 provide a continuous flow path in the column direction of the figure, enabling a single sample to be distributed evenly among multiple sample chambers.

[0174] Fig. 6 illustrates assay input lines 518, which provide for assay flow from assay input lines 420 and 422 illustrated in Fig. 5B to the assay chambers 512. Although Fig. 6 only illustrates assay input lines entering the unit cells from the right side of the figure, it will be evident to one of skill in the art that in the implementation illustrated in Fig. 5B, even and odd assays are loaded from opposing sides of the microfluidic device. The illustration provided in Fig. 6 is merely simplified for purposes of clarity. In a specific embodiment, the assays load through vias connecting the input lines and the assay chambers in a manner similar to the filling of the sample chambers. The loading of assays along the rows of the microfluidic device

provide a different assay for each of the samples, resulting in a number of pairwise combinations appropriate for an M x N array.

[0175] As described more fully throughout the present specification, reaction products are recovered from the microfluidic device using the sample input lines 516 and pumps (not shown). Containment valves 540 provide for containment between the various sample and assay chambers in each row. Utilizing the interface valves 530 and the containment valves 540, each of the sample chambers can be isolated from each of the other sample chambers as well as the assay chambers. The assay chambers can be isolated from the other assay chambers using the containment valves. Both the isolation and containment valves are actuated by application of pressure to a corresponding control line in fluid communication with sources 130-134 or by other means, for example, electrostatic actuation.

[0176] In Fig. 6, four sample chambers 510 are illustrated in an array configuration. The four illustrated chambers are merely shown by way of example and implementations of the present invention are not limited to the four illustrated chambers, but typically provide 2,304 chambers in a 48 x 48 array configuration, 4,096 chambers in a 64 x 64 array configuration, 9,216 chambers in a 96 x 96 array configuration, or the like.

[0177] Embodiments of the present invention provide unit cells with dimensions on the order of several hundred microns, for example unit cells with dimension of 500 x 500 μm, 525 x 525 μm, 550 x 550 μm, 575 x 575 μm, 600 x 600 μm, 625 x 625 μm, 650 x 650 μm, 675 x 675, μm, 700 x 700 μm, or the like. The dimensions of the sample chambers and the assay chambers are selected to provide amounts of materials sufficient for desired processes while reducing sample and assay usage. As examples, sample chambers can have dimensions on the order of 100-400 μm in width x 200-600 μm in length x 100-500 μm in height. For example, the width can be 100 μm, 125 μm, 150 μm, 175 μm, 200 μm, 225 μm, 250 μm, 275 μm, 300 μm, 325 μm, 350 μm, 375 μm, 400 μm, or the like. For example, the length can be 200 μm, 225 μm, 250 μm, 275 μm, 300 μm, 325 μm, 350 μm, 375 μm, 400 μm, 425 μm, 450 μm, 475 μm, 500 μm, 525 μm, 550 μm, 575 μm, 600 μm, or the like. For example, the height can be 100 μm, 125 μm, 150 μm, 175 μm, 200 μm, 225 μm, 250 μm, 275 μm, 300 μm, 325 μm, 350 μm, 375 μm, 400 μm, 425 μm, 450 μm, 475 μm, 500 μm, 525 μm, 550 μm, 575 μm, 600 μm, or the like. Assay chambers can have

similar dimensional ranges, typically providing similar steps sizes over smaller ranges than the smaller chamber volumes. In some embodiments, the ratio of the sample chamber volume to the assay chamber volume is about 5:1, 10:1, 15:1, 20:1, 25:1, or 30:1. Smaller chamber volumes than the listed ranges are included within the scope of the invention and are readily fabricated using microfluidic device fabrication techniques.

[0178] Higher density microfluidic devices will typically utilize smaller chamber volumes in order to reduce the footprint of the unit cells. In applications for which very small sample sizes are available, reduced chamber volumes will facilitate testing of such small samples.

[0179] The dimensions of the interface valves 530 are selected to provide for complete obstruction of the fluid lines 514 connecting the sample and assay chambers. In some embodiments, the valve dimensions range from about 10-200 μm x 10-200 μm, for example, 50 x 50 μm, 50 x 65 μm, 50 x 80 μm, 50 x 100 μm, 65 x 50 μm, 65 x 65 μm, 65 x 80 μm, 65 x 100 μm, 80 x 50 μm, 80 x 65 μm, 80 x 80 μm, 80 x 100 μm, 100 x 50 μm, 100 x 65 μm, 100 x 80 μm, 100 x 100 μm, or the like. The sample input lines may have various widths depending on the number of sample input lines and the sample chamber volumes, and desired flow rates for loading and product recovery. As examples, the sample input lines may have a cross-section of 1-20 μm in height and 50-100 μm in width. For example, the sample input lines may have heights of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 μm and widths of 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 μm.

[0180] Other device parameters, including layer to layer alignment, ranging from 20-100 μm, and via size, ranging from 50-200 microns, are selected to provide desired system performance characteristics. One of ordinary skill in the art would recognize many variations, modifications, and alternatives.

[0181] In some embodiments, an extra assay inlet is provided at the side of the microfluidic device adjacent the harvesting reagent input lines. Additionally, no assay inlet is provided at the side of the microfluidic device adjacent the sample input ports on the carrier. In this configuration, the extra assay inlet can be used for dehydration chamber loading. Typically, loading of the dehydration chambers will

use more than 5 μl of assay solution. Alternatively, a separate dehydration solution could be used to keep assay volumes uniform across the microfluidic device.

[0182] Fig. 7 is a simplified flowchart of a method of operating a microfluidic device according to an embodiment of the present invention. In the illustrated embodiment, the microfluidic device includes at least one assay chamber, at least one sample chamber, and at least one harvesting port. In a particular embodiment, the microfluidic device includes a plurality of assay chambers and a plurality of sample chambers. The method 600 includes closing a fluid line between the assay chamber and the sample chamber (610). Referring to Fig. 6, interface valves 530 are operable to close fluid lines 514 passing between the sample chambers 510 to the assay chambers 512. In some embodiments, the interface valves 530 are "push-up" valves as described more fully below. The interface valves are formed by the intersection of control line 532 or control channel that is at least partially contained in a first layer of the microfluidic device. The fluid lines are at least partially contained in a second layer of the microfluidic device. The control lines 532 are in fluid communication with one or more pressure actuators or accumulators as illustrated in Fig. 2.

[0183] The intersection of the control line 532 with the fluid line 514 forms a valve at the intersection, referred to as an interface valve 530 because the valve prevents mixing at the interface between the sample and the assay. The interface valve 530 is actuated in response to fluid pressure in the control line and is operative to prevent fluid flow through the fluid lines. Generally, the multilayer microfluidic device discussed herein includes a number of elastomeric layers and the valves 530 include a deflectable membrane between the first layer and the second layer. In a "push-up" configuration, the deflectable membrane of the valve is deflectable into the fluid line 514 positioned above the intersection with the control line 532. In this configuration, the deflectable membrane deflects up into the fluid line to close the fluid line at the position of the valve, thus the reference to "push-up" valves. Releasing the pressure in the control line will result in the deflectable membrane returning to the undeflected position and thereby opening of the closed valve. Additional description of microfluidic devices including valves is provided in U.S. Patent Application No. 2005/0226742, the entire disclosure of which is hereby incorporated by reference in its entirety for all purposes.

[0184] As illustrated in Fig. 6, actuation of control lines 532 will obstruct fluid lines 514. Typically, the control lines 532 are actuated concurrently by application of pressure to a pressure accumulator. Referring once again to Fig. 7, after closing of the interface valves 530, samples flow into sample chambers 510 via sample input lines 516 (612). As illustrated in Fig. 6, each sample chamber 510 is in fluid communication with multiple (e.g., two) sample input lines 516. In other embodiments, other numbers of sample input lines can be utilized. One of ordinary skill in the art would recognize many variations, modifications, and alternatives. Typically, the sample input lines, which are at least partially contained in a second layer of the microfluidic device, pass under the sample chambers, which are at least partially contained in a third layer of the microfluidic device. A via (not illustrated) passing from the sample input line up to the sample chamber, provide for fluid flow from the sample input line to the sample chamber. As shown in Fig. 6, samples flow in, for example, up the columns, past containment valves 540, which are open, through the vias extending out of the plane of the figure, and into the various sample chambers. Fluids such as air present in the sample chambers are expelled during loading of the samples as a result of the permeability of the elastomeric material used to fabricate the microfluidic device.

[0185] Referring once again to Fig. 7, assays flow into assay chambers 512 via assay input lines 518 (614). The assays flow through assay input lines 518, past containment valves 540, which are open, and through vias (not shown) passing from the assay input lines to the assay chambers. The closure of the interface valves 530 prevent the samples in the sample chambers and the assays in the assay chambers from mixing. Once the sample and assay are loaded, the fluid line between the assay chamber and the sample chamber is opened (616). In embodiments of the present invention, multiple fluid lines 514 are opened concurrently by opening of interface valves 530. At least a portion of the sample and at least a portion of the assay are combined to form a mixture (618). The mixture of the sample and assay is formed throughout the sample and assay chambers as well as the fluid lines connecting these chambers. Free interface diffusion is a process in which mixing is slow and the rate of species equilibration depends on the species' diffusion constants. Small molecules such as salts have large diffusion constants, and hence equilibrate quickly. Large molecules (e.g., proteins) have small diffusion constants, and equilibrate more slowly.

[0186] The mixture is reacted to form a reaction product (620). A typical reaction included within the scope of the present invention is PCR, which involves thermocycling of the microfluidic device through a number of cycles as will be evident to one of skill in the art. The fluid line between the assay chamber and the sample chamber is closed (622) by actuation of interface valves 530. Closure of the interface valves separates the reaction product present in the sample chambers from the reaction products present in the assay chambers. Additionally, the containment valves 540 can be closed during thermocycling in order to prevent precipitation during the thermocycling process. A harvesting reagent flows from the harvesting port to the sample chamber (624) in order to begin the process of harvesting the reaction products present in the sample chambers. The harvesting port 136 is an example of a fluid input port useful in the harvesting process. As illustrated in Fig. 4, the reaction products flow down through the sample input lines 516 toward the sample input ports from which the samples were originally provides. Thus, in the illustrated method, removing the reaction products from the microfluidic device includes flowing the reaction products through at least a portion of the sample input line that was used to load the samples to the sample input port. Thus, the reaction product are removed from the microfluidic device (626) and output to the sample input ports, for example, sample input ports 120 illustrated in Fig. 2.

[0187] Dilation pumping is used in the illustrated embodiment to remove the reaction products from the microfluidic device as discussed in additional detail in relation to Figs. 9A-D. Referring once again to Fig. 2, control ports 130 and 132 or pressure accumulators 150 and 152 can be used to actuate the valves used in dilation pumping. Thus, embodiments provide valves for dilation pumping on the microfluidic device, which provides for removal of the reaction products from the microfluidic device. This contrasts with conventional designs in which such valves were not provided as part of the microfluidic device.

[0188] It should be appreciated that the specific steps illustrated in Fig. 7 provide a particular method of operating a microfluidic device according to an embodiment of the present invention. Other sequences of steps may also be performed according to alternative embodiments. For example, alternative embodiments of the present invention may perform the steps outlined above in a different order. Moreover, the individual steps illustrated in Fig. 7 may include

multiple sub-steps that may be performed in various sequences as appropriate to the individual step. Furthermore, additional steps may be added or removed depending on the particular applications. One of ordinary skill in the art would recognize many variations, modifications, and alternatives.

[0189] Figs. 8A - 8D are simplified schematic diagrams illustrating fluid flow through unit cells of a microfluidic device during operation according to an embodiment of the present invention. Referring to Fig. 8A, the microfluidic device is illustrated during the sample and assay loading process. Containment valves 540 are open, allowing fluid flow along the sample input lines 516 to the various sample chambers 510. The open state of the containment valves also enables the assays to flow in through the assay input lines 518 to the various assay chambers 512. Using a single sample for each set of sample input lines (m of M samples) enables loading of a single sample in each column of sample chambers. Additionally, using a single assay for each assay input line (n of N assays) enables loading of a single assay in each row of assay chambers. The closed state of the interface valves 530 prevent mixing of the samples and the assays.

[0190] Fig. 8B illustrates the microfluidic device during a sample and assay mixing process as well as a subsequent reaction process (e.g., amplification). The containment valves 540 are closed, preventing fluid flow along the sample input lines 516. The closure of the containment valves thus isolates the sample chambers along a column from one another (with each column potentially containing a different sample). The closing of the containment valves 540 additionally isolates the assay chambers from other assay chambers in each row. After this chamber isolation is provided, the interface valves 530 are opened, enabling the samples and assays to mix via free interface diffusion (FID) and form M x N pairwise combinations. Although the materials in the pairs of sample/assay chambers are illustrated with the same shading in Fig. 8B, it will be appreciated that four different pairwise combinations are illustrated, one for each pair of sample/assay chambers. The multiple steps involved in mixing the samples and assays, then performing PCR amplification, are represented by a single drawing in Fig. 8B although it will be apparent to one of skill in the art that numerous steps, for example, multiple thermocycling steps, are involved in these processes.

[0191] Fig. 8C illustrates the microfluidic device during sample chamber isolation and initial loading of the harvesting reagent. The interface valves 530 are closed to maintain isolation between the sample chambers and the assay chambers. Thus, the reaction products present in the sample chambers are recovered while the reaction products in the assay chambers are not recovered. The containment valves 540 are opened to allow the harvesting reagent to flow into the sample input lines 516 from the harvesting reagent input lines 430 illustrated in Fig. 5B. The harvesting reagent flows through the sample input lines, and into the sample chambers. In the illustrated embodiment, the reaction products are removed as the harvesting reagent flows through the sample input lines and the sample chambers in response to a dilation pumping process described in additional detail in relation to Figs. 9A-9D. As illustrated in Fig. 8C, the harvesting reagent has only reached the middle region of the upper reaction chambers. As the dilation pumping process continues, the harvesting reagent will be progressively introduced into subsequent sample chambers, thereby displacing the reaction products. Eventually, the reaction products associated with each sample will be recovered as a pooled fluid including the reaction products and the harvesting reagent in the sample input port from which the sample was originally dispensed.

[0192] It should be noted that the straight line representing the interface between the harvesting reagent and the reaction products is shown merely for purposes of simplicity and it will be apparent to one of skill in the art that in practice, a more complicated interface will be present.

[0193] Fig. 8D illustrates the microfluidic device during final loading of the harvesting reagent and recovery of the reaction products. As the dilation pumping process continues, harvesting reagent is introduced into additional sample chambers progressively farther from the harvesting reagent input lines. The state of the recovery process illustrated in Fig. 8D shows that the reaction products have been flushed from the sample chambers, which are now filled with harvesting reagent. Although only four sample chambers are illustrated in Fig. 8D, it will be appreciated that recovery of the reaction products from all the sample chambers in the array is provided by the embodiments described herein.

[0194] Figs. 9A-9D are simplified schematic diagrams illustrating fluid flow through a microfluidic device during operation according to an embodiment of the

present invention. Fig. 9A illustrates a portion of a microfluidic device according to an embodiment of the present invention during loading of samples and assays. The illustrated portion includes a harvesting reagent input line 810, vent and loading bowl portions 830, and isolation valve 840. As described more fully throughout the present specification, valves 820 and 822 are used to perform dilation pumping of reaction products present in sample chambers 510. As illustrated in Fig. 9A, valve 820 is closed and valve 822 is open. Valves 820 and 822 are typically "push-up" valves described elsewhere in the present specification.

[0195] Samples are loaded into sample chambers 510 and assays are loaded into assay chambers 512 as described in relation to Fig. 6. Interface valves 530 are closed, preventing mixing of the samples and assays. Vents and loading bowls are provided in some embodiments to allow for reductions in effects related to depletion fronts. The inventors have observed that in loading samples into microfluidic devices (e.g., through the vertical sample input lines illustrated in Fig. 9A), binding of a portion of the sample present at the leading edge of the flow path to the material of the microfluidic device will produce a depletion front in which one or more components of the sample are depleted as a result of this binding process. The provision of the vents and loading bowls 830 enables the user to push the depletion front through the various sample chambers of the microfluidic device and store the depleted sample material in the loading bowls 830. Eventually, as the depleted sample material is flushed through the device into the loading bowls, the sample contained in the microfluidic device will be substantially undepleted.

[0196] Isolation valve 840 is open during the sample and assay loading process to enable the depletion front to flow into the loading bowls 830. Valve 822 is open, allowing the samples to flow through the sample input lines to the various sample chambers. Since valve 820 is closed, samples are not allowed to pass into the harvesting reagent input line 810. It should be noted that containment valves 540 are illustrated in the closed state in Fig. 9A. Containment valves are open during sample and assay loading and then are closed as illustrated after sample and assay loading is complete. The containment valves isolate the various pairs of reaction and assay chambers from other pairs containing the various pairwise combinations.

[0197] In Fig. 9A, only a single column of the microfluidic device is illustrated for purposes of clarity. It is understood that additional columns are

provided by the microfluidic devices as illustrated, for example, in Fig. 6. Moreover, much of the column is not illustrated for the purposes of clarity. The two sets of sample/assay chambers illustrated are those at the top and bottom of Fig. 4A, respectively. The set adjacent valve 820 is the topmost set and the set adjacent valve 822 is the bottommost set. Thus, these diagrams are merely representative and not intended to limit the scope of the present invention.

[0198] Fig. 9B illustrates mixing of the samples and assays and a subsequent reaction (e.g., amplification) process. In order to mix the samples and reagents, interface valves 530 are placed in the open position as shown. Closure of containment valves 540 seals the reaction products in the sample and assay chambers along with the connecting fluid lines. As illustrated in Fig. 9B, isolation valve 840 is closed, preventing fluid flow between the sample input lines and the loading bowls 830. Actuation of valve 840 to place it in the open or closed position is performed using a pressure accumulator (not shown) in some embodiments and using other actuation techniques in other embodiments, for example, mechanical, electrostatic, electromechanical, thermodynamic, piezoelectric, or the like. Such additional techniques may also be applicable to other valves described herein. One of ordinary skill in the art would recognize many variations, modifications, and alternatives.

[0199] Although Fig. 9B illustrates mixing and reaction using a single image, one of skill in the art will appreciate that multiple thermal cycles may be used to amplify DNA using the PCR process. Thus, this simple figure is intended to show mixing and subsequent reactions that can occur in the microfluidic device.

[0200] Fig. 9C illustrates a portion of the microfluidic device in a first stage of a reaction product harvesting process. A harvesting reagent flows from a harvesting port as illustrated in Fig. 5A through harvesting reagent input line 810 toward the sample chambers. As shown in Fig. 9A, valve 820 is open, allowing the harvesting reagent to flow into the topmost sample chamber. Closure of the interface valves 530 prevents the harvesting reagent from flowing into the assay chambers, which also contain reaction products. The extent to which the harvesting reagent initially fills the sample input lines and sample input chambers is limited by the closure of valve 822. As illustrated, the harvesting reagent has only partially filled a portion of the first sample chamber. The illustration of a side of the sample chamber being filled with harvesting reagent is merely provided by way of example, since the flow of the

harvesting reagent into the sample chamber is actually through vias extending from the plane of the figure. Closure of isolation valve 840 prevents harvesting reagent from flowing into the loading bowls, although other embodiments may enable such a flow if desired.

[0201] Fluid pressure resulting from the flow of the harvesting reagent into the array portion of the microfluidic device results in expansion of the sample input lines and sample chambers above the valve 822. The pump cycle is initiated by this pressurization of the sample chambers. As described below, closing of valve 820 and opening of valve 822 will enable the pressurized harvesting reagent and reaction products to be recovered from the microfluidic device as it flows through the microfluidic device.

[0202] Fig. 9D illustrates a portion of the microfluidic device in a second stage of the reaction product harvesting process. Although a second stage is illustrated, this is not intended to imply that the second stage immediately follows the first stage. As described below, the second stage is typically separated from the first stage by one or more intermediate stages of dilation pumping.

[0203] Dilation pumping (also know as volumetric capacitive pumping) is a method of operating a properly configured integrated fluidic circuit (microfluidic device) to obtain precise, low rate, low volume pumping through all configured elements of the microfluidic device. Dilation pumping is unique to microfluidic circuits that utilize channels that have one or more channel walls formed from an elastomeric material. As an example, the flow of the harvesting reagent through the sample input lines and sample chambers is considered volumetric capacitive pumping. Pumping proceeds by the closure of valves 822 and the opening of valves 820. As discussed above, harvesting reagent ports (not illustrated) are pressurized to introduce the harvesting reagent into the topmost sample input lines and sample chambers, which can be considered as a channel. The pressurization of microfluidic channels with at least one channel wall formed from an elastomeric material results in expansion of the elastomeric wall(s) outward from the channel with a resulting increase in channel volume that is proportional to the fluidic pressure (or gaseous pressure in alternate embodiments) within the channel, the elastic properties of the elastomeric channel wall material such as Young's modulus, and the length and cross sectional area of the channel. The sample input lines and sample chambers are

allowed to pressurize and then valves 820 is closed as illustrated in Fig. 9D. Following closure of valves 820, valves 822 are opened. The pumped volume through the sample input lines and the sample chambers is equal to the expanded volume of the channel when under pressure minus the native volume of the channel when pressure is released and the expanded elastomeric channel wall(s) is allow to relax. Dilation pumping is continued through repetitive cycles of closing 822, opening 820, pressurizing the sample input lines and sample chambers, closing 820, and opening 822. In this manner, continuous or discontinuous low volume pumping may be accomplished at precisely controlled flow rates.

[0204] Thus embodiments provide a method of dilation pumping that includes closing a first valve disposed between the sample chamber and the sample input port (i.e., valve 822), opening a second valve disposed between the harvesting port and the sample chamber (i.e., valve 820), closing the second valve, opening the first valve, and repeating these steps a predetermined number of times. Between the steps of opening the second valve and closing the second valve, the harvesting reagent flows into the sample input lines and sample chambers, pressurizing the channel as described above. After the dilation pumping process is complete, harvesting reagent substantially fills the sample input lines and sample chambers (e.g., recovery rates > 95%), thereby pooling the reaction products associated with a given sample in the sample input port from which the given sample was initially dispensed.

[0205] Dilation pumping provides benefits not typically available using conventional techniques. For example, dilation pumping enables for a slow removal of the reaction products from the microfluidic device. In an exemplary embodiment, the reaction products are recovered at a fluid flow rate of less than 100 μl per hour. In this example, for 48 reaction products distributed among the reaction chambers in each column, with a volume of each reaction product of about 1.5 μl, removal of the reaction products in a period of about 30 minutes, will result in a fluid flow rate of 72 μl/hour. (i.e., 48 * 1.5 / 0.5 hour). In other embodiments, the removal rate of the reaction products is performed at a rate of less than 90 μl/hr, 80 μl/hr, 70 μl/hr, 60 μl/hr, 50 μl/hr, 40 μl/hr, 30 μl/hr, 20 μl/hr, 10 μl/hr, 9 μl/hr, less than 8 μl/hr, less than 7 μl/hr, less than 6 μl/hr, less than 5 μl/hr, less than 4 μl/hr, less than 3 μl/hr, less than 2 μl/hr, less than 1 μl/hr, or less than 0.5 μl/hr.

[0206] Dilation pumping results in clearing of substantially a high percentage and potentially all the reaction products present in the microfluidic device. Some embodiments remove more than 75% of the reaction products present in the reaction chambers (e.g., sample chambers) of the microfluidic device. As an example, some embodiments remove more than 80%, 85%, 90%, 92 %, 95%, 96%, 97%, 98%, or 99% of the reaction products present in the reaction chambers.

[0207] In some embodiments, a harvesting valve is provided on the microfluidic device to obstruct the flow of harvesting reagent through the device. Application of a pressure source to a harvesting input port results in flow of harvesting fluid (e.g., a harvesting liquid) through harvest reagent input lines up to the harvesting valve. The permeability of the materials utilized to fabricate the microfluidic device enables such a harvesting fluid to fill the harvest reagent input lines, typically expelling air initially present in such lines. The presence of the harvesting valve will obstruct the flow of the harvest reagent at the location of the harvesting valve. Actuation (i.e., opening) of the harvesting valve will result in the harvesting fluid flowing through the harvest reagent input lines downstream of the harvesting valve. In other embodiments, a harvesting valve is replaced with one or more other suitable valves as appropriate to the particular application. For example, in the embodiment illustrated in Figs. 9A-9D, valve 820 serves to prevent flow of harvesting reagent until the dilation pumping process is initiated.

[0208] Fabrication methods using elastomeric materials and methods for design of devices and their components have been described in detail in the scientific and patent literature. See, *e.g.*, Unger *et al.* (2000) Science 288:113-116; U.S. Pat. Nos. US 6,960,437 (Nucleic acid amplification utilizing microfluidic devices); 6,899,137 (Microfabricated elastomeric valve and pump systems); 6,767,706 (Integrated active flux microfluidic devices and methods); 6,752,922 (Microfluidic chromatography); 6,408,878 (Microfabricated elastomeric valve and pump systems); 6,645,432 (Microfluidic systems including three-dimensionally arrayed channel networks); U.S. Patent Application Publication Nos. 2004/0115838; 2005/0072946; 2005/0000900; 2002/0127736; 2002/0109114; 2004/0115838; 2003/0138829; 2002/0164816; 2002/0127736; and 2002/0109114; PCT Publication Nos. WO 2005/084191; WO 05/030822A2; and WO 01/01025; Quake & Scherer, 2000, "From micro to nanofabrication with soft materials" Science 290: 1536-40; Unger *et al.*,

2000, "Monolithic microfabricated valves and pumps by multilayer soft lithography" Science 288:113-116; Thorsen *et al.*, 2002, "Microfluidic large-scale integration" Science 298:580-584; Chou *et al.*, 2000, "Microfabricated Rotary Pump" Biomedical Microdevices 3:323-330; Liu *et al.*, 2003, "Solving the "world-to-chip" interface problem with a microfluidic matrix" Analytical Chemistry 75, 4718-23, Hong et al, 2004, "A nanoliter-scale nucleic acid processor with parallel architecture" Nature Biotechnology 22:435-39.

[0209] According to certain embodiments describer herein, the detection and/or quantification of one or more target nucleic acids from one or more samples may generally be carried out on a microfluidic device by obtaining a sample, optionally pre-amplifying the sample, and distributing the optionally pre-amplified sample, or aliquots thereof, into reaction chambers of a microfluidic device containing the appropriate buffers, primers, optional probe(s), and enzyme(s), subjecting these mixtures to amplification, and querying the aliquots for the presence of amplified target nucleic acids. The sample aliquots may have a volume of less than 1 picoliter or, in various embodiments, in the range of about 1 picoliter to about 500 nanoliters, in a range of about 2 picoliters to about 50 picoliters, in a range of about 5 picoliters to about 25 picoliters, in the range of about 100 picoliters to about 20 nanoliters, in the range of about 1 nanoliter to about 20 nanoliters, and in the range of about 5 nanoliters to about 15 nanoliters. In many embodiments, sample aliquots account for the majority of the volume of the amplification mixtures. Thus, amplification mixtures can have a volume of less than 1 picoliter or, in various embodiments about 2, about 5 about 7, about 10, about 15, about 20, about 25, about 50, about 100, about 250, about 500, and about 750 picoliters; or about 1, about 2, about 5, about 7, about 15, about 20, about 25, about 50, about 250, and about 500 nanoliters. The amplification mixtures can also have a volume within any range bounded by any of these values (e.g., about 2 picoliters to about 50 picoliters).

[0210] In certain embodiments, multiplex detection is carried out in individual amplification mixture, *e.g.*, in individual reaction chambers of a microfluidic device, which can be used to further increase the number of samples and/or targets that can be analyzed in a single assay or to carry out comparative methods, such as comparative genomic hybridization (CGH). In various embodiments, up to 2, 3, 4, 5, 6, 7, 8, 9, 10,

50, 100, 500, 1000, 5000, 10000 or more amplification reactions are carried out in each individual reaction chamber.

[0211] In specific embodiments, the assay usually has a dynamic range of at least 3 orders of magnitude, more often at least 4, at least 5, at least 6, at least 7, or at least 8 orders of magnitude.

Quantitative Real-Time PCR and Other Detection and Quantification Methods

[0212] Any method of detection and/or quantification of nucleic acids can be used in the invention to detect amplification products. In one embodiment, PCR (polymerase chain reaction) is used to amplify and/or quantify target nucleic acids. In other embodiments, other amplification systems or detection systems are used, including, e.g., systems described in U.S. Pat. No. 7,118,910 (which is incorporated herein by reference in its entirety for its description of amplification/detection systems) and Invader assays; PE BioSystems). In particular embodiments, real-time quantification methods are used. For example, "quantitative real-time PCR" methods can be used to determine the quantity of a target nucleic acid present in a sample by measuring the amount of amplification product formed during the amplification process itself.

[0213] Fluorogenic nuclease assays are one specific example of a real-time quantification method that can be used successfully in the methods described herein. This method of monitoring the formation of amplification product involves the continuous measurement of PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe--an approach frequently referred to in the literature as the "TaqMan® method." See U.S. Pat. No. 5,723,591; Heid et al., 1996, Real-time quantitative PCR Genome Res. 6:986-94, each incorporated herein by reference in their entireties for their descriptions of fluorogenic nuclease assays. It will be appreciated that while "TaqMan® probes" are the most widely used for qPCR, the invention is not limited to use of these probes; any suitable probe can be used.

[0214] Other detection/quantification methods that can be employed in the present invention include FRET and template extension reactions, molecular beacon detection, Scorpion detection, Invader detection, and padlock probe detection.

[0215] FRET and template extension reactions utilize a primer labeled with one member of a donor/acceptor pair and a nucleotide labeled with the other member of the donor/acceptor pair. Prior to incorporation of the labeled nucleotide into the primer during a template-dependent extension reaction, the donor and acceptor are spaced far enough apart that energy transfer cannot occur. However, if the labeled nucleotide is incorporated into the primer and the spacing is sufficiently close, then energy transfer occurs and can be detected. These methods are particularly useful in conducting single base pair extension reactions in the detection of single nucleotide polymorphisms and are described in U.S. Patent No. 5,945,283 and PCT Publication WO 97/22719.

[0216] With molecular beacons, a change in conformation of the probe as it hybridizes to a complementary region of the amplified product results in the formation of a detectable signal. The probe itself includes two sections: one section at the 5' end and the other section at the 3' end. These sections flank the section of the probe that anneals to the probe binding site and are complementary to one another. One end section is typically attached to a reporter dye and the other end section is usually attached to a quencher dye. In solution, the two end sections can hybridize with each other to form a hairpin loop. In this conformation, the reporter and quencher dye are in sufficiently close proximity that fluorescence from the reporter dye is effectively quenched by the quencher dye. Hybridized probe, in contrast, results in a linearized conformation in which the extent of quenching is decreased. Thus, by monitoring emission changes for the two dyes, it is possible to indirectly monitor the formation of amplification product. Probes of this type and methods of their use are described further, for example, by Piatek et al., 1998, Nat. Biotechnol. 16:359-63; Tyagi, and Kramer, 1996, Nat. Biotechnology 14:303-308; and Tyagi, et al., 1998, Nat. Biotechnol. 16:49-53 (1998).

[0217] The Scorpion detection method is described, for example, by Thelwell et al. 2000, Nucleic Acids Research, 28:3752-3761 and Solinas et al., 2001, "Duplex Scorpion primers in SNP analysis and FRET applications" Nucleic Acids Research 29:20. Scorpion primers are fluorogenic PCR primers with a probe element attached at the 5'-end via a PCR stopper. They are used in real-time amplicon-specific detection of PCR products in homogeneous solution. Two different formats are possible, the "stem–loop" format and the "duplex" format. In both cases the probing

mechanism is intramolecular. The basic elements of Scorpions in all formats are: (i) a PCR primer; (ii) a PCR stopper to prevent PCR read-through of the probe element; (iii) a specific probe sequence; and (iv) a fluorescence detection system containing at least one fluorophore and quencher. After PCR extension of the Scorpion primer, the resultant amplicon contains a sequence that is complementary to the probe, which is rendered single-stranded during the denaturation stage of each PCR cycle. On cooling, the probe is free to bind to this complementary sequence, producing an increase in fluorescence, as the quencher is no longer in the vicinity of the fluorophore. The PCR stopper prevents undesirable read-through of the probe by Taq DNA polymerase.

[0218] Invader assays (Third Wave Technologies, Madison, WI) are used particularly for SNP genotyping and utilize an oligonucleotide, designated the signal probe, that is complementary to the target nucleic acid (DNA or RNA) or polymorphism site. A second oligonucleotide, designated the Invader Oligo, contains the same 5' nucleotide sequence, but the 3' nucleotide sequence contains a nucleotide polymorphism. The Invader Oligo interferes with the binding of the signal probe to the target nucleic acid such that the 5' end of the signal probe forms a "flap" at the nucleotide containing the polymorphism. This complex is recognized by a structure specific endonuclease, called the Cleavase enzyme. Cleavase cleaves the 5' flap of the nucleotides. The released flap binds with a third probe bearing FRET labels, thereby forming another duplex structure recognized by the Cleavase enzyme. This time, the Cleavase enzyme cleaves a fluorophore away from a quencher and produces a fluorescent signal. For SNP genotyping, the signal probe will be designed to hybridize with either the reference (wild type) allele or the variant (mutant) allele. Unlike PCR, there is a linear amplification of signal with no amplification of the nucleic acid. Further details sufficient to guide one of ordinary skill in the art are provided by, for example, Neri, B.P., et al., *Advances in Nucleic Acid and Protein Analysis* 3826:117-125, 2000) and U.S. Patent No. 6,706,471.

[0219] Padlock probes (PLPs) are long (e.g., about 100 bases) linear oligonucleotides. The sequences at the 3' and 5' ends of the probe are complementary to adjacent sequences in the target nucleic acid. In the central, noncomplementary region of the PLP there is a "tag" sequence that can be used to identify the specific PLP. The tag sequence is flanked by universal priming sites,

which allow PCR amplification of the tag. Upon hybridization to the target, the two ends of the PLP oligonucleotide are brought into close proximity and can be joined by enzymatic ligation. The resulting product is a circular probe molecule catenated to the target DNA strand. Any unligated probes (i.e., probes that did not hybridize to a target) are removed by the action of an exonuclease. Hybridization and ligation of a PLP requires that both end segments recognize the target sequence. In this manner, PLPs provide extremely specific target recognition.

[0220] The tag regions of circularized PLPs can then be amplified and resulting amplicons detected. For example, TaqMan® real-time PCR can be carried out to detect and quantify the amplicon. The presence and amount of amplicon can be correlated with the presence and quantity of target sequence in the sample. For descriptions of PLPs see, e.g., Landegren et al., 2003, Padlock and proximity probes for in situ and array-based analyses: tools for the post-genomic era, *Comparative and Functional Genomics* 4:525-30; Nilsson et al., 2006, Analyzing genes using closing and replicating circles *Trends Biotechnol*. 24:83-8; Nilsson et al., 1994, Padlock probes: circularizing oligonucleotides for localized DNA detection, *Science* 265:2085-8.

[0221] In particular embodiments, fluorophores that can be used as detectable labels for probes include, but are not limited to, rhodamine, cyanine 3 (Cy 3), cyanine 5 (Cy 5), fluorescein, Vic™, Liz™., Tamra™, 5-Fam™, 6-Fam™, and Texas Red (Molecular Probes). (Vic™, Liz™, Tamra™, 5-Fam™, 6-Fam™ are all available from Applied Biosystems, Foster City, Calif.).

[0222] Devices have been developed that can perform a thermal cycling reaction with compositions containing a fluorescent indicator, emit a light beam of a specified wavelength, read the intensity of the fluorescent dye, and display the intensity of fluorescence after each cycle. Devices comprising a thermal cycler, light beam emitter, and a fluorescent signal detector, have been described, e.g., in U.S. Pat. Nos. 5,928,907; 6,015,674; and 6,174,670.

[0223] In some embodiments, each of these functions can be performed by separate devices. For example, if one employs a Q-beta replicase reaction for amplification, the reaction may not take place in a thermal cycler, but could include a

light beam emitted at a specific wavelength, detection of the fluorescent signal, and calculation and display of the amount of amplification product.

[0224] In particular embodiments, combined thermal cycling and fluorescence detecting devices can be used for precise quantification of target nucleic acids. In some embodiments, fluorescent signals can be detected and displayed during and/or after one or more thermal cycles, thus permitting monitoring of amplification products as the reactions occur in "real-time." In certain embodiments, one can use the amount of amplification product and number of amplification cycles to calculate how much of the target nucleic acid sequence was in the sample prior to amplification.

[0225] According to some embodiments, one can simply monitor the amount of amplification product after a predetermined number of cycles sufficient to indicate the presence of the target nucleic acid sequence in the sample. One skilled in the art can easily determine, for any given sample type, primer sequence, and reaction condition, how many cycles are sufficient to determine the presence of a given target nucleic acid.

[0226] According to certain embodiments, one can employ an internal standard to quantify the amplification product indicated by the fluorescent signal. See, e.g., U.S. Pat. No. 5,736,333.

[0227] In various embodiments, employing preamplification, the number of preamplification cycles is sufficient to add one or more nucleotide tags to the target nucleotide sequences, so that the relative copy numbers of the tagged target nucleotide sequences is substantially representative of the relative copy numbers of the target nucleic acids in the sample. For example, preamplification can be carried out for 2-20 cycles to introduce the sample-specific or set-specific nucleotide tags. In other embodiments, detection is carried out at the end of exponential amplification, i.e., during the "plateau" phase, or endpoint PCR is carried out. In this instance, preamplification will normalize amplicon copy number across targets and across samples. In various embodiments, preamplification and/or amplification can be carried out for about: 2, 4, 10, 15, 20, 25, 30, 35, or 40 cycles or for a number of cycles falling within any range bounded by any of these values.

Labeling Strategies

[0228] Any suitable labeling strategy can be employed in the methods of the invention. Where the assay mixture is aliquoted, and each aliquot is analyzed for presence of a single amplification product, a universal detection probe can be employed in the amplification mixture. In particular embodiments, real-time PCR detection can be carried out using a universal qPCR probe. Suitable universal qPCR probes include double-stranded DNA dyes, such as SYBR Green, Pico Green (Molecular Probes, Inc., Eugene, OR), Eva Green (Biotinum), ethidium bromide, and the like (see Zhu et al., 1994, *Anal. Chem.* 66:1941-48). Suitable universal qPCR probes also include sequence-specific probes that bind to a nucleotide sequence present in all amplification products. Binding sites for such probes can be conveniently introduced into the tagged target nucleic acids during amplification.

[0229] Alternatively, one or more target-specific qPCR probes (i.e., specific for a target nucleotide sequence to be detected) is employed in the amplification mixtures to detect amplification products. Target-specific probes could be useful, e.g., when only a few target nucleic acids are to be detected in a large number of samples. For example, if only three targets were to be detected, a target-specific probe with a different fluorescent label for each target could be employed. By judicious choice of labels, analyses can be conducted in which the different labels are excited and/or detected at different wavelengths in a single reaction. See, e.g., Fluorescence Spectroscopy (Pesce et al., Eds.) Marcel Dekker, New York, (1971); White et al., Fluorescence Analysis: A Practical Approach, Marcel Dekker, New York, (1970); Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd ed., Academic Press, New York, (1971); Griffiths, Colour and Constitution of Organic Molecules, Academic Press, New York, (1976); Indicators (Bishop, Ed.). Pergamon Press, Oxford, 19723; and Haugland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Eugene (1992).

Removal of Undesired Reaction Components

[0230] It will be appreciated that reactions involving complex mixtures of nucleic acids in which a number of reactive steps are employed can result in a variety of unincorporated reaction components, and that removal of such unincorporated reaction components, or reduction of their concentration, by any of a variety of clean-

up procedures can improve the efficiency and specificity of subsequently occurring reactions. For example, it may be desirable, in some embodiments, to remove, or reduce the concentration of preamplification primers prior to carrying out the amplification steps described herein.

[0231] In certain embodiments, the concentration of undesired components can be reduced by simple dilution. For example, preamplified samples can be diluted about 2-, 5-, 10-, 50-, 100-, 500-, 1000-fold prior to amplification to improve the specificity of the subsequent amplification step.

[0232] In some embodiments, undesired components can be removed by a variety of enzymatic means. Alternatively, or in addition to the above-described methods, undesired components can be removed by purification. For example, a purification tag can be incorporated into any of the above-described primers (e.g., into the barcode nucleotide sequence) to facilitate purification of the tagged target nucleotides.

[0233] In particular embodiments, clean-up includes selective immobilization of the desired nucleic acids. For example, desired nucleic acids can be preferentially immobilized on a solid support. In an illustrative embodiment, an affinity moiety, such as biotin (e.g., photo-biotin), is attached to desired nucleic acid, and the resulting biotin-labeled nucleic acids immobilized on a solid support comprising an affinity moiety-binder such as streptavidin. Immobilized nucleic acids can be queried with probes, and non-hybridized and/or non-ligated probes removed by washing (See, e.g., Published P.C.T. Application WO 03/006677 and USSN 09/931,285.) Alternatively, immobilized nucleic acids can be washed to remove other components and then released from the solid support for further analysis. This approach can be used, for example, in recovering target amplicons from amplification mixtures after the addition of primer binding sites for DNA sequencing. In particular embodiments, an affinity moiety, such as biotin, can be attached to an amplification primer such that amplification produces an affinity moiety-labeled (e.g., biotin-labeled) amplicon. Thus, for example, where three primers are employed to add barcode and nucleotide tag elements to a target nucleotide sequence, as described above, at least one of the barcode or reverse primers can include an affinity moiety. Where four primers (two inner primers and two outer primers) are employed to add desired element to a target nucleotide sequence, at least one of the outer primers can include an affinity moiety.

Data Output and Analysis

[0234] In certain embodiments, when the methods of the invention are carried out on a matrix-type microfluidic device, the data can be output as a heat matrix (also termed "heat map"). In the heat matrix, each square, representing a reaction chamber on the DA matrix, has been assigned a color value which can be shown in gray scale, but is more typically shown in color. In gray scale, black squares indicate that no amplification product was detected, whereas white squares indicate the highest level of amplification produce, with shades of gray indicating levels of amplification product in between. In a further aspect, a software program may be used to compile the data generated in the heat matrix into a more reader-friendly format.

Applications

[0235] The methods of the invention are applicable to any technique aimed at detecting the presence or amount of one or more target nucleic acids in a nucleic acid sample. Thus, for example, these methods are applicable to identifying the presence of particular polymorphisms (such as SNPs), alleles, or haplotypes, or chromosomal abnormalities, such as amplifications, deletions, or aneuploidy. The methods may be employed in genotyping, which can be carried out in a number of contexts, including diagnosis of genetic diseases or disorders, pharmacogenomics (personalized medicine), quality control in agriculture (e.g., for seeds or livestock), the study and management of populations of plants or animals (e.g., in aquaculture or fisheries management or in the determination of population diversity), or paternity or forensic identifications. The methods of the invention can be applied in the identification of sequences indicative of particular conditions or organisms in biological or environmental samples. For example, the methods can be used in assays to identify pathogens, such as viruses, bacteria, and fungi). The methods can also be used in studies aimed at characterizing environments or microenvironments, e.g., characterizing the microbial species in the human gut.

[0236] These methods can also be employed in determinations DNA or RNA copy number. Determinations of aberrant DNA copy number in genomic DNA is useful, for example, in the diagnosis and/or prognosis of genetic defects and diseases, such as cancer. Determination of RNA "copy number," i.e., expression level is useful for expression monitoring of genes of interest, e.g., in different individuals, tissues, or

cells under different conditions (e.g., different external stimuli or disease states) and/or at different developmental stages.

[0237] In addition, the methods can be employed to prepare nucleic acid samples for further analysis, such as, e.g., DNA sequencing.

[0238] Finally, nucleic acid samples can be tagged as a first step, prior subsequent analysis, to reduce the risk that mislabeling or cross-contamination of samples will compromise the results. For example, any physician's office, laboratory, or hospital could tag samples immediately after collection, and the tags could be confirmed at the time of analysis. Similarly, samples containing nucleic acids collected at a crime scene could be tagged as soon as practicable, to ensure that the samples could not be mislabeled or tampered with. Detection of the tag upon each transfer of the sample from one party to another could be used to establish chain of custody of the sample.

Kits

[0239] Kits according to the invention include one or more reagents useful for practicing one or more assay methods of the invention. A kit generally includes a package with one or more containers holding the reagent(s) (e.g., primers and/or probe(s)), as one or more separate compositions or, optionally, as admixture where the compatibility of the reagents will allow. The kit can also include other material(s) that may be desirable from a user standpoint, such as a buffer(s), a diluent(s), a standard(s), and/or any other material useful in sample processing, washing, or conducting any other step of the assay.

[0240] Kits according to the invention generally include instructions for carrying out one or more of the methods of the invention. Instructions included in kits of the invention can be affixed to packaging material or can be included as a package insert. While the instructions are typically written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to, electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), RF tags, and the like. As used herein, the term "instructions" can include the address of an internet site that provides the instructions.

[0241] 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.

[0242] In addition, all other publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

Example 1

Multi-Primer Amplification Method For Barcoding Of Target Nucleic Acids in Preparation for DNA Sequencing

[0243] Genomic DNA samples (BioChain, USA) at 100 and 0 ng/ml (negative control ["NTC"]) were amplified for 25 cycles 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) with the following primer pairs at 200 nM per primer: 1) 454 tails; 2) A5 specific primers; and 3) the three primers shown in Fig. 10. PCR was performed in 15 μl reaction volumes containing 7.5 μl of FastStart TaqMan® Probe Master (Roche Diagnostics, USA), 0.75 μl of DA sample loading reagent (Fluidigm Corp, USA) and 6.75 μl of sample. Thermal cycling condition included an initial hot start at 50 °C for 2 minutes and at 94 °C for 10 minutes, followed by 25 cycles at 94 °C for 15 s, 70 °C for 5s, 60°C for 30 s and 72°C for 90 s. The resulting amplification products were run on an electrophoresis gel (Invitrogen, USA) using 8 μl of the reaction mixture per lane following the manufacturer instruction. See Fig. 11, which shows that the 3-primer method produced an amplicon of the correct size. The amplicons generated from PCR amplification were purified using Ampure Beads® and then re-amplified on a PCR plate with 454 tail primers, followed by Sanger sequencing with either 454 tail primers, which showed that the 3-primer method generated an amplicon having the correct sequence.

Example 2

Multi-Primer Amplification Method For Quantifying Target Nucleic Acids in Preparation for DNA Sequencing

[0244] Primers for preparing genomic DNA for sequencing using various DNA conventional DNA sequencing methods are shown below.

ShotGun Forward: 5'-CCATCTCATCCCTGCGTGTC-3' (SEQ ID NO:1)

ShotGun Reverse: 5'-CCTATCCCCTGTGTGCCTTG-3' (SEQ ID NO:2)

ShotGun UPR Forward: 5'-GGCGGCGACCATCTCATCCCTGCGTGTC-3' (SEQ ID NO:3)

MID Forward: 5'-GCCTCCCTCGCGCCATCAG-3' (SEQ ID NO:4)

MID Reverse: 5'-GCCTTGCCAGCCCGCTCAG-3' (SEQ ID NO:5)

MID UPR Forward: 5'-GGCGGCGAGCCTCCCTCGCGCCATCAG-3' (SEQ ID NO:6)

Solexa Forward: 5'-ACACTCTTTCCCTACACGA-3' (SEQ ID NO:7)

Solexa Reverse: 5'-CAAGCAGAAGACGGCATA-3' (SEQ ID NO:8)

Solexa UPR Forward: 5'-GGCGGCGAACACTCTTTCCCTACACGA-3' (SEQ ID NO:9)

Solid Forward: 5'-CCACTACGCCTCCGCTTTCCTCTCTATG-3' (SEQ ID NO:10)

Solid Reverse: 5'-CTGCCCCGGGTTCCTCATTCT-3' (SEQ ID NO:11)

Solid UPR Forward: 5'-GGCGGCGACCACTACGCCTCCGCTTTCCTCTCTATG-3' (SEQ ID NO:12)

454 Titanium Forward: 5'-CCATCTCATCCCTGCGTG-3' (SEQ ID NO:13)

454 Titanium Reverse: 5'-CCTATCCCCTGTGTGCCTTG-3' (SEQ ID NO:14)

454 Titanium UPR Forward: 5'-GGCGGCGACCATCTCATCCCTGCGTG-3' (SEQ ID NO:15)

Solexa smRNA Forward: 5'-TAATGATACGGCGACCACC-3' (SEQ ID NO:16)

Solexa smRNA Reverse: 5'-ACAAGCAGAAGACGGCATAC-3' (SEQ ID NO:17)

Solexa smRNA UPL Forward: 5'-GGCGGCGATAATGATACGGCGACCAC-3' (SEQ ID NO:18)

[0245] The properties of these primers is shown in Table 1 below.

Table 1

454-standard (ShotGun)	Primer	Length (nt)	CG%	Tm (°C)	Primer-Dimer
	ShotGun Forward:	20	60	68.4	No self/cross-dimer, 1.5°C diff in Tm
	ShotGun Reverse:	20	60	66.9	
	ShotGun UPR Forward:	28	67.8	84.8	
454-MID					
	MID Forward:	19	73.6	74.9	4-bases of self-dimer(F.UPL) & cross-dimer(F./UPL , R/UPL)
	MID Reverse:	19	73.6	74.9	High GC
	MID UPR Forward:	27	77.7	88.5	
Solexa					
	Solexa Forward:	19	47.3	57.8	No dimer, 2.1°C diff in Tm
	Solexa Reverse:	18	50	60.6	Low GC
	Solexa UPR Forward:	27	59.2	78.4	
Solid					
	Solid Forward:	28	57.1	74.7	Strong self-dimer & cross-dimer
	Solid Reverse:	21	61.9	72.5	variety of GC & Tm
	Solid UPR Forward:	36	63.8	85.6	

[0246] The reaction mixture used for amplification of genomic DNA to incorporate primer sequences is given below in Table 2.

Table 2

	Add V µl of TE into dry probe tube	
100uM stock solution	V= "Total nmol" value of the dry probe * 10	
10X Fluidigm Assay		
	100 µMol	
Forward:	4	2000 nM
UPR Forward:	4	2000 nM
Reverse:	8	4000 nM
TE:	184	
Total:	200	

Example 3

Additional Illustrative Primers For Barcoding Of Target Nucleic Acids in Preparation for 454 DNA Sequencing

[0247] Tables 3 and 4 below show additional illustrative primers for barcoding of target nucleic acids in preparation for 454 DNA sequencing. "454F" refers to a 454 forward primer binding site; "454R" refers to 454 reverse primer binding site. "BC" refers to a nucleotide barcode. "TAG" refers to a nucleotide tag. "P53" refers to a target-specific primer sequence.

Table 3

Sequence Name	Sequence	SEQ ID
454F-BC1-TAG8	GCCTCCCTCGCGCCATCAGGCATGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:19)
454F-BC2-TAG8	GCCTCCCTCGCGCCATCAGCGTACGACACTGACGACA TGGTTCTACA	(SEQ ID NO:20)
454F-BC3-TAG8	GCCTCCCTCGCGCCATCAGGTCAGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:21)
454F-BC4-TAG8	GCCTCCCTCGCGCCATCAGAGCTGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:22)
454F-BC5-TAG8	GCCTCCCTCGCGCCATCAGTGCATCACACTGACGACA TGGTTCTACA	(SEQ ID NO:23)
454F-BC6-TAG8	GCCTCCCTCGCGCCATCAGCTGATGACACTGACGACA TGGTTCTACA	(SEQ ID NO:24)
454F-BC7-TAG8	GCCTCCCTCGCGCCATCAGGTAGTCACACTGACGACA TGGTTCTACA	(SEQ ID NO:25)

454F-BC8-TAG8	GCCTCCCTCGCGCCATCAGGTCGATACACTGACGACA TGGTTCTACA	(SEQ ID NO:26)
454F-BC9-TAG8	GCCTCCCTCGCGCCATCAGGATACGACACTGACGACA TGGTTCTACA	(SEQ ID NO:27)
454F-BC10-TAG8	GCCTCCCTCGCGCCATCAGTGATGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:28)
454F-BC11-TAG8	GCCTCCCTCGCGCCATCAGAGCTGAACACTGACGACA TGGTTCTACA	(SEQ ID NO:29)
454F-BC12-TAG8	GCCTCCCTCGCGCCATCAGACTGTAACACTGACGACA TGGTTCTACA	(SEQ ID NO:30)
454F-BC13-TAG8	GCCTCCCTCGCGCCATCAGTGCATGACACTGACGACA TGGTTCTACA	(SEQ ID NO:31)
454F-BC14-TAG8	GCCTCCCTCGCGCCATCAGAGTCTAACACTGACGACA TGGTTCTACA	(SEQ ID NO:32)
454F-BC15-TAG8	GCCTCCCTCGCGCCATCAGTGTCTGACACTGACGACA TGGTTCTACA	(SEQ ID NO:33)
454F-BC16-TAG8	GCCTCCCTCGCGCCATCAGGCTAGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:34)
454F-BC17-TAG8	GCCTCCCTCGCGCCATCAGGATAGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:35)
454F-BC18-TAG8	GCCTCCCTCGCGCCATCAGGCTACTACACTGACGACA TGGTTCTACA	(SEQ ID NO:36)
454F-BC19-TAG8	GCCTCCCTCGCGCCATCAGCTATGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:37)
454F-BC20-TAG8	GCCTCCCTCGCGCCATCAGGCTATGACACTGACGACA TGGTTCTACA	(SEQ ID NO:38)
454F-BC21-TAG8	GCCTCCCTCGCGCCATCAGCGTGCAACACTGACGACA TGGTTCTACA	(SEQ ID NO:39)
454F-BC22-TAG8	GCCTCCCTCGCGCCATCAGATAGCTACACTGACGACA TGGTTCTACA	(SEQ ID NO:40)
454F-BC23-TAG8	GCCTCCCTCGCGCCATCAGTGTAGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:41)
454F-BC24-TAG8	GCCTCCCTCGCGCCATCAGGTGCTAACACTGACGACA TGGTTCTACA	(SEQ ID NO:42)
454F-BC25-TAG8	GCCTCCCTCGCGCCATCAGGTCATGACACTGACGACA TGGTTCTACA	(SEQ ID NO:43)
454F-BC26-TAG8	GCCTCCCTCGCGCCATCAGATCGTGACACTGACGACA TGGTTCTACA	(SEQ ID NO:44)
454F-BC27-TAG8	GCCTCCCTCGCGCCATCAGTGTACGACACTGACGACA TGGTTCTACA	(SEQ ID NO:45)
454F-BC28-TAG8	GCCTCCCTCGCGCCATCAGAGTGTAACACTGACGACA TGGTTCTACA	(SEQ ID NO:46)
454F-BC29-TAG8	GCCTCCCTCGCGCCATCAGTGACAGACACTGACGACA TGGTTCTACA	(SEQ ID NO:47)
454F-BC30-TAG8	GCCTCCCTCGCGCCATCAGGATCACACACTGACGACA TGGTTCTACA	(SEQ ID NO:48)
454F-BC31-TAG8	GCCTCCCTCGCGCCATCAGCTAGAGACACTGACGACA TGGTTCTACA	(SEQ ID NO:49)
454F-BC32-TAG8	GCCTCCCTCGCGCCATCAGCTAGTCACACTGACGACA TGGTTCTACA	(SEQ ID NO:50)
454F-BC33-TAG8	GCCTCCCTCGCGCCATCAGAGCTAGACACTGACGACA TGGTTCTACA	(SEQ ID NO:51)
454F-BC34-TAG8	GCCTCCCTCGCGCCATCAGTGACTGACACTGACGACA TGGTTCTACA	(SEQ ID NO:52)
454F-BC35-TAG8	GCCTCCCTCGCGCCATCAGTGATAGACACTGACGACA TGGTTCTACA	(SEQ ID NO:53)
454F-BC36-TAG8	GCCTCCCTCGCGCCATCAGCGTATCACACTGACGACA TGGTTCTACA	(SEQ ID NO:54)
454F-BC37-TAG8	GCCTCCCTCGCGCCATCAGGTCTGAACACTGACGACA	(SEQ ID NO:55)

	TGGTTCTACA	
454F-BC38-TAG8	GCCTCCCTCGCGCCATCAGCATGACACACTGACGACA TGGTTCTACA	(SEQ ID NO:56)
454F-BC39-TAG8	GCCTCCCTCGCGCCATCAGCGATGAACACTGACGACA TGGTTCTACA	(SEQ ID NO:57)
454F-BC40-TAG8	GCCTCCCTCGCGCCATCAGGCTGATACACTGACGACA TGGTTCTACA	(SEQ ID NO:58)
454F-BC41-TAG8	GCCTCCCTCGCGCCATCAGCAGTACACACTGACGACA TGGTTCTACA	(SEQ ID NO:59)
454F-BC42-TAG8	GCCTCCCTCGCGCCATCAGGCGACTACACTGACGACA TGGTTCTACA	(SEQ ID NO:60)
454F-BC43-TAG8	GCCTCCCTCGCGCCATCAGGTACGAACACTGACGACA TGGTTCTACA	(SEQ ID NO:61)
454F-BC44-TAG8	GCCTCCCTCGCGCCATCAGACGCTAACACTGACGACA TGGTTCTACA	(SEQ ID NO:62)
454F-BC45-TAG8	GCCTCCCTCGCGCCATCAGAGCATCACACTGACGACA TGGTTCTACA	(SEQ ID NO:63)
454F-BC46-TAG8	GCCTCCCTCGCGCCATCAGGATGCTACACTGACGACA TGGTTCTACA	(SEQ ID NO:64)
454F-BC47-TAG8	GCCTCCCTCGCGCCATCAGGTCTGCACACTGACGACA TGGTTCTACA	(SEQ ID NO:65)
454F-BC48-TAG8	GCCTCCCTCGCGCCATCAGATGCGAACACTGACGACA TGGTTCTACA	(SEQ ID NO:66)

Table 4

Sequence Name	Sequence	SEQ ID
TAG8-P53-1+	ACACTGACGACATGGTTCTACAACTGTCCAGCTTTGT GCC	(SEQ ID NO:67)
TAG8-P53-2+	ACACTGACGACATGGTTCTACAGATCATCATAGGAGT TGCATTGTTG	(SEQ ID NO:68)
TAG8-P53-3+	ACACTGACGACATGGTTCTACACGGACCTTTGTCCTT CCT	(SEQ ID NO:69)
TAG8-P53-4+	ACACTGACGACATGGTTCTACAATGCAAACCTCAATC CCTCC	(SEQ ID NO:70)
TAG8-P53-5+	ACACTGACGACATGGTTCTACAAGTTTCTTCCCATGC ACCTG	(SEQ ID NO:71)
TAG8-P53-6+	ACACTGACGACATGGTTCTACAGTGAATCCCCGTCTC TACTAAAA	(SEQ ID NO:72)
TAG8-P53-7+	ACACTGACGACATGGTTCTACATGTTTCCCATTTGCG GTTATGA	(SEQ ID NO:73)
TAG8-P53-8+	ACACTGACGACATGGTTCTACAAGTTGTGGGACTGCT TTATACATT	(SEQ ID NO:74)
454R-P53-1-	GCCTTGCCAGCCCGCTCAGTCCTCTGCCTAGGCGTT	(SEQ ID NO:75)
454R-P53-2-	GCCTTGCCAGCCCGCTCAGGAAATGTAAATGTGGAGC CAAACA	(SEQ ID NO:76)
454R-P53-3-	GCCTTGCCAGCCCGCTCAGACTCATTCTTGAAAATAC CTCCGG	(SEQ ID NO:77)
454R-P53-4-	GCCTTGCCAGCCCGCTCAGAAATGCCACCTCGATTTA GGAAA	(SEQ ID NO:78)
454R-P53-5-	GCCTTGCCAGCCCGCTCAGTCACCCTCCCGAATAGCT	(SEQ ID NO:79)
454R-P53-6-	GCCTTGCCAGCCCGCTCAGAGTGTAAAATGGTACAAC CGCT	(SEQ ID NO:80)
454R-P53-7-	GCCTTGCCAGCCCGCTCAGCCTCTTAAGATACTGTAA ACTCTGTAAAGC	(SEQ ID NO:81)
454R-P53-8-	GCCTTGCCAGCCCGCTCAGATTGTGCCATTGTACTCT	(SEQ ID NO:82)

	AGCC	
TAG8-P53-9+	ACACTGACGACATGGTTCTACACTTCCTTTCTCTACTG AATGCTTTTAATTT	(SEQ ID NO:83)
TAG8-P53-10+	ACACTGACGACATGGTTCTACATCTTACACAAACTCT TCAGAAAACAGA	(SEQ ID NO:84)
TAG8-P53-11+	ACACTGACGACATGGTTCTACAGTACCAAAACCAAA CAAGGACAT	(SEQ ID NO:85)
TAG8-P53-12+	ACACTGACGACATGGTTCTACAGGTGAAACGCCATCT CTACTAA	(SEQ ID NO:86)
TAG8-P53-13+	ACACTGACGACATGGTTCTACATCATGATTGTAGCTG ATTCAACATTCA	(SEQ ID NO:87)
TAG8-P53-14+	ACACTGACGACATGGTTCTACAACTAGCATGCTGAAA CCCC	(SEQ ID NO:88)
TAG8-P53-15+	ACACTGACGACATGGTTCTACATCAGGAGATCGAGA CCATCC	(SEQ ID NO:89)
TAG8-P53-16+	ACACTGACGACATGGTTCTACATCATGCCTGTAATCC CAGC	(SEQ ID NO:90)
454R-P53-9-	GCCTTGCCAGCCCGCTCAGACCTCAAATGATCCCCTG C	(SEQ ID NO:91)
454R-P53-10-	GCCTTGCCAGCCCGCTCAGATTACAGGCGTGAGCCAC	(SEQ ID NO:92)
454R-P53-11-	GCCTTGCCAGCCCGCTCAGTTTTGAGATGAAGTCTTG CTCTGT	(SEQ ID NO:93)
454R-P53-12-	GCCTTGCCAGCCCGCTCAGTAAAGACCAGTCTGACTA TGTTGC	(SEQ ID NO:94)
454R-P53-13-	GCCTTGCCAGCCCGCTCAGACCATGCCCGGCTAATTT T	(SEQ ID NO:95)
454R-P53-14-	GCCTTGCCAGCCCGCTCAGAGTTCACGCCATTCTCCT G	(SEQ ID NO:96)
454R-P53-15-	GCCTTGCCAGCCCGCTCAGCACTACGCCCGGCTAATT TT	(SEQ ID NO:97)
454R-P53-16-	GCCTTGCCAGCCCGCTCAGTGGCCCCATTAGGACATG TAT	(SEQ ID NO:98)
TAG8-P53-17+	ACACTGACGACATGGTTCTACATTGTCCCATTGCACT CCAG	(SEQ ID NO:99)
TAG8-P53-18+	ACACTGACGACATGGTTCTACATGGGCAACAAGAGT GAAACT	(SEQ ID NO:100)
TAG8-P53-19+	ACACTGACGACATGGTTCTACAAAATAAATATAGCA GGGTTGCAGGT	(SEQ ID NO:101)
TAG8-P53-20+	ACACTGACGACATGGTTCTACATGCATTTCTCTTGGC TCCC	(SEQ ID NO:102)
TAG8-P53-21+	ACACTGACGACATGGTTCTACAACTTTCCTCAACTCT ACATTTCCC	(SEQ ID NO:103)
TAG8-P53-22+	ACACTGACGACATGGTTCTACATCAGTGCAAACAACA GAAAAGTG	(SEQ ID NO:104)
TAG8-P53-23+	ACACTGACGACATGGTTCTACACATGTTTCTTAGCAA ATCTGATGACA	(SEQ ID NO:105)
TAG8-P53-24+	ACACTGACGACATGGTTCTACATCTGTGGTCCCAGCT ACT	(SEQ ID NO:106)
454R-P53-17-	GCCTTGCCAGCCCGCTCAGTTTCACCATGTTAGGTTG GTCTC	(SEQ ID NO:107)
454R-P53-18-	GCCTTGCCAGCCCGCTCAGTGTAGGTTAAATCCAAAT ACTATACCGTC	(SEQ ID NO:108)
454R-P53-19-	GCCTTGCCAGCCCGCTCAGTCTCAAATCTTCAGTAGC AACTAAAATCT	(SEQ ID NO:109)
454R-P53-20-	GCCTTGCCAGCCCGCTCAGTCCCGACCTCAGGTGATC	(SEQ ID NO:110)
454R-P53-21-	GCCTTGCCAGCCCGCTCAGTGGTCTTGAACTCCCAAC TTC	(SEQ ID NO:111)
454R-P53-22-	GCCTTGCCAGCCCGCTCAGCCTCCGACTCCCAAAGTG	(SEQ ID NO:112)

454R-P53-23-	GCCTTGCCAGCCCGCTCAGACTACAGCCTCGGACTCC	(SEQ ID NO:113)
454R-P53-24-	GCCTTGCCAGCCCGCTCAGATCTTGCACGAAGTTATG CAACTA	(SEQ ID NO:114)
TAG8-P53-25+	ACACTGACGACATGGTTCTACAACCACTGCACTCCAG C	(SEQ ID NO:115)
TAG8-P53-26+	ACACTGACGACATGGTTCTACAACAAGGAAAAGTAT CAGACAATGTAAGT	(SEQ ID NO:116)
TAG8-P53-27+	ACACTGACGACATGGTTCTACAACGGTAGCTCACACC TGTAAT	(SEQ ID NO:117)
TAG8-P53-28+	ACACTGACGACATGGTTCTACATGGAAGTCCCTCTCT GATTGT	(SEQ ID NO:118)
TAG8-P53-29+	ACACTGACGACATGGTTCTACAACTGACTTTCTGCTC TTGTCTTTC	(SEQ ID NO:119)
TAG8-P53-30+	ACACTGACGACATGGTTCTACAATTCTGGGACAGCCA AGTC	(SEQ ID NO:120)
TAG8-P53-31+	ACACTGACGACATGGTTCTACAAGGAGTTCAAGACC AGCCT	(SEQ ID NO:121)
TAG8-P53-32+	ACACTGACGACATGGTTCTACATCTGTCTCCTTCCTCT TCCTAC	(SEQ ID NO:122)
454R-P53-25-	GCCTTGCCAGCCCGCTCAGCCTCTTCCCCAAAAGCTC T	(SEQ ID NO:123)
454R-P53-26-	GCCTTGCCAGCCCGCTCAGTCTCGAACTCCTTACTTC AGGT	(SEQ ID NO:124)
454R-P53-27-	GCCTTGCCAGCCCGCTCAGCCCAACACCATGCCAGTG	(SEQ ID NO:125)
454R-P53-28-	GCCTTGCCAGCCCGCTCAGTCCCCAGCCCTCCAG	(SEQ ID NO:126)
454R-P53-29-	GCCTTGCCAGCCCGCTCAGATTGAAGTCTCATGGAAG CCAG	(SEQ ID NO:127)
454R-P53-30-	GCCTTGCCAGCCCGCTCAGTCAAGTGATCTTCCCACC TCA	(SEQ ID NO:128)
454R-P53-31-	GCCTTGCCAGCCCGCTCAGACAACCTCCGTCATGTGC	(SEQ ID NO:129)
454R-P53-32-	GCCTTGCCAGCCCGCTCAGACCCATTTACTTTGCACA TCTCA	(SEQ ID NO:130)
TAG8-P53-33+	ACACTGACGACATGGTTCTACATTAAGGGTGGTTGTC AGTGG	(SEQ ID NO:131)
TAG8-P53-34+	ACACTGACGACATGGTTCTACATTGCAGTGAGCTGAG ATCAC	(SEQ ID NO:132)
TAG8-P53-35+	ACACTGACGACATGGTTCTACAATCTCCTTACTGCTC CCACT	(SEQ ID NO:133)
TAG8-P53-36+	ACACTGACGACATGGTTCTACATTTTATCACCTTTCCT TGCCTCTT	(SEQ ID NO:134)
TAG8-P53-37+	ACACTGACGACATGGTTCTACAACTCGTCGTAAGTTG AAAATATTGTAAGT	(SEQ ID NO:135)
TAG8-P53-38+	ACACTGACGACATGGTTCTACATCCCAAAGTGCTGGG ATTAC	(SEQ ID NO:136)
TAG8-P53-39+	ACACTGACGACATGGTTCTACATCCATCCTCCCAGCT CAG	(SEQ ID NO:137)
TAG8-P53-40+	ACACTGACGACATGGTTCTACAATCTCAGCTCACTGC AGC	(SEQ ID NO:138)
454R-P53-33-	GCCTTGCCAGCCCGCTCAGAGCCAACCTAGGAGATA ACACA	(SEQ ID NO:139)
454R-P53-34-	GCCTTGCCAGCCCGCTCAGAGGCTCCATCTACTCCCA A	(SEQ ID NO:140)
454R-P53-35-	GCCTTGCCAGCCCGCTCAGTTGATAAGAGGTCCCAAG ACTTAGTA	(SEQ ID NO:141)
454R-P53-36-	GCCTTGCCAGCCCGCTCAGTGGGTGACAGAGTGAGA CT	(SEQ ID NO:142)
454R-P53-37-	GCCTTGCCAGCCCGCTCAGACATCACTGTAATCCAGC CTG	(SEQ ID NO:143)

454R-P53-38-	GCCTTGCCAGCCCGCTCAGAGATCATGCCACTGCACTC	(SEQ ID NO:144)
454R-P53-39-	GCCTTGCCAGCCCGCTCAGGGCATGTGCCTGTAGTCC	(SEQ ID NO:145)
454R-P53-40-	GCCTTGCCAGCCCGCTCAGTGGTCTTGAACTCCTGACCT	(SEQ ID NO:146)
TAG8-P53-41+	ACACTGACGACATGGTTCTACAAAACAGCATGGTTGCATGAAAG	(SEQ ID NO:147)
TAG8-P53-42+	ACACTGACGACATGGTTCTACAAGTCGCATGCACATGTAGTC	(SEQ ID NO:148)
TAG8-P53-43+	ACACTGACGACATGGTTCTACAAAAAGTCAGCTGTATAGGTACTTGAAG	(SEQ ID NO:149)
TAG8-P53-44+	ACACTGACGACATGGTTCTACACCTCAGTGTATCCACAGAACA	(SEQ ID NO:150)
TAG8-P53-45+	ACACTGACGACATGGTTCTACAATGCATGCCTGTAATCCCAG	(SEQ ID NO:151)
TAG8-P53-46+	ACACTGACGACATGGTTCTACAAACTCATGTTCAAGACAGAAGGG	(SEQ ID NO:152)
TAG8-P53-47+	ACACTGACGACATGGTTCTACAATTTTCTCTAACTTCAAGGCCCATAT	(SEQ ID NO:153)
TAG8-P53-48+	ACACTGACGACATGGTTCTACATGGATCCACCAAGACTTGTTTTAT	(SEQ ID NO:154)
454R-P53-41-	GCCTTGCCAGCCCGCTCAGGATTACAGGTGTGAGCCACT	(SEQ ID NO:155)
454R-P53-42-	GCCTTGCCAGCCCGCTCAGACAGTACCTGAGTTAAAAGATGGTTC	(SEQ ID NO:156)
454R-P53-43-	GCCTTGCCAGCCCGCTCAGTGAGACCCTCCAGCTCTG	(SEQ ID NO:157)
454R-P53-44-	GCCTTGCCAGCCCGCTCAGATCTTCCCTTACCCCATTTTACTTTATT	(SEQ ID NO:158)
454R-P53-45-	GCCTTGCCAGCCCGCTCAGTTCAAAGACCCAAAACCCAAAATG	(SEQ ID NO:159)
454R-P53-46-	GCCTTGCCAGCCCGCTCAGGTCAAGTTCTAGACCCCATGTAATA	(SEQ ID NO:160)
454R-P53-47-	GCCTTGCCAGCCCGCTCAGTGTGGTCCCAGCTACTCC	(SEQ ID NO:161)
454R-P53-48-	GCCTTGCCAGCCCGCTCAGAGCAAAGTTTTATTGTAAAATAAGAGATCGAT	(SEQ ID NO:162)

Example 4

4-Primer Barcoding Of Target Nucleic Acids in Preparation for 454 DNA Sequencing Using a Microfluidic Device that Permits Recovery of Amplication Products

[0248] Target-specific primers were designed for 48 genomic regions associated with prostate cancer. In addition to the target-specific regions, the primers were designed to contain additional tag sequences at the 5' end. Forward primers contained the sequence ACACTGACGACATGGTTCTACA (SEQ ID NO:163). Reverse primers contained the sequence TACGGTAGCAGAGACTTGGTCT (SEQ ID NO:164). The sequences of the primers containing both tag sequences and the target-specific regions are listed in Table 5.

Table 5

Assay #	Assay Name	Tagged Forward primer sequence	Tagged Reverse primer sequence	Amplicon position	Amplicon size (no tags)	SEQ ID NO.
1	MSMB-1	ACACTGACGACATGGTTCTACAGTGGTTGCCCTCTCCAGTA	TACGGTAGCAGAGACTTGGTCTGCACACGCATATTAAAATAGGAA	chr10:51219512+51219668	157	(SEQ ID NO:165)
2	MSMB-2	ACACTGACGACATGGTTCTACATCATTCTCCACCCTGACCTT	TACGGTAGCAGAGACTTGGTCTTTCATCTGCAGACAGGTCCA	chr10:51225703+51225910	208	(SEQ ID NO:166)
3	MSMB-3	ACACTGACGACATGGTTCTACAAGGCCTTGTTCTCATTGCAT	TACGGTAGCAGAGACTTGGTCTCCAGCACTGGCTTGAGACTT	chr10:51226702+51226884	183	(SEQ ID NO:167)
4	MSMB-4	ACACTGACGACATGGTTCTACAGGGTCCTTTCTCTTCTAACAGG	TACGGTAGCAGAGACTTGGTCTAGGCCAGAGGAGAATGAGG	chr10:51232232+51232460	229	(SEQ ID NO:168)
5	HNF1B-1	ACACTGACGACATGGTTCTACACAGAGGGTGATGGTGTGGA	TACGGTAGCAGAGACTTGGTCTATGACCCTGCCAAATGACAC	chr17:33121423+33121560	138	(SEQ ID NO:169)
6	HNF1B-5	ACACTGACGACATGGTTCTACATGCTTCCCATTCTTCTTCTCC	TACGGTAGCAGAGACTTGGTCTTGGAAACTGCTCTTTGTGGTC	chr17:33138980+33139231	252	(SEQ ID NO:170)
7	HNF1B-6	ACACTGACGACATGGTTCTACATGCCTCTTATCTTATCAGCTCCA	TACGGTAGCAGAGACTTGGTCTTGGTGGCACTAATGTTCCCTA	chr17:33144574+33144827	254	(SEQ ID NO:171)
8	HNF1B-7	ACACTGACGACATGGTTCTACATAAGATCCGTGGCAAGAACC	TACGGTAGCAGAGACTTGGTCTGAGGTCCGTGTCTACAACTGG	chr17:33165634+33165867	234	(SEQ ID NO:172)
9	HNF1B-8	ACACTGACGACATGGTTCTACAGTCCATGGCCAGCTTTTG	TACGGTAGCAGAGACTTGGTCTCCCCTCACTCACCATCTCC	chr17:33165796+33165970	175	(SEQ ID NO:173)
10	HNF1B-9	ACACTGACGACATGGTTCTACAGGGTTCCTGGGTCTGTGTA	TACGGTAGCAGAGACTTGGTCTAGTCCGATGATGCCTGCT	chr17:33167605+33167819	215	(SEQ ID NO:174)
11	HNF1B-10	ACACTGACGACATGGTTCTACACTTCTTGTTGGTGGGCTCAG	TACGGTAGCAGAGACTTGGTCTTGAGTGAAGGCTACAGACCCTA	chr17:33167782+33167975	194	(SEQ ID NO:175)

12	HNF1B-11	ACACTGACGACATGGTTCTACATGAGAGGGCAAAGGTCACTT	TACGGTAGCAGAGACTTGGTCTAGAGGGAGGTGGTCGATGT	chr17:33173490+33173681	192	(SEQ ID NO:176)
13	HNF1B-12	ACACTGACGACATGGTTCTACAGTTGAGATGCTGGGAGAGGT	TACGGTAGCAGAGACTTGGTCTTCTCCCACTAGTACCCTAACCATC	chr17:33173623+33173782	160	(SEQ ID NO:177)
14	MYC-1	ACACTGACGACATGGTTCTACAGACCCGCTTCTCTGAAAGG	TACGGTAGCAGAGACTTGGTCTGCATTCGACTCATCTCAGCA	chr8:128817980+128818121	142	(SEQ ID NO:178)
15	MYC-2	ACACTGACGACATGGTTCTACACAGGTTTCCGCACCAAGA	TACGGTAGCAGAGACTTGGTCTCAGCAGCTCGAATTTCTTCC	chr8:128819612+128819858	247	(SEQ ID NO:179)
16	MYC-6	ACACTGACGACATGGTTCTACAAACCTTGCTAAAGGAGTGATTTCT	TACGGTAGCAGAGACTTGGTCTCCTCTTGGCAGCAGGATAGT	chr8:128821784+128822038	255	(SEQ ID NO:180)
17	MYC-7	ACACTGACGACATGGTTCTACAACGTCTCCACACATCAGCAC	TACGGTAGCAGAGACTTGGTCTAACTCCGGGATCTGGTCAC	chr8:128821968+128822217	250	(SEQ ID NO:181)
18	MYC-8	ACACTGACGACATGGTTCTACACCAGAGGAGGAACGAGCTAA	TACGGTAGCAGAGACTTGGTCTTTCTGTTAGAAGGAATCGTTTTCC	chr8:128822158+128822420	263	(SEQ ID NO:182)
19	JAZF1-2	ACACTGACGACATGGTTCTACATTCCATGTGGTTATGCCAAG	TACGGTAGCAGAGACTTGGTCTCTCCTGACAGTCCTTGCACTT	chr7:27846803+27847046	244	(SEQ ID NO:183)
20	JAZF1-4	ACACTGACGACATGGTTCTACACAATAAGCAGCAGATATAAGGTTGTT	TACGGTAGCAGAGACTTGGTCTCTTTGTGTTAGGTAGCCTCATATATTC	chr7:27998002+27998196	195	(SEQ ID NO:184)
21	NCOA4-1	ACACTGACGACATGGTTCTACATTCAAAGGTGGTTTTTGGTTG	TACGGTAGCAGAGACTTGGTCTGCCCTGTGTCAAGAGTCCAG	chr10:51249073+51249337	265	(SEQ ID NO:185)
22	NCOA4-2	ACACTGACGACATGGTTCTACATTGGGAAACATCATTCTTTGG	TACGGTAGCAGAGACTTGGTCTACCAGAAGCCATGCTCAAAC	chr10:51250503+51250748	246	(SEQ ID NO:186)
23	NCOA4-3	ACACTGACGACATGGTTCTACATGGTGTCATTGTGGCTAGTTG	TACGGTAGCAGAGACTTGGTCTTGATCTTATCCTAGCAACACAGAAG	chr10:51250847+51251096	250	(SEQ ID NO:187)

24	NCOA4-4	ACACTGACGACATGGTTCTACATGAAGTTGATGAAACAGATATTCCTT	TACGGTAGCAGAGACTTGGTCTAGAAGTGCCCAGTGAAGCAT	chr10:51251218+51251418	201	(SEQ ID NO:188)
25	NCOA4-5	ACACTGACGACATGGTTCTACATTGGCAGCATAGCATAAATAACA	TACGGTAGCAGAGACTTGGTCTCCCAAAGGAAGTATAAGCCAAG	chr10:51252141+51252337	197	(SEQ ID NO:189)
26	NCOA4-6	ACACTGACGACATGGTTCTACACTGCATTTGACATTCCTTGTTT	TACGGTAGCAGAGACTTGGTCTTCCACCTACTGCTGTGTCTACTG	chr10:51252768+51252994	227	(SEQ ID NO:190)
27	NCOA4-7	ACACTGACGACATGGTTCTACAGCAGACAGAATCTCCAAAGCA	TACGGTAGCAGAGACTTGGTCTTCTGATAGGTCCATCTCATCTTGA	chr10:51254556+51254815	260	(SEQ ID NO:191)
28	NCOA4-8	ACACTGACGACATGGTTCTACAGGTTGGAGATCAAGAGCTTCCT	TACGGTAGCAGAGACTTGGTCTTGGTCATTCAGGCACTTCAG	chr10:51254768+51255022	255	(SEQ ID NO:192)
29	NCOA4-9	ACACTGACGACATGGTTCTACAGAAACCAGCCCAAAGGTGT	TACGGTAGCAGAGACTTGGTCTCCTTCTTTCTTCAGAAGCCACT	chr10:51254962+51255214	253	(SEQ ID NO:193)
30	NCOA4-10	ACACTGACGACATGGTTCTACAGAATTGTGAGAAGGAGGCTCTG	TACGGTAGCAGAGACTTGGTCTTGGGACTTCCTTCTTTGTATGG	chr10:51255167+51255432	266	(SEQ ID NO:194)
31	NCOA4-11	ACACTGACGACATGGTTCTACACCTTGTCGGAGTGGCTTATC	TACGGTAGCAGAGACTTGGTCTCCAGTGCTATTTTGATGTTTATGC	chr10:51255385+51255633	249	(SEQ ID NO:195)
32	NCOA4-13	ACACTGACGACATGGTTCTACAGGAGCTTTAAGGCAGGGAAA	TACGGTAGCAGAGACTTGGTCTTTGGCAAGCTGCAGTCAC	chr10:51259156+51259310	155	(SEQ ID NO:196)
33	NUDT11-1	ACACTGACGACATGGTTCTACAAGCGAGGCAGACAAATAGAAG	TACGGTAGCAGAGACTTGGTCTGTACTGACTGTCACGGAGCTG	chrX:51255496+51255748	253	(SEQ ID NO:197)
34	SLC22A3-4	ACACTGACGACATGGTTCTACATCTGCATTCTGGCATGTCTC	TACGGTAGCAGAGACTTGGTCTTCCCCGTATTAATGCATGGTAT	chr6:160738955+160739163	209	(SEQ ID NO:198)
35	SLC22A3-5	ACACTGACGACATGGTTCTACAAGGTGAGCTCTTTTCCTGTCTT	TACGGTAGCAGAGACTTGGTCTTTGTTGGCTATCTGGCCCTA	chr6:160748030+160748274	245	(SEQ ID NO:199)

36	SLC22A3-6	ACACTGACGACATGGTTCTACATGCTTCTGTGACCTCTTGTGT	TACGGTAGCAGAGACTTGGTCTGTCTGTTTGGAGTCTAATTTCTGC	chr6:160749740+160750007	268	(SEQ ID NO:200)
37	SLC22A3-7	ACACTGACGACATGGTTCTACACATAACTCACAACAGCCTCCTTC	TACGGTAGCAGAGACTTGGTCTAATCAATTCACCAGCTTTAGCAA	chr6:160751720+160751920	201	(SEQ ID NO:201)
38	SLC22A3-10	ACACTGACGACATGGTTCTACAGTGGTGGAACTGCCAGGA	TACGGTAGCAGAGACTTGGTCTGGCTCCCTATACTTGATTGTGG	chr6:160778107+160778308	202	(SEQ ID NO:202)
39	SLC22A3-11	ACACTGACGACATGGTTCTACACCTCCCTTTCAAACTTTCTGTG	TACGGTAGCAGAGACTTGGTCTCGCTGGTCTACAGAGTTACTTAGGA	chr6:160783754+160783942	189	(SEQ ID NO:203)
40	SLC22A3-12	ACACTGACGACATGGTTCTACATGATTATCTTGAAGTCACTTGTTGAA	TACGGTAGCAGAGACTTGGTCTTGAAGGCTCTTAAGAATAGCAAATG	chr6:160784591+160784798	208	(SEQ ID NO:204)
41	SLC22A3-13	ACACTGACGACATGGTTCTACAGTGTCTTCCTGGAGCGGTAA	TACGGTAGCAGAGACTTGGTCTTTCCCTGTGGATATTCAATTTTTCT	chr6:160788700+160788934	235	(SEQ ID NO:205)
42	SLC22A3-14	ACACTGACGACATGGTTCTACATCTTTCCTAAAGACTTTCTCCTTTG	TACGGTAGCAGAGACTTGGTCTATCTCTGCAAGGCACAGCTT	chr6:160791984+160792152	169	(SEQ ID NO:206)
43	KLK3-1	ACACTGACGACATGGTTCTACAAGTCCTGGGGAATGAAGGTT	TACGGTAGCAGAGACTTGGTCTGGAAAGAGCCTCAGCTTGAC	chr19:56049936+56050140	205	(SEQ ID NO:207)
44	KLK3-2	ACACTGACGACATGGTTCTACAGTTCCTCCTGTCAACCCTGA	TACGGTAGCAGAGACTTGGTCTCCTCTGGGACACAGACACCT	chr19:56051260+56051515	256	(SEQ ID NO:208)
45	KLK3-3	ACACTGACGACATGGTTCTACATCCTTATCATCCTCGCTCCT	TACGGTAGCAGAGACTTGGTCTTTCACAGCATCCGTGAGC	chr19:56053051+56053300	250	(SEQ ID NO:209)
46	KLK3-4	ACACTGACGACATGGTTCTACAACTCCAGCCACGACCTCAT	TACGGTAGCAGAGACTTGGTCTCCCTCAGACCCAGGCATC	chr19:56053237+56053436	200	(SEQ ID NO:210)
47	KLK3-5	ACACTGACGACATGGTTCTACAGGTCCAGCCCACAACAGT	TACGGTAGCAGAGACTTGGTCTCCCAGCCCAGAATTAAGGT	chr19:56053490+56053729	240	(SEQ ID NO:211)
48	KLK3-8	ACACTGACGACATGGTTCTACA	TACGGTAGCAGAGACTTGGT	chr19:56054924+56055115	192	(SEQ ID NO:212)

		TCTTCCAAAGC TGGGAACTG	CTGGGCACAT GGTTCACTGC			

Preparation of Reaction Mixtures

[0249] Primers were synthesized by IDT at 10nmol scale, and provided resuspended in water at a concentration of 100uM. The forward and reverse primer for each region in Table 5 were combined in separate wells in a 96-well PCR plate (USA scientific) to a final concentration of 1μM of each primer in PCR-quality water (Teknova) containing 0.05% Tween-20.

[0250] 48 human genomic DNA samples from the HapMap sample collection were resuspended at 50ng/μl in low-EDTA TE buffer (Teknova), and prepared for PCR as follows.

[0251] A pre-sample mixture was prepared as follows:

Table 6

Pre-sample mixture	**Volume per sample (μl)**	**Volume for 64 samples (μl)**
Faststart High Fidelity reaction Buffer with $MgCl_2$	0.5	32
DMSO	0.1	6.4
PCR-Grade Nucleotide Mixture	0.1	6.4
Faststart High-Fidelity Enzyme Blend (Roche 04 738 292 001)	0.05	3.2
20x Access Array Loading Reagent (PN: 100-0883)	0.25	16
20x Evagreen (Biotium-31000)	0.25	16
20x ROX dye (Invitrogen 12223-012)	0.25	16
PCR-Grade water	0.5	32
Total	2	128

[0252] For each sample, a sample mixture containing forward and reverse barcode primers, genomic DNA, and pre-sample mix was prepared in an individual well in a 96-well PCR plate.

Table 7

Sample Mixture	Volume (μl)
Pre-sample Mixture	2
2 μM forward barcode primer	0.5
2 μm reverse barcode primer	0.5
Genomic DNA (50ng/μl)	1
PCR-grade water	1

[0253] Each sample was mixed with one pair of barcode primers selected from Table 8.

Table 8

	Reverse barcode primer (454B-BC#-CS1)	Reverse barcode primer SEQ ID NO.	Forward barcode primer (454A-BC#-CS2)	Forward barcode primer SEQ ID NO.
1	GCCTTGCCAGCCCGCTCAGGCATGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:213)	GCCTCCCTCGCGCCATCAGGCATGCACACTGACGACATGGTTCTACA	(SEQ ID NO:214)
2	GCCTTGCCAGCCCGCTCAGCGTACGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:215)	GCCTCCCTCGCGCCATCAGCGTACGACACTGACGACATGGTTCTACA	(SEQ ID NO:216)
3	GCCTTGCCAGCCCGCTCAGGTCAGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:217)	GCCTCCCTCGCGCCATCAGGTCAGCACACTGACGACATGGTTCTACA	(SEQ ID NO:218)
4	GCCTTGCCAGCCCGCTCAGAGCTGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:219)	GCCTCCCTCGCGCCATCAGAGCTGCACACTGACGACATGGTTCTACA	(SEQ ID NO:220)
5	GCCTTGCCAGCCCGCTCAGTGCATCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:221)	GCCTCCCTCGCGCCATCAGTGCATCACACTGACGACATGGTTCTACA	(SEQ ID NO:222)
6	GCCTTGCCAGCCCGCTCAGCTGATGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:223)	GCCTCCCTCGCGCCATCAGCTGATGACACTGACGACATGGTTCTACA	(SEQ ID NO:224)
7	GCCTTGCCAGCCCGCTCAGGTAGTCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:225)	GCCTCCCTCGCGCCATCAGGTAGTCACACTGACGACATGGTTCTACA	(SEQ ID NO:226)

8	GCCTTGCCAGCCCGCTCAGGTCGATTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:227)	GCCTCCCTCGCGCCATCAGGTCGATACACTGACGACATGGTTCTACA	(SEQ ID NO:228)
9	GCCTTGCCAGCCCGCTCAGGATACGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:229)	GCCTCCCTCGCGCCATCAGGATACGACACTGACGACATGGTTCTACA	(SEQ ID NO:230)
10	GCCTTGCCAGCCCGCTCAGTGATGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:231)	GCCTCCCTCGCGCCATCAGTGATGCACACTGACGACATGGTTCTACA	(SEQ ID NO:232)
11	GCCTTGCCAGCCCGCTCAGAGCTGATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:233)	GCCTCCCTCGCGCCATCAGAGCTGAACACTGACGACATGGTTCTACA	(SEQ ID NO:234)
12	GCCTTGCCAGCCCGCTCAGACTGTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:235)	GCCTCCCTCGCGCCATCAGACTGTAACACTGACGACATGGTTCTACA	(SEQ ID NO:236)
13	GCCTTGCCAGCCCGCTCAGTGCATGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:237)	GCCTCCCTCGCGCCATCAGTGCATGACACTGACGACATGGTTCTACA	(SEQ ID NO:238)
14	GCCTTGCCAGCCCGCTCAGAGTCTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:239)	GCCTCCCTCGCGCCATCAGAGTCTAACACTGACGACATGGTTCTACA	(SEQ ID NO:240)
15	GCCTTGCCAGCCCGCTCAGTGTCTGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:241)	GCCTCCCTCGCGCCATCAGTGTCTGACACTGACGACATGGTTCTACA	(SEQ ID NO:242)
16	GCCTTGCCAGCCCGCTCAGGCTAGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:243)	GCCTCCCTCGCGCCATCAGGCTAGCACACTGACGACATGGTTCTACA	(SEQ ID NO:244)
17	GCCTTGCCAGCCCGCTCAGGATAGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:245)	GCCTCCCTCGCGCCATCAGGATAGCACACTGACGACATGGTTCTACA	(SEQ ID NO:246)
18	GCCTTGCCAGCCCGCTCAGGCTACTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:247)	GCCTCCCTCGCGCCATCAGGCTACTACACTGACGACATGGTTCTACA	(SEQ ID NO:248)
19	GCCTTGCCAGCCCGCTCAGCTATGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:249)	GCCTCCCTCGCGCCATCAGCTATGCACACTGACGACATGGTTCTACA	(SEQ ID NO:250)
20	GCCTTGCCAGCCCGCTCAGGCTATGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:251)	GCCTCCCTCGCGCCATCAGGCTATGACACTGACGACATGGTTCTACA	(SEQ ID NO:252)
21	GCCTTGCCAGCCCGCTCAGCGTGCATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:253)	GCCTCCCTCGCGCCATCAGCGTGCAACACTGACGACATGGTTCTACA	(SEQ ID NO:254)
22	GCCTTGCCAGCCCGCTCAGATAGCTTACGGTAGCAGAGACTTGGT	(SEQ ID NO:255)	GCCTCCCTCGCGCCATCAGATAGCTACACTGACGACATGGTTCTACA	(SEQ ID NO:256)

	CT			
23	GCCTTGCCAGCCCGCTCAGTGTAGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:257)	GCCTCCCTCGCGCCATCAGTGTAGCACACTGACGACATGGTTCTACA	(SEQ ID NO:258)
24	GCCTTGCCAGCCCGCTCAGGTGCTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:259)	GCCTCCCTCGCGCCATCAGGTGCTAACACTGACGACATGGTTCTACA	(SEQ ID NO:260)
25	GCCTTGCCAGCCCGCTCAGGTCATGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:261)	GCCTCCCTCGCGCCATCAGGTCATGACACTGACGACATGGTTCTACA	(SEQ ID NO:262)
26	GCCTTGCCAGCCCGCTCAGATCGTGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:263)	GCCTCCCTCGCGCCATCAGATCGTGACACTGACGACATGGTTCTACA	(SEQ ID NO:264)
27	GCCTTGCCAGCCCGCTCAGTGTACGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:265)	GCCTCCCTCGCGCCATCAGTGTACGACACTGACGACATGGTTCTACA	(SEQ ID NO:266)
28	GCCTTGCCAGCCCGCTCAGAGTGTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:267)	GCCTCCCTCGCGCCATCAGAGTGTAACACTGACGACATGGTTCTACA	(SEQ ID NO:268)
29	GCCTTGCCAGCCCGCTCAGTGACAGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:269)	GCCTCCCTCGCGCCATCAGTGACAGACACTGACGACATGGTTCTACA	(SEQ ID NO:270)
30	GCCTTGCCAGCCCGCTCAGGATCACTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:271)	GCCTCCCTCGCGCCATCAGGATCACACACTGACGACATGGTTCTACA	(SEQ ID NO:272)
31	GCCTTGCCAGCCCGCTCAGCTAGAGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:273)	GCCTCCCTCGCGCCATCAGCTAGAGACACTGACGACATGGTTCTACA	(SEQ ID NO:274)
32	GCCTTGCCAGCCCGCTCAGCTAGTCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:275)	GCCTCCCTCGCGCCATCAGCTAGTCACACTGACGACATGGTTCTACA	(SEQ ID NO:276)
33	GCCTTGCCAGCCCGCTCAGAGCTAGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:277)	GCCTCCCTCGCGCCATCAGAGCTAGACACTGACGACATGGTTCTACA	(SEQ ID NO:278)
34	GCCTTGCCAGCCCGCTCAGTGACTGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:279)	GCCTCCCTCGCGCCATCAGTGACTGACACTGACGACATGGTTCTACA	(SEQ ID NO:280)
35	GCCTTGCCAGCCCGCTCAGTGATAGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:281)	GCCTCCCTCGCGCCATCAGTGATAGACACTGACGACATGGTTCTACA	(SEQ ID NO:282)
36	GCCTTGCCAGCCCGCTCAGCGTATCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:283)	GCCTCCCTCGCGCCATCAGCGTATCACACTGACGACATGGTTCTACA	(SEQ ID NO:284)
37	GCCTTGCCAGCCCGCTCAGGTCTGATACGG	(SEQ ID NO:285)	GCCTCCCTCGCGCCATCAGGTCTGAACACTGACGACATGGTTCTAC	(SEQ ID NO:286)

	TAGCAGAGACTTGGTCT		A	
38	GCCTTGCCAGCCCGCTCAGCATGACTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:287)	GCCTCCCTCGCGCCATCAGCATGACACACTGACGACATGGTTCTACA	(SEQ ID NO:288)
39	GCCTTGCCAGCCCGCTCAGCGATGATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:289)	GCCTCCCTCGCGCCATCAGCGATGAACACTGACGACATGGTTCTACA	(SEQ ID NO:290)
40	GCCTTGCCAGCCCGCTCAGGCTGATTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:291)	GCCTCCCTCGCGCCATCAGGCTGATACACTGACGACATGGTTCTACA	(SEQ ID NO:292)
41	GCCTTGCCAGCCCGCTCAGCAGTACTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:293)	GCCTCCCTCGCGCCATCAGCAGTACACACTGACGACATGGTTCTACA	(SEQ ID NO:294)
42	GCCTTGCCAGCCCGCTCAGGCGACTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:295)	GCCTCCCTCGCGCCATCAGGCGACTACACTGACGACATGGTTCTACA	(SEQ ID NO:296)
43	GCCTTGCCAGCCCGCTCAGGTACGATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:297)	GCCTCCCTCGCGCCATCAGGTACGAACACTGACGACATGGTTCTACA	(SEQ ID NO:298)
44	GCCTTGCCAGCCCGCTCAGACGCTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:299)	GCCTCCCTCGCGCCATCAGACGCTAACACTGACGACATGGTTCTACA	(SEQ ID NO:300)
45	GCCTTGCCAGCCCGCTCAGAGCATCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:301)	GCCTCCCTCGCGCCATCAGAGCATCACACTGACGACATGGTTCTACA	(SEQ ID NO:302)
46	GCCTTGCCAGCCCGCTCAGGATGCTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:303)	GCCTCCCTCGCGCCATCAGGATGCTACACTGACGACATGGTTCTACA	(SEQ ID NO:304)
47	GCCTTGCCAGCCCGCTCAGGTCTGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:305)	GCCTCCCTCGCGCCATCAGGTCTGCACACTGACGACATGGTTCTACA	(SEQ ID NO:306)
48	GCCTTGCCAGCCCGCTCAGATGCGATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:307)	GCCTCCCTCGCGCCATCAGATGCGAACACTGACGACATGGTTCTACA	(SEQ ID NO:308)

Running the Access Array IFC

[0254] The containment and interface accumulator reservoirs were filled with 300 µl of Control Line Fluid (Fluidigm PN 89000020) and the H1-H4 reagent wells were loaded with 500 µl of 0.05% Tween-20 in PCR-grade water prior to Access Array IFC loading. 5µl of each sample mixture was loaded into the sample ports, and

5 µl of each primer mixture was loaded into the primer inlets on the Access Array IFC.

[0255] The Access Array IFC was thermal cycled and imaged using a BioMark ™ Real-Time PCR system manufactured by Fluidigm Corporation. The Access Array IFC thermal cycling protocol contains a thermal mix step [50 °C for 2 min, 70 °C for 20 min], a hotstart step [95 °C for 10 min], a 35 cycle touch down PCR strategy [2 cycles of 95 °C for 15 sec and 63 °C for 1 min, 2 cycles of 95 °C for 15 sec and 62 °C for 1 min, 2 cycles of 95 °C for 15 sec and 61 °C for 1 min, 2 cycles of 95 °C for 15 sec and 60 °C for 1 min, 2 cycles of 95 °C for 15 sec and 58 °C for 1 min, 25 cycles of 95 °C for 15 sec and 72 °C for 1 min], and an elongation step [72 °C for 3 min]. The real-time data was analyzed with Fluidigm Real-Time PCR Analysis software to obtain C_T values for each reaction chamber.

[0256] After amplification, the PCR products were harvested from the Access Array IFC using the Post-PCR IFC Loader AX. Before harvesting, each sample port was filled with 2 µl of 0.05% Tween-20. Residual solution was removed from the H1-H4 reagent wells, and they were refilled with 600 µl of 1X Access Array Harvesting Reagent (0.05% tween-20). After harvesting ,each sample port became a PCR product outlet that contained 10 µl (± 10%) of 48 pooled PCR products. The pooled PCR products were removed from the Access Array IFC and stored in a microtiter plate at 4 °C.

[0257] 1 µl of each PCR product pool for each sample was taken and loaded onto an Agilent 1K Bioanalyzer chip. Figure 12 shows the electropherograms from each of the 48 individual product pools. Figure 13 shows the distribution of product size within a single product pool. All products fall within the predicted size range, and there is no evidence of any small-sized PCR by-products.

[0258] PCR Products for each sample were pooled based on concentrations calculated from the Agilent Bioanalyzer traces. The product pool was purified using AMPure beads (Agencourt) according to the manufacturer's instructions.

[0259] The purified product pool was subjected to emulsion PCR followed by pyrosequencing on a 454 FLX sequencer (Roche Analytical Sciences) according to manufacturer's instructions. The sequence file output by the sequencer was then analyzed for the presence of barcoded PCR products.

[0260] The number of sequences obtained for each barcode were counted, and plotted (Figure 14(A)). On average ~3400 sequences were counted for each barcode. All samples were represented at >50% of average and < 2-fold of average.

[0261] The number of sequences counted for each individual PCR product in each sample were then analyzed (figure 14(B)). Only one of 2304 PCR products was not observed on the sequencer. The vast majority of sequences were present at >50% of average and <2 fold of average. 2303/2304 products were counted >5 times. Figure 14(C) shows the distribution of PCR products from all 2304 PCR reactions in the Access Array IFC. >95% of sequences were measured between 50% and 2 fold of the average coverage. >99% of sequences were measured between 50% and 2 fold of the average coverage.

Example 5

Multi-Primer Amplification Using Four Outer Primers With Different Combinations Of Primer Binding Site And Nucleotide Tags

[0262] Sets of primer pairs were designed to amplify specific regions from the EGFR and MET genes. These were then combined in an Access Array IFC with human genomic DNA and four outer primers (Figure 15).

Preparation of Reaction Mixtures

[0263] Primers were synthesized by Eurofins MWG Operon at 10nmol scale and provided resuspended in water at a concentration of 100μM. The forward and reverse primer for each region in Table 9 were combined in separate wells in a 96-well PCR plate (USA scientific) to a final concentration of 1μM of each primer in PCR-quality water (Teknova) containing 0.05% Tween-20.

Table 9

Assay	Forward Primer	Forward Primer SEQ ID NO.	Reverse Primer	Reverse Primer SEQ ID NO.
EGFR_Exon3	ACACTGACGACATGGTTCTACATTCTTAGACCATCCAGGAGGTG	(SEQ ID NO:309)	TACGGTAGCAGAGACTTGGTCTCCAGCCTCTCACCCTGTAAA	(SEQ ID NO:310)
EGFR_Exon4	ACACTGACGACATGGTTCTACAAGCTGGAAAGAGTGCTC	(SEQ ID NO:311)	TACGGTAGCAGAGACTTGGTCTTAGGAGCTGGAGGCAGAGAT	(SEQ ID NO:312)

	ACC			
EGFR_Exon5	ACACTGACGACATGGTTCTACAGCGTCATCAGTTTCTCATCATT	(SEQ ID NO:313)	TACGGTAGCAGAGACTTGGTCTACATGGGTCTGAGGCTGTTC	(SEQ ID NO:314)
EGFR_Exon6	ACACTGACGACATGGTTCTACACCCTGGGAAATGATCCTACC	(SEQ ID NO:315)	TACGGTAGCAGAGACTTGGTCTTCTTACCAGGCAGTCGCTCT	(SEQ ID NO:316)
EGFR_Exon7	ACACTGACGACATGGTTCTACACCAGCGTGTCCTCTCTCCT	(SEQ ID NO:317)	TACGGTAGCAGAGACTTGGTCTGACAAGGATGCCTGACCAGT	(SEQ ID NO:318)
EGFR_Exon8	ACACTGACGACATGGTTCTACACAAAGGAGGATGGAGCCTTTC	(SEQ ID NO:319)	TACGGTAGCAGAGACTTGGTCTGATGTGTTCCTTTGGAGGTGG	(SEQ ID NO:320)
EGFR_Exon9	ACACTGACGACATGGTTCTACATCCAACAAATGTGAACGGAAT	(SEQ ID NO:321)	TACGGTAGCAGAGACTTGGTCTCAAGCAACTGAACCTGTGACTC	(SEQ ID NO:322)
EGFR_Exon10	ACACTGACGACATGGTTCTACAGATCAATAATCACCCTGTTGTTTG	(SEQ ID NO:323)	TACGGTAGCAGAGACTTGGTCTTTCCAAGGGAACAGGAAATATG	(SEQ ID NO:324)
EGFR_Exon11	ACACTGACGACATGGTTCTACATCCTACGTGGTGTGTGTCTGA	(SEQ ID NO:325)	TACGGTAGCAGAGACTTGGTCTGCTTTGGCTGTGGTCAACTT	(SEQ ID NO:326)
EGFR_Exon12	ACACTGACGACATGGTTCTACACCACATGATTTTTCTTCTCCA	(SEQ ID NO:327)	TACGGTAGCAGAGACTTGGTCTCGGTGACTTACTGCAGCTGTT	(SEQ ID NO:328)
EGFR_Exon13	ACACTGACGACATGGTTCTACAGCTCTGTCACTGACTGCTGTG	(SEQ ID NO:329)	TACGGTAGCAGAGACTTGGTCTGCTATAACAACAACCTGGAGCCT	(SEQ ID NO:330)
EGFR_Exon14	ACACTGACGACATGGTTCTACAGCTGACGGGTTTCCTCTTC	(SEQ ID NO:331)	TACGGTAGCAGAGACTTGGTCTGACGTGGATAGCAGCAAGG	(SEQ ID NO:332)
EGFR_Exon15	ACACTGACGACATGGTTCTACAGCATGAACATTTTTCTCCACCT	(SEQ ID NO:333)	TACGGTAGCAGAGACTTGGTCTTTCTGTTCTCCTTCACTTTCCAC	(SEQ ID NO:334)
EGFR_Exon16	ACACTGACGACATGGTTCTACATTTCTCTTTCACTTCCTACAGATGC	(SEQ ID NO:335)	TACGGTAGCAGAGACTTGGTCTCCACAGCAGTGTGGTCATTC	(SEQ ID NO:336)
EGFR_Exon17	ACACTGACGACATGGTTCTACATGGAATCTGTCAGCAACCTC	(SEQ ID NO:337)	TACGGTAGCAGAGACTTGGTCTCCCAGGACTGGCACTCA	(SEQ ID NO:338)
EGFR_Exon18	ACACTGACGACATGGTTCTACAGCTGAGGTGACCCTTGTCTC	(SEQ ID NO:339)	TACGGTAGCAGAGACTTGGTCTCCCACCAGACCATGAGAGG	(SEQ ID NO:340)
EGFR_Exon19	ACACTGACGACATGGTTCTACATCAC	(SEQ ID NO:341)	TACGGTAGCAGAGACTTGGTCTCCACACAGCAAAGCAG	(SEQ ID NO:342)

	AATTGCCAGTTAACGTCT		AAAC	
EGFR_Exon20	ACACTGACGACATGGTTCTACACCACACTGACGTGCCTCTC	(SEQ ID NO:343)	TACGGTAGCAGAGACTTGGTCTCCGTATCTCCCTTCCCTGAT	(SEQ ID NO:344)
EGFR_Exon21	ACACTGACGACATGGTTCTACACCTCACAGCAGGGTCTTCTC	(SEQ ID NO:345)	TACGGTAGCAGAGACTTGGTCTCTGACCTAAAGCCACCTCCTT	(SEQ ID NO:346)
EGFR_Exon22	ACACTGACGACATGGTTCTACACACTGCCTCATCTCTCACCA	(SEQ ID NO:347)	TACGGTAGCAGAGACTTGGTCTCCAGCTTGGCCTCAGTACA	(SEQ ID NO:348)
EGFR_Exon23	ACACTGACGACATGGTTCTACACATGATCCCACTGCCTTCTT	(SEQ ID NO:349)	TACGGTAGCAGAGACTTGGTCTAGTGTGGACAGACCCACCA	(SEQ ID NO:350)
EGFR_Exon24	ACACTGACGACATGGTTCTACATTCCAGTGTTCTAATTGCACTGTT	(SEQ ID NO:351)	TACGGTAGCAGAGACTTGGTCTGAGGGACTCTTCCCAATGGA	(SEQ ID NO:352)
EGFR_Exon25	ACACTGACGACATGGTTCTACACTAATAGCCTCAAAATCTCTGCAC	(SEQ ID NO:353)	TACGGTAGCAGAGACTTGGTCTTTTGTTCAAATGAGTAGACACAGC	(SEQ ID NO:354)
EGFR_Exon26	ACACTGACGACATGGTTCTACACATTCCATGGGCAACTTCTC	(SEQ ID NO:355)	TACGGTAGCAGAGACTTGGTCTTTCTGGCTTATAAGGTGTTCATACA	(SEQ ID NO:356)
EGFR_Exon27	ACACTGACGACATGGTTCTACACCTTCCCTCATTTCCTCCTG	(SEQ ID NO:357)	TACGGTAGCAGAGACTTGGTCTTCCAGACAAGCCACTCACC	(SEQ ID NO:358)
EGFR_Exon28-1	ACACTGACGACATGGTTCTACAcctctgatttctttccactttca	(SEQ ID NO:359)	TACGGTAGCAGAGACTTGGTCTCTAATTTGGTGGCTGCCTTT	(SEQ ID NO:360)
EGFR_Exon28-2	ACACTGACGACATGGTTCTACATGTCAACAGCACATTCGACAG	(SEQ ID NO:361)	TACGGTAGCAGAGACTTGGTCTGGTCCTGGGTATCGAAAGAGT	(SEQ ID NO:362)
EGFR_Exon2	ACACTGACGACATGGTTCTACATTTCTTCCAGTTTGCCAAGG	(SEQ ID NO:363)	TACGGTAGCAGAGACTTGGTCTAGGAAAATCAAAGTCACCAACC	(SEQ ID NO:364)
MET_Exon1-1	ACACTGACGACATGGTTCTACACTCTCGCCTTGAACCTGTTT	(SEQ ID NO:365)	TACGGTAGCAGAGACTTGGTCTCAGCACAGGCCCAGTCTT	(SEQ ID NO:366)
MET_Exon1-2	ACACTGACGACATGGTTCTACATTCCTTGGTGCCACTAACTACA	(SEQ ID NO:367)	TACGGTAGCAGAGACTTGGTCTGGGAGAATATGCAGTGAACCTC	(SEQ ID NO:368)
MET_Exon2	ACACTGACGACATGGTTCTACATGGATTCACATTAACTCTATGACCA	(SEQ ID NO:369)	TACGGTAGCAGAGACTTGGTCTTTGCACAATACCAGATAGAACAGAC	(SEQ ID NO:370)
MET_Exon3	ACACTGACGACATGGTTCTACATGAG	(SEQ ID NO:371)	TACGGTAGCAGAGACTTGGTCTCGTCTATGGAAATTCC	(SEQ ID NO:372)

	CTTGTTGGAATAAGGATG		CTGTG	
MET_Exon4	ACACTGACGACATGGTTCTACAGAAGCTCTTTCCACCCCTTC	(SEQ ID NO:373)	TACGGTAGCAGAGACTTGGTCTTGCCAGCTGTTAGAGATTCCT	(SEQ ID NO:374)
MET_Exon5	ACACTGACGACATGGTTCTACATGTCCTTGTAGGTTTTCCCAAA	(SEQ ID NO:375)	TACGGTAGCAGAGACTTGGTCTCCCCAGCAAAGCATTTTAAG	(SEQ ID NO:376)
MET_Exon6	ACACTGACGACATGGTTCTACAGAAAATTCCTTGGATTTGTCATG	(SEQ ID NO:377)	TACGGTAGCAGAGACTTGGTCTCATGATAGGATAGAATCTTCCTTACCA	(SEQ ID NO:378)
MET_Exon7	ACACTGACGACATGGTTCTACAGTTTTGTTTTTATCTCCCCTCCA	(SEQ ID NO:379)	TACGGTAGCAGAGACTTGGTCTTTCAAATTGACAGATGCAACAA	(SEQ ID NO:380)
MET_Exon8	ACACTGACGACATGGTTCTACAGGAACCATTGAGTTATATCCTTTTG	(SEQ ID NO:381)	TACGGTAGCAGAGACTTGGTCTTTGTTTTCTTATACCCATCAGAAGC	(SEQ ID NO:382)
MET_Exon9	ACACTGACGACATGGTTCTACATTGGTGGAAAGAACCTCTCAA	(SEQ ID NO:383)	TACGGTAGCAGAGACTTGGTCTCAGGTACCATGAAAGCCACA	(SEQ ID NO:384)
MET_Exon10	ACACTGACGACATGGTTCTACATGTTGCCAAGCTGTATTCTGTT	(SEQ ID NO:385)	TACGGTAGCAGAGACTTGGTCTTTTGAGCTGATGATTTAAGACAGTG	(SEQ ID NO:386)
MET_Exon12	ACACTGACGACATGGTTCTACAGGACCCAAAGTGCTACAACC	(SEQ ID NO:387)	TACGGTAGCAGAGACTTGGTCTCAAGAATCGACGACAATCTTAAAC	(SEQ ID NO:388)
MET_Exon13	ACACTGACGACATGGTTCTACAGCCCATGATAGCCGTCTTTA	(SEQ ID NO:389)	TACGGTAGCAGAGACTTGGTCTCAACAATGTCACAACCCACTG	(SEQ ID NO:390)
MET_Exon14	ACACTGACGACATGGTTCTACACCTTCATCTTACAGATCAGTTTCCT	(SEQ ID NO:391)	TACGGTAGCAGAGACTTGGTCTGCTTACTGGAAAATCGTATTTAACAAA	(SEQ ID NO:392)
MET_Exon15	ACACTGACGACATGGTTCTACAACGCAGTGCTAACCAAGTTCT	(SEQ ID NO:393)	TACGGTAGCAGAGACTTGGTCTTCCACAAGGGGAAAGTGTAAA	(SEQ ID NO:394)
MET_Exon16	ACACTGACGACATGGTTCTACATGTCTCCACCACTGGATTTCT	(SEQ ID NO:395)	TACGGTAGCAGAGACTTGGTCTGGCTTACAGCTAGTTTGCCAGT	(SEQ ID NO:396)
MET_Exon17	ACACTGACGACATGGTTCTACATGCTTTTCTAACTCTCTTTGACTGC	(SEQ ID NO:397)	TACGGTAGCAGAGACTTGGTCTTCCTCCTTGTCACTTAATTTGGA	(SEQ ID NO:398)
MET_Exon18	ACACTGACGACATGGTTCTACATTCTATTTCAGCCACGGGTAA	(SEQ ID NO:399)	TACGGTAGCAGAGACTTGGTCTAGAGGAGAAACTCAGAGATAACCAA	(SEQ ID NO:400)
MET_Exon19	ACACTGACGACAT	(SEQ ID	TACGGTAGCAGAGACTTGG	(SEQ ID

	GGTTCTACACTCA CCTCATCTGTCCTG TTTCT	NO:401)	TCTGGCATTTCTGTAAAAG TAAAGAACG	NO:402)
MET_Exon20	ACACTGACGACAT GGTTCTACACCTG CCTTCAAAGGGTC TCT	(SEQ ID NO:403)	TACGGTAGCAGAGACTTGG TCTGTGTGGACTGTTGCTT TGACA	(SEQ ID NO:404)

[0264] A single human Genomic DNA sample (Coriell NA10830) was resuspended at 50ng/μl in low-EDTA TE buffer (Teknova) and prepared for PCR as follows.

[0265] A pre-sample mixture was prepared as follows:

Table 10

Pre-sample mixture	**Volume per sample (μl)**	**Volume for 64 samples (μl)**
Faststart High Fidelity reaction Buffer with $MgCl_2$	0.5	32
DMSO	0.1	6.4
PCR-Grade Nucleotide Mixture	0.1	6.4
Faststart High-Fidelity Enzyme Blend (Roche 04 738 292 001)	0.05	3.2
20x Access Array Loading Reagent (PN: 100-0883)	0.25	16
PCR-Grade water	0.5	32
Total	2.5	160

[0266] For each sample replicate, a sample mixture containing forward and reverse barcode primers, genomic DNA and pre-sample mix was prepared in an individual well in a 96-well PCR plate.

Table 11

Sample Mixture	**Volume (μl)**
Pre-sample Mixture	2
4 μM forward barcode primer	0.5

4 μm reverse barcode primer	0.5
Genomic DNA (50ng/μl)	1
PCR-grade water	1

[0267] Four replicate samples were prepared by mixing each sample with one pair of barcode primers selected from Table 12.

Table 12

	Reverse barcode primer (454B-BC#-CS1)	Reverse barcode primer SEQ ID NO.	Forward barcode primer (454A-BC#-CS2)	Forward barcode primer SEQ ID NO.
1	GCCTTGCCAGCCC GCTCAGGCATGC TACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:405)	GCCTCCCTCGCGCC ATCAGGCATGCAC ACTGACGACATGGT TCTACA	(SEQ ID NO:406)
2	GCCTTGCCAGCCC GCTCAGCGTACG TACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:407)	GCCTCCCTCGCGCC ATCAGCGTACGAC ACTGACGACATGGT TCTACA	(SEQ ID NO:408)
3	GCCTTGCCAGCCC GCTCAGGTCAGC TACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:409)	GCCTCCCTCGCGCC ATCAGGTCAGCAC ACTGACGACATGGT TCTACA	(SEQ ID NO:410)
4	GCCTTGCCAGCCC GCTCAGAGCTGC TACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:411)	GCCTCCCTCGCGCC ATCAGAGCTGCAC ACTGACGACATGGT TCTACA	(SEQ ID NO:412)
5	GCCTTGCCAGCCC GCTCAGTGCATCT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:413)	GCCTCCCTCGCGCC ATCAGTGCATCACA CTGACGACATGGTT CTACA	(SEQ ID NO:414)
6	GCCTTGCCAGCCC GCTCAGCTGATGT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:415)	GCCTCCCTCGCGCC ATCAGCTGATGACA CTGACGACATGGTT CTACA	(SEQ ID NO:416)
7	GCCTTGCCAGCCC GCTCAGGTAGTCT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:417)	GCCTCCCTCGCGCC ATCAGGTAGTCACA CTGACGACATGGTT CTACA	(SEQ ID NO:418)
8	GCCTTGCCAGCCC GCTCAGGTCGATT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:419)	GCCTCCCTCGCGCC ATCAGGTCGATACA CTGACGACATGGTT CTACA	(SEQ ID NO:420)
9	GCCTTGCCAGCCC GCTCAGGATACG TACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:421)	GCCTCCCTCGCGCC ATCAGGATACGAC ACTGACGACATGGT TCTACA	(SEQ ID NO:422)
10	GCCTTGCCAGCCC GCTCAGTGATGCT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:423)	GCCTCCCTCGCGCC ATCAGTGATGCACA CTGACGACATGGTT CTACA	(SEQ ID NO:424)

11	GCCTTGCCAGCCC GCTCAGAGCTGA TACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:425)	GCCTCCCTCGCGCC ATCAGAGCTGAAC ACTGACGACATGGT TCTACA	(SEQ ID NO:426)
12	GCCTTGCCAGCCC GCTCAGACTGTAT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:427)	GCCTCCCTCGCGCC ATCAGACTGTAACA CTGACGACATGGTT CTACA	(SEQ ID NO:428)
13	GCCTTGCCAGCCC GCTCAGTGCATGT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:429)	GCCTCCCTCGCGCC ATCAGTGCATGACA CTGACGACATGGTT CTACA	(SEQ ID NO:430)
14	GCCTTGCCAGCCC GCTCAGAGTCTAT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:431)	GCCTCCCTCGCGCC ATCAGAGTCTAACA CTGACGACATGGTT CTACA	(SEQ ID NO:432)
15	GCCTTGCCAGCCC GCTCAGTGTCTGT ACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:433)	GCCTCCCTCGCGCC ATCAGTGTCTGACA CTGACGACATGGTT CTACA	(SEQ ID NO:434)
16	GCCTTGCCAGCCC GCTCAGGCTAGC TACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:435)	GCCTCCCTCGCGCC ATCAGGCTAGCAC ACTGACGACATGGT TCTACA	(SEQ ID NO:436)

Running the Access Array IFC

[0268] The containment and interface accumulator reservoirs were filled with 300 μl of Control Line Fluid (Fluidigm PN 89000020), and the H1-H4 reagent wells were loaded with 500 μl of 0.05% Tween-20 in PCR-grade water prior to Access Array IFC loading. 5μl of each sample mixture was loaded into the sample ports, and 5 μl of each primer mixture was loaded into the primer inlets on the Access Array IFC.

[0269] The Access Array IFC was thermal cycled and imaged using an IFC Stand-Alone Thermal Cycler (Fluidigm Corporation). The thermal cycling protocol contains a thermal mix step [50 °C for 2 min, 70 °C for 20 min], a hotstart step [95 °C for 10 min], a 35 cycle PCR strategy [2 cycles of 95 °C for 15 sec and 60 °C for 4 min, 33 cycles of 95 °C for 15 sec, 60 °C for 15 sec, 72°C for 1 min, and an elongation step [72 °C for 3 min].

[0270] After amplification, the PCR products were harvested from the Access Array IFC using the Post-PCR IFC Loader AX. Before harvesting, each sample port was filled with 2 μl of 0.05% Tween-20. Residual solution was removed from the H1-H4 reagent wells, and they were refilled with 600 μl of 1X Access Array

Harvesting Reagent (0.05% tween-20). After harvesting each sample port became a PCR product outlet that contained 10 μl (± 10%) of 48 pooled PCR products. The pooled PCR products were removed from the Access Array IFC and stored in a microtiter plate at 4 °C.

[0271] PCR products for each sample were pooled based on concentrations calculated from the Agilent Bioanalyzer traces. The purified product pool was subjected to emulsion PCR followed by pyrosequencing on a 454 FLX sequencer (Roche Analytical Sciences) according to manufacturer's instructions. Emulsion PCR reactions were run with beads containing both A and B primer sequences attached, enabling sequence reads for both strands of the amplicon.

[0272] The number of sequences counted for each individual PCR product in each sample were analyzed to demonstrate representation of the PCR products shown in Figure 15. Sequences could be counted for each of the amplicons shown in Figure 15B, by summing tag 5 sequences from emulsion A with tag 8 sequences from emulsion B (Figure 16A) or tag 5 sequences from emulsion B with tag 8 sequences from emulsion A. Figure 16 shows that representation of all amplicons mostly lies between 2x and 0.5x of average coverage. Furthermore, representation of both amplicons for each primer pair is very similar, although the amplicon represented in Figure 16B shows less variation within samples.

Example 6

4-Primer Barcoding Of Target Nucleic Acids for Illumina DNA Sequencing Using a Microfluidic Device that Permits Recovery of Amplication Products

[0273] Sequences designed for a 4-primer tagging scheme to be used on the Illumina Genome Analyzer II are shown in Tables 13 and 14. The tag sequence is the inner primer sequence.

Table 13

Inner primers		
Tag Sequence	**Target-Specific Sequence (Forward)**	**Oligonucleotide Sequence**
ACACTCTTTCCCTACACGACGCTCTTCCGATCT (SEQ ID NO:437)	ACTGTCCAGCTTTGTGCC (SEQ ID NO:438)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTGTCCAGCTTTGTGCC (SEQ ID NO:439)
ACACTCTTTCCCTACACGACGCTCTTCCGAT	GATCATCATAGGAGTTGCATTGTTG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCATCATAGGAGTTGCATTGTTG

CT (SEQ ID NO:440)	(SEQ ID NO:441)	(SEQ ID NO:442)
Tag Sequence	**Target-Specific Sequence (Reverse)**	**Oligonucleotide Sequence**
CTCGGCATTCCTGCTG AACCGCTCTTCCGAT CT (SEQ ID NO:443)	TCCTCTGCCTAGG CGTT (SEQ ID NO:444)	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTTCCTCTGCCTAGGCGTT (SEQ ID NO:445)
CTCGGCATTCCTGCTG AACCGCTCTTCCGAT CT (SEQ ID NO:446)	GAAATGTAAATGT GGAGCCAAACA (SEQ ID NO:447)	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTGAAATGTAAATGTGGAGCCAAACA (SEQ ID NO:448)

Table 14

Barcode Primers	**Direction**	
ILMN_PE1sh_F	Forward	AATGATACGGCGACCAC**CGAGATC**TACACTCTTT CCCTACACGA (SEQ ID NO:449)
ILMN_PE2sh_R	Reverse	CAAGCAGAAGACGGCATA**CGAGATC**GGTCTCGG CATTCCTGCTGAAC (SEQ ID NO:450)

[0274] The successful amplication of a PCR product using the 4-primer strategy designed for use on the Illumina GA II sequencer is shown in Figure 17.

Example 7

Barcoding Of Target Nucleic Acids for Titanium Chemistry on the 454 FLX Sequencer (Roche Analytical Sciences)

[0275] Table 15 shows forward barcode sequences for use with Titanium chemistry on the 454 FLX Sequencer (Roche Analytical Sciences). Table 16 shows reverse barcode sequences for use with Titanium chemistry on the 454 FLX Sequencer (Roche Analytical Sciences).

Table 15

Well	**Barcode**	**Forward Oligo Name**	**Forward Oligo sequence**	**SEQ ID NO.**
A1	TI-MID1	TI-F-MID1-TAG8	CGTATCGCCTCCCTCGCGCCATCA GACGAGTGCGTACACTGACGACA TGGTTCTACA	(SEQ ID NO:451)
B1	TI-MID2	TI-F-MID2-TAG8	CGTATCGCCTCCCTCGCGCCATCA GACGCTCGACAACACTGACGACA TGGTTCTACA	(SEQ ID NO:452)

C1	TI-MID3	TI-F-MID3-TAG8	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCACACTGACGACATGGTTCTACA	(SEQ ID NO:453)
D1	TI-MID67	TI-F-MID67-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCGATAGTGAACACTGACGACATGGTTCTACA	(SEQ ID NO:454)
E1	TI-MID5	TI-F-MID5-TAG8	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGACACTGACGACATGGTTCTACA	(SEQ ID NO:455)
F1	TI-MID6	TI-F-MID6-TAG8	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGACACTGACGACATGGTTCTACA	(SEQ ID NO:456)
G1	TI-MID7	TI-F-MID7-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCGTGTCTCTAACACTGACGACATGGTTCTACA	(SEQ ID NO:457)
H1	TI-MID8	TI-F-MID8-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCTCGCGTGTCACACTGACGACATGGTTCTACA	(SEQ ID NO:458)
A2	TI-MID10	TI-F-MID10-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCTCTATGCGACACTGACGACATGGTTCTACA	(SEQ ID NO:459)
B2	TI-MID11	TI-F-MID11-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTGATACGTCTACACTGACGACATGGTTCTACA	(SEQ ID NO:460)
C2	TI-MID13	TI-F-MID13-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCATAGTAGTGACACTGACGACATGGTTCTACA	(SEQ ID NO:461)
D2	TI-MID14	TI-F-MID14-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCGAGAGATACACACTGACGACATGGTTCTACA	(SEQ ID NO:462)
E2	TI-MID15	TI-F-MID15-TAG8	CGTATCGCCTCCCTCGCGCCATCAGATACGACGTAACACTGACGACATGGTTCTACA	(SEQ ID NO:463)
F2	TI-MID16	TI-F-MID16-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCACGTACTAACACTGACGACATGGTTCTACA	(SEQ ID NO:464)
G2	TI-MID17	TI-F-MID17-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCGTCTAGTACACACTGACGACATGGTTCTACA	(SEQ ID NO:465)
H2	TI-MID18	TI-F-MID18-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCTACGTAGCACACTGACGACATGGTTCTACA	(SEQ ID NO:466)
A3	TI-MID19	TI-F-MID19-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTGTACTACTCACACTGACGACATGGTTCTACA	(SEQ ID NO:467)
B3	TI-MID20	TI-F-MID20-TAG8	CGTATCGCCTCCCTCGCGCCATCAGACGACTACAGACACTGACGACATGGTTCTACA	(SEQ ID NO:468)
C3	TI-MID21	TI-F-MID21-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCGTAGACTAGACACTGACGACATGGTTCTACA	(SEQ ID NO:469)
D3	TI-MID22	TI-F-MID22-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTACGAGTATGACACTGACGACATGGTTCTACA	(SEQ ID NO:470)
E3	TI-MID23	TI-F-MID23-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTACTCTCGTGACACTGACGACATGGTTCTACA	(SEQ ID NO:471)
F3	TI-MID24	TI-F-MID24-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTAGAGACGAGACACTGACGACA	(SEQ ID NO:472)

			TGGTTCTACA	
G3	TI-MID25	TI-F-MID25-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCGTCGCTCGACACTGACGACATGGTTCTACA	(SEQ ID NO:473)
H3	TI-MID26	TI-F-MID26-TAG8	CGTATCGCCTCCCTCGCGCCATCAGACATACGCGTACACTGACGACATGGTTCTACA	(SEQ ID NO:474)
A4	TI-MID27	TI-F-MID27-TAG8	CGTATCGCCTCCCTCGCGCCATCAGACGCGAGTATACACTGACGACATGGTTCTACA	(SEQ ID NO:475)
B4	TI-MID28	TI-F-MID28-TAG8	CGTATCGCCTCCCTCGCGCCATCAGACTACTATGTACACTGACGACATGGTTCTACA	(SEQ ID NO:476)
C4	TI-MID68	TI-F-MID68-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCGCTGCGTAACACTGACGACATGGTTCTACA	(SEQ ID NO:477)
D4	TI-MID30	TI-F-MID30-TAG8	CGTATCGCCTCCCTCGCGCCATCAGAGACTATACTACACTGACGACATGGTTCTACA	(SEQ ID NO:478)
E4	TI-MID31	TI-F-MID31-TAG8	CGTATCGCCTCCCTCGCGCCATCAGAGCGTCGTCTACACTGACGACATGGTTCTACA	(SEQ ID NO:479)
F4	TI-MID32	TI-F-MID32-TAG8	CGTATCGCCTCCCTCGCGCCATCAGAGTACGCTATACACTGACGACATGGTTCTACA	(SEQ ID NO:480)
G4	TI-MID33	TI-F-MID33-TAG8	CGTATCGCCTCCCTCGCGCCATCAGATAGAGTACTACACTGACGACATGGTTCTACA	(SEQ ID NO:481)
H4	TI-MID34	TI-F-MID34-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCACGCTACGTACACTGACGACATGGTTCTACA	(SEQ ID NO:482)
A5	TI-MID35	TI-F-MID35-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCAGTAGACGTACACTGACGACATGGTTCTACA	(SEQ ID NO:483)
B5	TI-MID36	TI-F-MID36-TAG8	CGTATCGCCTCCCTCGCGCCATCAGCGACGTGACTACACTGACGACATGGTTCTACA	(SEQ ID NO:484)
C5	TI-MID37	TI-F-MID37-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTACACACACTACACTGACGACATGGTTCTACA	(SEQ ID NO:485)
D5	TI-MID38	TI-F-MID38-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTACACGTGATACACTGACGACATGGTTCTACA	(SEQ ID NO:486)
E5	TI-MID39	TI-F-MID39-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTACAGATCGTACACTGACGACATGGTTCTACA	(SEQ ID NO:487)
F5	TI-MID40	TI-F-MID40-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTACGCTGTCTACACTGACGACATGGTTCTACA	(SEQ ID NO:488)
G5	TI-MID69	TI-F-MID69-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCTGACGTCAACACTGACGACATGGTTCTACA	(SEQ ID NO:489)
H5	TI-MID42	TI-F-MID42-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCGATCACGTACACTGACGACATGGTTCTACA	(SEQ ID NO:490)
A6	TI-MID43	TI-F-MID43-TAG8	CGTATCGCCTCCCTCGCGCCATCAGTCGCACTAGTACACTGACGACATGGTTCTACA	(SEQ ID NO:491)
B6	TI-MID44	TI-F-MID44-	CGTATCGCCTCCCTCGCGCCATCA	(SEQ ID NO:492)

		TAG8	GTCTAGCGACTACACTGACGACAT GGTTCTACA	
C6	TI-MID45	TI-F-MID45-TAG8	CGTATCGCCTCCCTCGCGCCATCA GTCTATACTATACACTGACGACAT GGTTCTACA	(SEQ ID NO:493)
D6	TI-MID46	TI-F-MID46-TAG8	CGTATCGCCTCCCTCGCGCCATCA GTGACGTATGTACACTGACGACAT GGTTCTACA	(SEQ ID NO:494)
E6	TI-MID47	TI-F-MID47-TAG8	CGTATCGCCTCCCTCGCGCCATCA GTGTGAGTAGTACACTGACGACA TGGTTCTACA	(SEQ ID NO:495)
F6	TI-MID48	TI-F-MID48-TAG8	CGTATCGCCTCCCTCGCGCCATCA GACAGTATATAACACTGACGACA TGGTTCTACA	(SEQ ID NO:496)
G6	TI-MID49	TI-F-MID49-TAG8	CGTATCGCCTCCCTCGCGCCATCA GACGCGATCGAACACTGACGACA TGGTTCTACA	(SEQ ID NO:497)
H6	TI-MID50	TI-F-MID50-TAG8	CGTATCGCCTCCCTCGCGCCATCA GACTAGCAGTAACACTGACGACA TGGTTCTACA	(SEQ ID NO:498)

Table 16

Well	Barcode	Reverse Oligo Name	Reverse Oligo Sequence	SEQ ID NO.
A1	TI-MID1	TI-R-MID1-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GACGAGTGCGTTACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:499)
B1	TI-MID2	TI-R-MID2-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GACGCTCGACATACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:500)
C1	TI-MID3	TI-R-MID3-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GAGACGCACTCTACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:501)
D1	TI-MID67	TI-R-MID67-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GTCGATAGTGATACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:502)
E1	TI-MID5	TI-R-MID5-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GATCAGACACGTACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:503)
F1	TI-MID6	TI-R-MID6-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GATATCGCGAGTACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:504)
G1	TI-MID7	TI-R-MID7-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GCGTGTCTCTATACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:505)
H1	TI-MID8	TI-R-MID8-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GCTCGCGTGTCTACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:506)
A2	TI-MID10	TI-R-MID10-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GTCTCTATGCGTACGGTAGCAGAG ACTTGGTCT	(SEQ ID NO:507)
B2	TI-MID11	TI-R-MID11-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GTGATACGTCTTACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:508)
C2	TI-MID13	TI-R-MID13-TAG5	CTATGCGCCTTGCCAGCCCGCTCA GCATAGTAGTGTACGGTAGCAGA GACTTGGTCT	(SEQ ID NO:509)

D2	TI-MID14	TI-R-MID14-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGCGAGAGATACTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:510)
E2	TI-MID15	TI-R-MID15-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGATACGACGTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:511)
F2	TI-MID16	TI-R-MID16-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCACGTACTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:512)
G2	TI-MID17	TI-R-MID17-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGCGTCTAGTACTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:513)
H2	TI-MID18	TI-R-MID18-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCTACGTAGCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:514)
A3	TI-MID19	TI-R-MID19-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTGTACTACTCTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:515)
B3	TI-MID20	TI-R-MID20-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGACGACTACAGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:516)
C3	TI-MID21	TI-R-MID21-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGCGTAGACTAGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:517)
D3	TI-MID22	TI-R-MID22-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTACGAGTATGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:518)
E3	TI-MID23	TI-R-MID23-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTACTCTCGTGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:519)
F3	TI-MID24	TI-R-MID24-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTAGAGACGAGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:520)
G3	TI-MID25	TI-R-MID25-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCGTCGCTCGTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:521)
H3	TI-MID26	TI-R-MID26-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGACATACGCGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:522)
A4	TI-MID27	TI-R-MID27-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGACGCGAGTATTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:523)
B4	TI-MID28	TI-R-MID28-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGACTACTATGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:524)
C4	TI-MID68	TI-R-MID68-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCGCTGCGTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:525)
D4	TI-MID30	TI-R-MID30-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGAGACTATACTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:526)
E4	TI-MID31	TI-R-MID31-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGAGCGTCGTCTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:527)
F4	TI-MID32	TI-R-MID32-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGAGTACGCTATTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:528)
G4	TI-MID33	TI-R-MID33-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGATAGAGTACTTACGGTAGCAGA	(SEQ ID NO:529)

			GACTTGGTCT	
H4	TI-MID34	TI-R-MID34-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGCACGCTACGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:530)
A5	TI-MID35	TI-R-MID35-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGCAGTAGACGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:531)
B5	TI-MID36	TI-R-MID36-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGCGACGTGACTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:532)
C5	TI-MID37	TI-R-MID37-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTACACACACTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:533)
D5	TI-MID38	TI-R-MID38-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTACACGTGATTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:534)
E5	TI-MID39	TI-R-MID39-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTACAGATCGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:535)
F5	TI-MID40	TI-R-MID40-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTACGCTGTCTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:536)
G5	TI-MID69	TI-R-MID69-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCTGACGTCATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:537)
H5	TI-MID42	TI-R-MID42-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCGATCACGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:538)
A6	TI-MID43	TI-R-MID43-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCGCACTAGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:539)
B6	TI-MID44	TI-R-MID44-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCTAGCGACTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:540)
C6	TI-MID45	TI-R-MID45-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTCTATACTATTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:541)
D6	TI-MID46	TI-R-MID46-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTGACGTATGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:542)
E6	TI-MID47	TI-R-MID47-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGTGTGAGTAGTTACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:543)
F6	TI-MID48	TI-R-MID48-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGACAGTATATATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:544)
G6	TI-MID49	TI-R-MID49-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGACGCGATCGATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:545)
H6	TI-MID50	TI-R-MID50-TAG5	CTATGCGCCTTGCCAGCCCGCTCAGACTAGCAGTATACGGTAGCAGAGACTTGGTCT	(SEQ ID NO:546)

Example 8

Muliplex Barcoding Of Target Nucleic Acids

[0276] Three pools of 10 primers were assembled from the primers listed in Table 9. PCR conditions were identical to those listed in Example 4, with the exception that primer concentrations were varied. Figure 18 shows the results of PCR reactions of three pools of 10 sets of PCR primers (A, B, C) when the PCR reactions were run for template-specific primers only and in 4-primer mode. The presence of higher molecular weight products in the 4-primer strategy dmonestrates successful 4-primer assembly.

CLAIMS

What is claimed is:

1. A method for amplifying a plurality of target nucleic acids in a plurality of samples, the method comprising:

preparing an amplification mixture for each target nucleic acid, said amplification mixture comprising:

a forward primer comprising a target-specific portion;

a reverse primer comprising a target-specific portion, wherein the forward primer additionally comprises a first nucleotide tag and/or the reverse primer additionally comprises a second nucleotide tag; and

at least one barcode primer comprising a barcode nucleotide sequence and a first and/or second nucleotide tag-specific portion, wherein the barcode primer is in excess of the forward and/or reverse primer(s);

subjecting each amplification mixture to amplification to produce a plurality of target amplicons comprising tagged target nucleotide sequences, each comprising first and/or second nucleotide tags flanking the target nucleotide sequence, and at least one barcode nucleotide sequence at the 5' or 3' end of the target amplicon.

2. The method of claim 1, wherein the forward primer additionally comprises a first nucleotide tag.

3. The method of claims 1 or 2, wherein the reverse primer additionally comprises a second nucleotide tag.

4. The method of claim 1, wherein the concentration of the barcode primer in the amplification mixtures is at least 4-fold the concentration of the forward and/or reverse primer(s).

5. The method of claim 4, wherein the concentration of the barcode primer in the amplification mixtures is at least 50-fold the concentration of the forward and/or reverse primer(s).

6. The method of claim 1, wherein the first and/or second nucleotide tags and/or the barcode nucleotide sequence are selected so as to avoid substantial annealing to the target nucleic acids.

7. The method of claim 1, wherein the barcode nucleotide sequence identifies a particular sample.

8. The method of claims 1 or 7, wherein the barcode primer comprises a barcode nucleotide sequence and a first nucleotide tag-specific portion.

9. The method of claim 8, wherein a plurality of forward primers comprise the same first nucleotide tag.

10. The method of claim 9, wherein the all forward primers used to amplify target sequences in each sample comprise the same first nucleotide tag.

11. The method of claim 7, wherein the forward and reverse primers for each target are initially combined separately from the sample, and each barcode primer is initially combined with its corresponding sample.

12. The method of claim 11, wherein T targets are amplified in S samples, T and S being integers greater than one, the method additionally comprising preparing S×T amplification mixtures wherein the initially combined forward and reverse primers are added to the initially combined samples and barcode primers.

13. The method of claim 1, wherein the amplification is carried out for at least 3 cycles to introduce the first and second nucleotide tags and the barcode nucleotide sequence.

14. The method of claim 13, wherein the amplification is carried out for between 5 and 50 cycles.

15. The method of claims 1, wherein the amplification is carried out for a sufficient number of cycles to normalize target amplicon copy number across targets and across samples.

16. The method of claim 1, wherein at least 50 percent of the target amplicons are present at greater than 50 percent of the average number of copies of

target amplicons and less than 2-fold the average number of copies of target amplicons.

17. A method for amplifying a plurality of target nucleic acids in a plurality of samples, the method comprising:

preparing an amplification mixture for each target nucleic acid, said amplification mixture comprising:

a forward primer comprising a target-specific sequence; and

a reverse primer comprising a target-specific sequence;

subjecting each amplification mixture to amplification to produce a plurality of target nucleotide sequences;

tagging the target nucleotide sequences to produce a plurality of target amplicons, each comprising first and/or second nucleotide tags flanking the target nucleotide sequence;

wherein at least 50 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

18. The method of claim 16 or 17, wherein at least 70 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

19. The method of claims 16, 17, or 18, wherein the average length of the target amplicons is at least 25 bases.

20. The method of claims 16, 17, or 18, wherein the average length of the target amplicons is at least 50 bases.

21. The method of claims 16, 17, or 18, wherein the average length of the target amplicons is at least 100 bases.

22. The method of claims 16, 17, or 18, wherein the average length of the target amplicons is at least 200 bases.

23. The method of claims 16, 17, or 18, wherein the average length of the target amplicons is at least 1 kilobase.

24. The method of claim 16 or 17, wherein at least 90 percent of the target amplicons are present at greater than 50 percent of the average number of copies of target amplicons and less than 2-fold the average number of copies of target amplicons.

25. The method of any of the preceding claims, wherein the volume of the amplification mixtures is in the range of about 1 picoliter to about 50 nanoliters.

26. The method of claim 17, wherein the volume of the amplification mixtures is in the range of about 5 picoliters to about 25 nanoliters.

27. The method of claims 1 or 17, wherein the amplification mixtures are formed in, or distributed into, separate compartments of a microfluidic device prior to amplification.

28. The method of claim 27, wherein the microfluidic device is fabricated, at least in part, from an elastomeric material.

29. The method of claims 1 or 17, wherein the amplification is carried out by polymerase chain reaction (PCR).

30. The method of claim 1 or 17, additionally comprising recovering the target amplicons from the amplification mixtures.

31. The method of claim 30, wherein the target amplicons are recovered in a volume and/or copy number that varies less than about 50% among the recovered target amplicons.

32. The method of claim 30, additionally comprising subjecting at least one target amplicon to amplification using primers specific for the first and second nucleotide tags to produce a target amplicon lacking the barcode nucleotide sequence.

33. The method of claims 1 or 17, wherein the target nucleic acids comprise genomic DNA.

34. The method of claim 1, wherein one or more of the forward primer, reverse primer, and barcode primer comprises at least one additional primer binding site.

35. The method of claim 34, wherein the barcode primer comprises at least a first additional primer binding site upstream of the barcode nucleotide sequence, which is upstream of the first nucleotide tag.

36. The method of claim 35, wherein the reverse primer comprises at least a second additional primer binding site downstream of the second nucleotide tag.

37. The method of claim 36, wherein the first and second additional primer binding sites are capable of being bound by DNA sequencing primers.

38. The method of claim 17, wherein the first and second nucleotide tags are capable of being bound by DNA sequencing primers.

39. The method of claim 37 or 38, additionally comprising subjecting at least one target amplicon to DNA sequencing.

40. The method of claims 1 or 17, additionally comprising quantifying the amount of target amplicons in the amplification mixtures.

41. The method of claim 40, wherein said quantifying comprises recovering the target amplicons and subjecting them to digital amplification.

42. The method of claim 41, wherein said digital amplification comprises:

distributing the preamplified target amplicons into discrete reaction mixtures, wherein each reaction mixture, on average, includes no more than one amplicon per reaction mixture; and

subjecting the reaction mixtures to amplification.

43. The method of claim 42, wherein said digital amplification comprises real-time PCR.

44. The method of claim 42, wherein said digital amplification comprises endpoint PCR.

45. The method of claim 42, wherein the amplification mixtures are formed in, or distributed into, separate compartments of a microfluidic device prior to amplification.

46. The method of claim 45, wherein the microfluidic device is fabricated, at least in part, from an elastomeric material.

47. The method of claims 1 or 17, wherein the presence of a target amplicon is determined by quantitative real-time polymerase chain reaction (qPCR).

48. The method of claims 1 or 17, wherein a universal qPCR probe is employed in the amplification mixtures to detect target amplicons.

49. The method of claims 1 or 17, wherein one or more target-specific qPCR probes is employed in the amplification mixtures to detect target amplicons.

50. The method of claims 1 or 17, wherein the presence of a target amplicon is detected using a fluorogenic nuclease assay.

51. The method of claims 1 or 17, wherein the presence of a target amplicon is detected using a dual-labeled fluorogenic hydrolysis oligonucleotide probe.

52. The method of claims 1 or 17, additionally comprising determining the amount of each target nucleic acid present in each sample.

53. The method of claims 1 or 17, wherein the method is performed in determining the copy numbers of the target nucleic acids in each sample.

54. The method of claims 1 or 17, wherein the method is performed in determining the genotypes at loci corresponding to the target nucleic acids.

55. The method of claims 1 or 17, wherein the method is performed in determining the expression levels of the target nucleic acids.

56. The method of claims 1 or 17, wherein the method is performed to prepare target nucleic acids for sequencing.

57. A microfluidic device comprising:

a plurality of first input lines;

a plurality of second input lines;

a plurality of sets of first chambers, wherein each set of first chambers is in fluid communication with one of the plurality of first input lines;

a plurality of sets of second chambers, wherein each set of second chambers is in fluid communication with one of the plurality of second input lines;

a plurality of first pump elements in fluid communication with a first portion of the plurality of second input lines; and

a plurality of second pump elements in fluid communication with a second portion of the plurality of second input lines.

58. The microfluidic device of claim 57 wherein the first chambers comprise assay chambers and the second chambers comprise sample chambers.

59. The microfluidic device of claim 57 further comprising a set of valves preventing fluid communication between sample chambers making up a set of sample chambers.

60. The microfluidic device of claim 59 further comprising a set of valves preventing fluid communication between assay chambers making up a set of assay chambers and a sample chamber from each of the sets of sample chambers.

61. The microfluidic device of claim 57 wherein the plurality of first pump elements comprise a plurality of first valves.

62. The microfluidic device of claim 57 wherein the plurality of second pump elements comprise a plurality of second valves.

63. The microfluidic device of claim 57 wherein the microfluidic device comprises elastomeric material.

64. The microfluidic device of claim 57 wherein for a set of the plurality of sets of first chambers:

one of the first chambers is in fluid communication with a second chamber of a first set of second chambers via a fluid line;

another of the first chambers is in fluid communication with a second chamber of a second set of second chambers via another fluid line;

a first valve is operable to obstruct fluid flow through the fluid line; and

a second valve is operable to obstruct fluid flow through the another fluid line.

65. The microfluidic device of claim 64 wherein the first valve and the second valve are actuated using a common source.

66. A method of operating a microfluidic device having an assay chamber, a sample chamber, and a harvesting port, the method comprising:

closing a fluid line between the assay chamber and the sample chamber;

flowing a sample into the sample chamber via a sample input line;

flowing an assay into the assay chamber via an assay input line;

opening the fluid line between the assay chamber and the sample chamber;

combining at least a portion of the sample and at least a portion of the assay to form a mixture;

reacting the mixture to form a reaction product;

closing the fluid line between the assay chamber and the sample chamber;

flowing a harvesting reagent from the harvesting port to the sample chamber; and

removing the reaction product from the microfluidic device.

67. The method of claim 66 further comprising thermocycling the mixture.

68. The method of claim 66 wherein removing the reaction product from the microfluidic device comprises flowing the reaction product through at least a portion of the sample input line to a sample input port.

69. The method of claim 68 wherein flowing the reaction product through at least a portion of the sample input line comprises performing dilation pumping.

70. The method of claim 69 wherein dilation pumping comprises a fluid flow rate of less than or equal to 10 μl per hour.

71. The method of claim 69 wherein performing dilation pumping comprises:

a) closing a first valve disposed between the sample chamber and the sample input port;

b) opening a second valve disposed between the harvesting port and the sample chamber;

c) closing the second valve;

d) opening the first valve; and

repeating steps (a) through (d) a predetermined number of times.

72. The method of claim 70 wherein the fluid flow rate is less than or equal to 5 μl per hour.

73. The method of claim 72 wherein the fluid flow rate is less than or equal to 1 μl per hour.

74. The method of claim 66 wherein combining at least a portion of the sample and at least a portion of the assay to form a mixture comprises a free interface diffusion process.

75. The method of claim 66 wherein removing the reaction product comprises a fluid flow rate of less than or equal to 10 microliters per hour.

76. The method of claim 75 wherein the fluid flow rate is less than or equal to 5 microliters per hour.

77. The method of claim 76 wherein the fluid flow rate is less than or equal to 2 microliters per hour.

78. The method of claim 77 wherein the fluid flow rate is less than or equal to 1 microliters per hour.

79. The method of claim 66 wherein removing the reaction product comprises removing at least 95% of the reaction product from the microfluidic device.

80. The method of claim 66 wherein flowing the sample into the sample chamber via the sample input line comprises applying pressure to a sample input port to pressurize the sample input line.

81. The method of claim 66 wherein flowing the assay into the assay chamber via the assay input line comprises applying pressure to an assay input port to pressurize the assay input line.

82. A method of preparing reaction products, the method comprising:

providing M samples;

providing N assays;

mixing the M samples and N assays to form MxN pairwise combinations, each of the MxN pairwise combinations being contained in a closed volume;

forming MxN reaction products from the MxN pairwise combinations; and

recovering the MxN reaction products.

83. The method of claim 82 wherein the M samples are contained in M sets of first chambers and the N assays are contained in N sets of second chambers.

84. The method of claim 83 wherein the first chambers containing one of the M samples are associated with a set of second chambers, each of the set of second chambers containing one of the N assays.

85. The method of claim 84 wherein mixing the M samples and N assays comprises opening fluid lines to provide for fluid communication between the firsrt chambers containing one of the M samples and each of the set of second chambers containing one of the N assays.

86. The method of claim 82 wherein the first chambers are characterized by a volume of less than or equal to 100 nl.

87. The method of claim 86 wherein the volume is less than or equal to 40 nl.

88. The method of claim 82 wherein the second chambers are characterized by a volume of less than or equal to 10 nl.

89. The method of claim 88 wherein the volume is less than or equal to 2 nl.

90. The method of claim 82 wherein the closed volume is characterized by a volume of less than or equal to 100 nl.

91. The method of claim 82 further comprising thermocycling the MxN pairwise combinations.

92. The method of claim 82 wherein the M samples are provided at M sample ports of a microfluidic device.

93. The method of claim 91 wherein the MxN samples are recovered at the M sample ports of the microfluidic device.

94. The method of claim 82 wherein forming the MxN pairwise combinations is performed concurrently.

95. The method of claim 82 wherein forming the MxN pairwise combinations is performed sequentially.

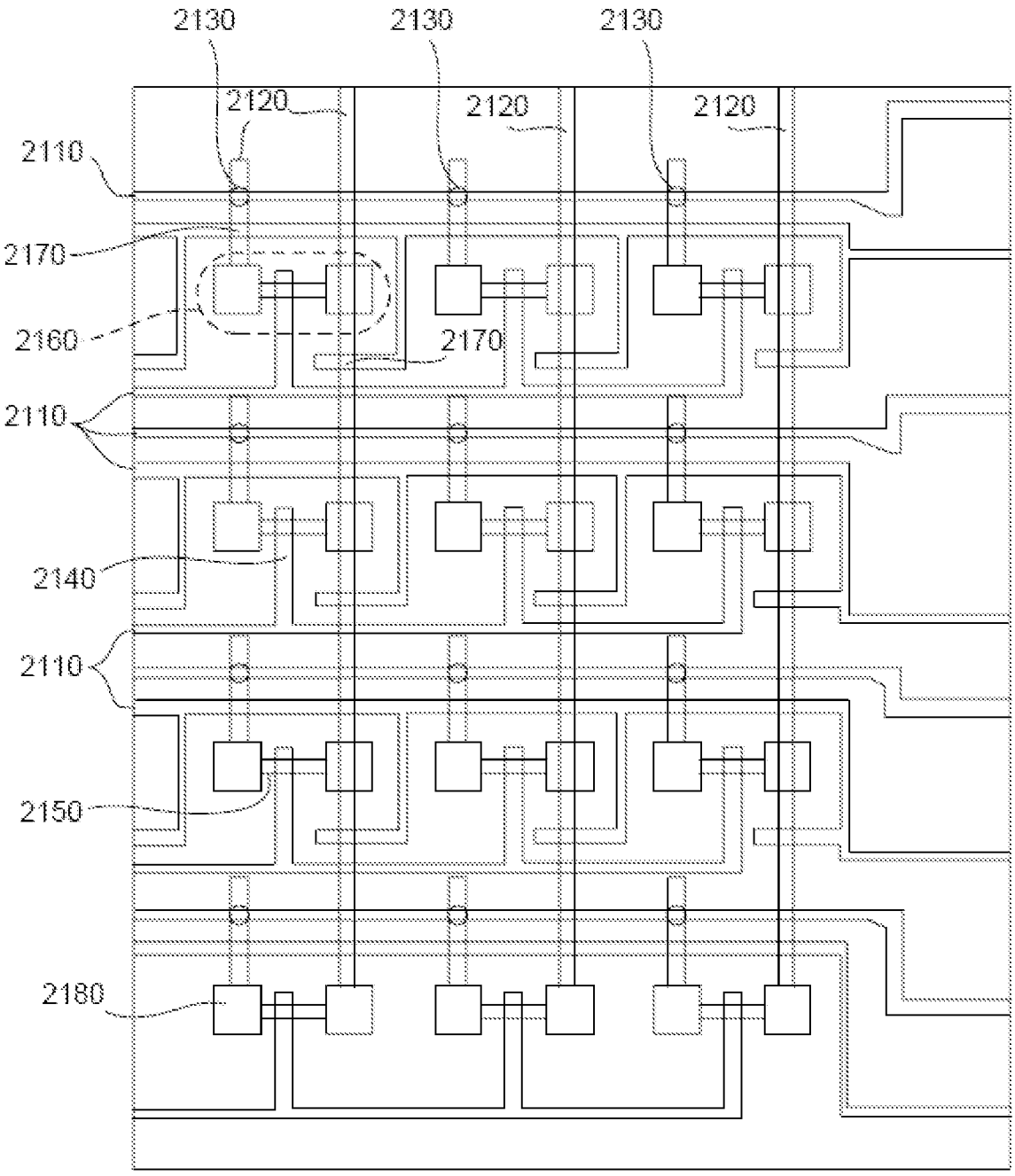

2130
2130
2130
2120
2120
2120
2110
2170
2160
2170
2110
2140
2110
2150
2180

Fig. 1

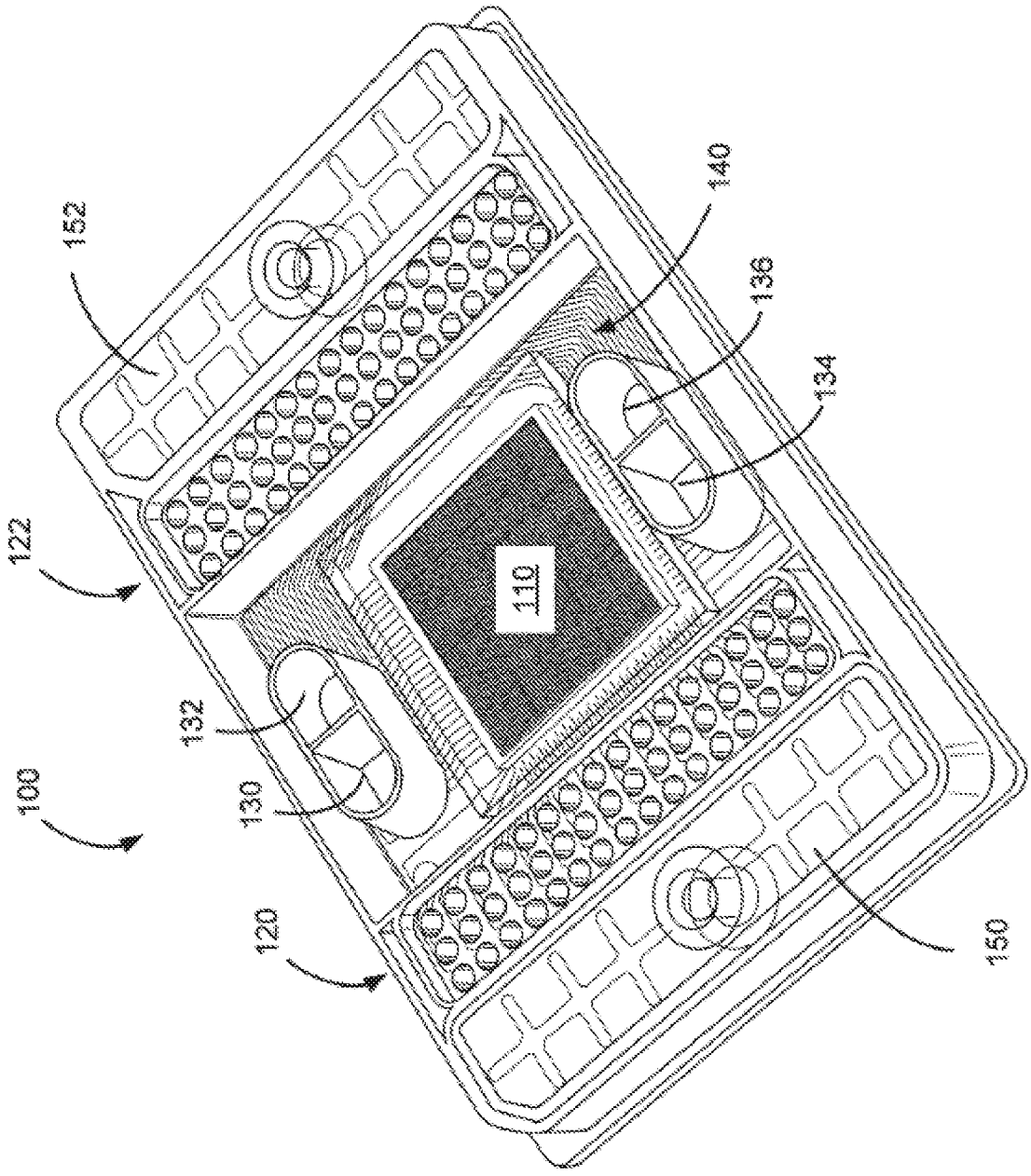


Fig. 2

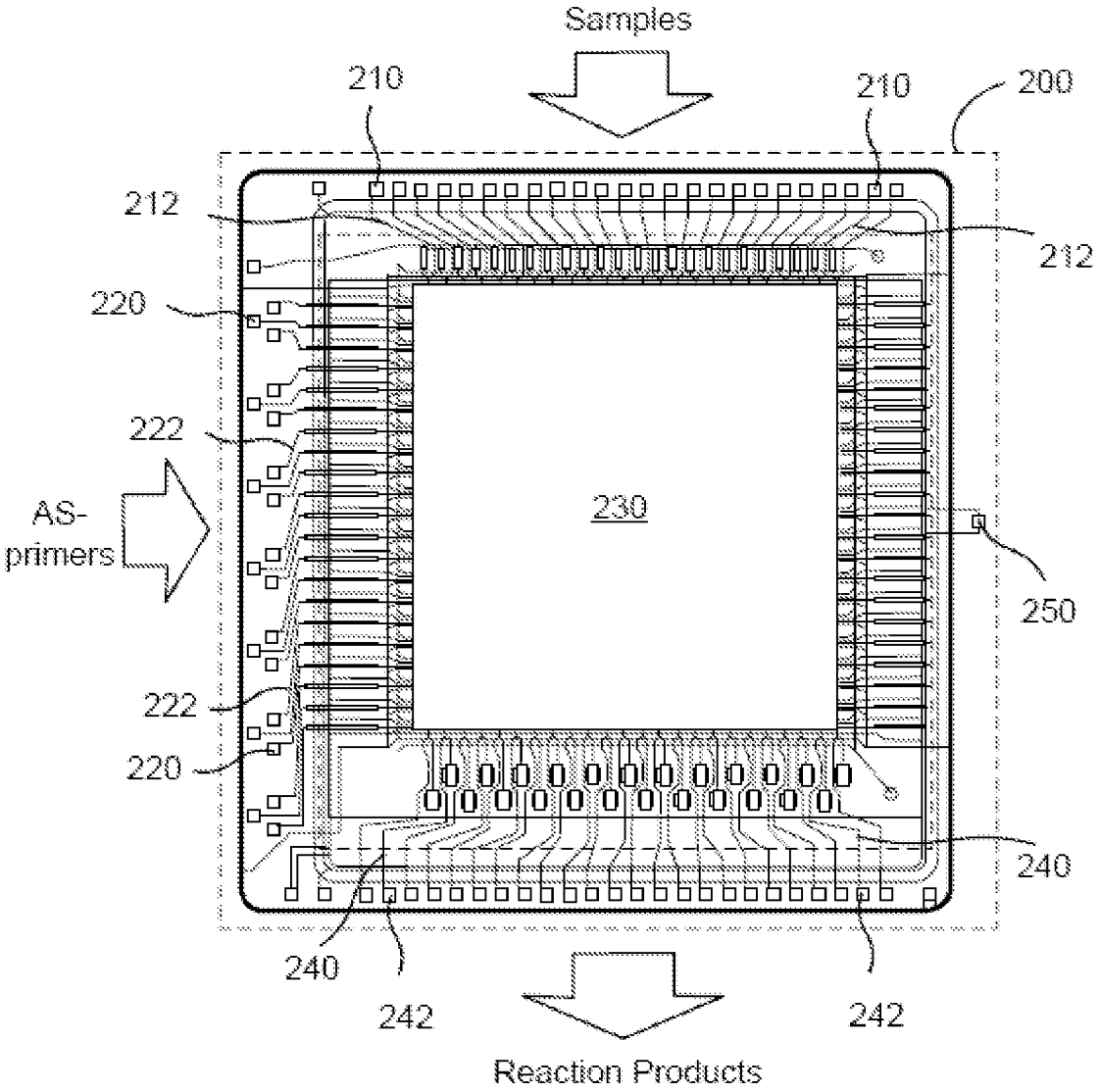

Samples
210
210
200
212
212
220
222
AS-
primers
230
250
222
220
240
240
242
242
Reaction Products

Fig. 3

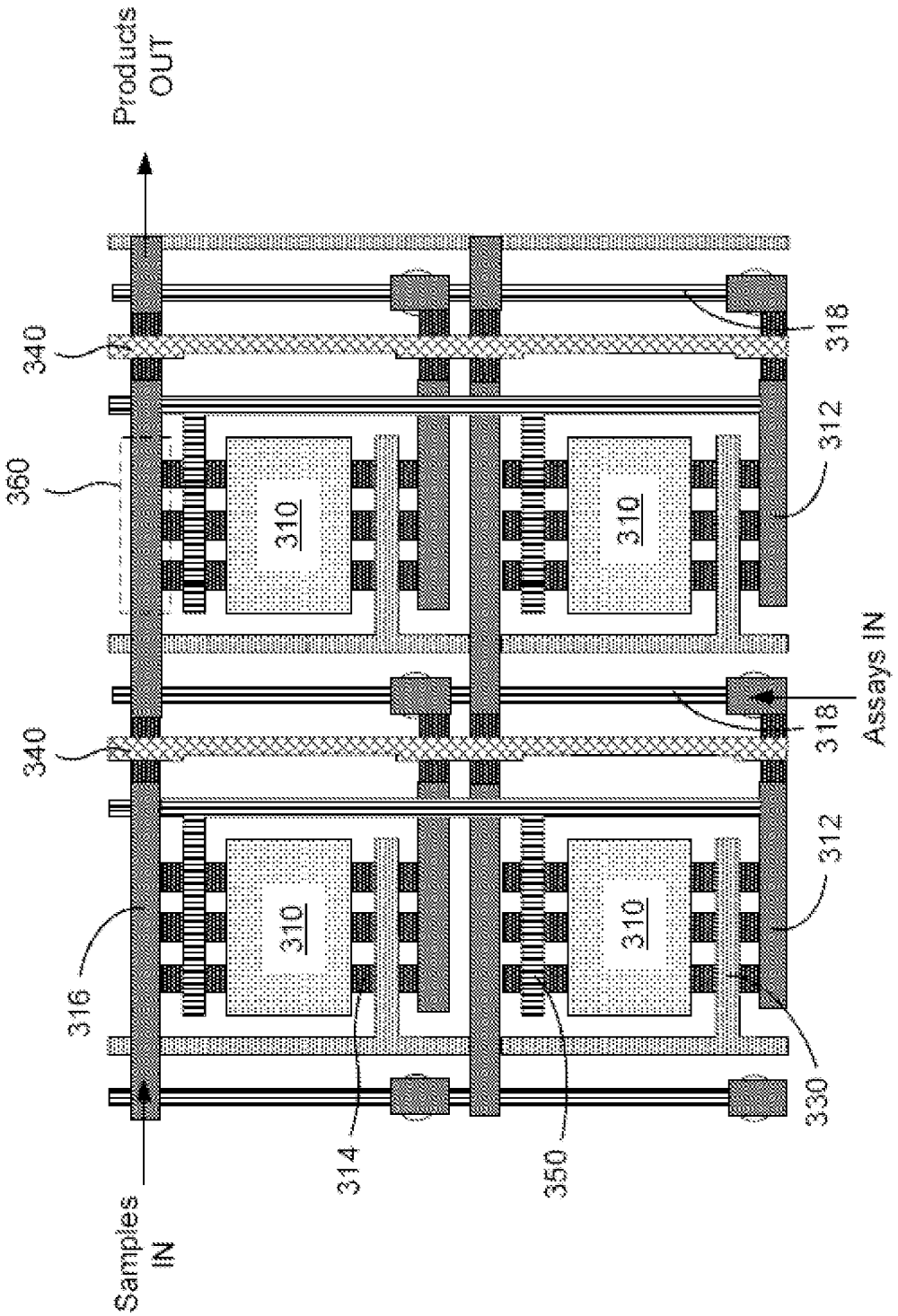

Products OUT
340
360
340
316
Samples IN
310
310
310
310
312
312
318
318
Assays IN
314
350
330

Fig. 4

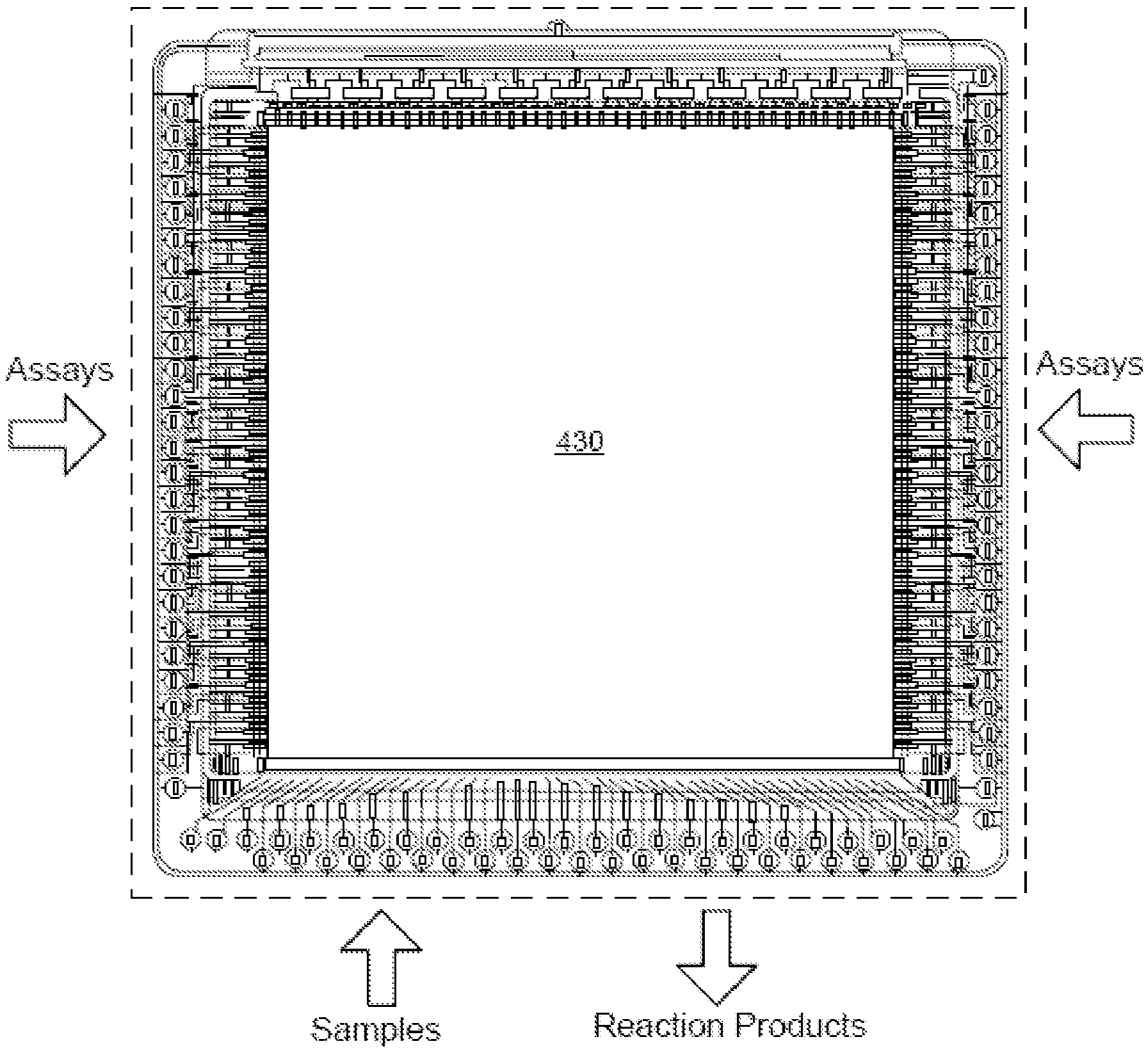


Fig. 5A

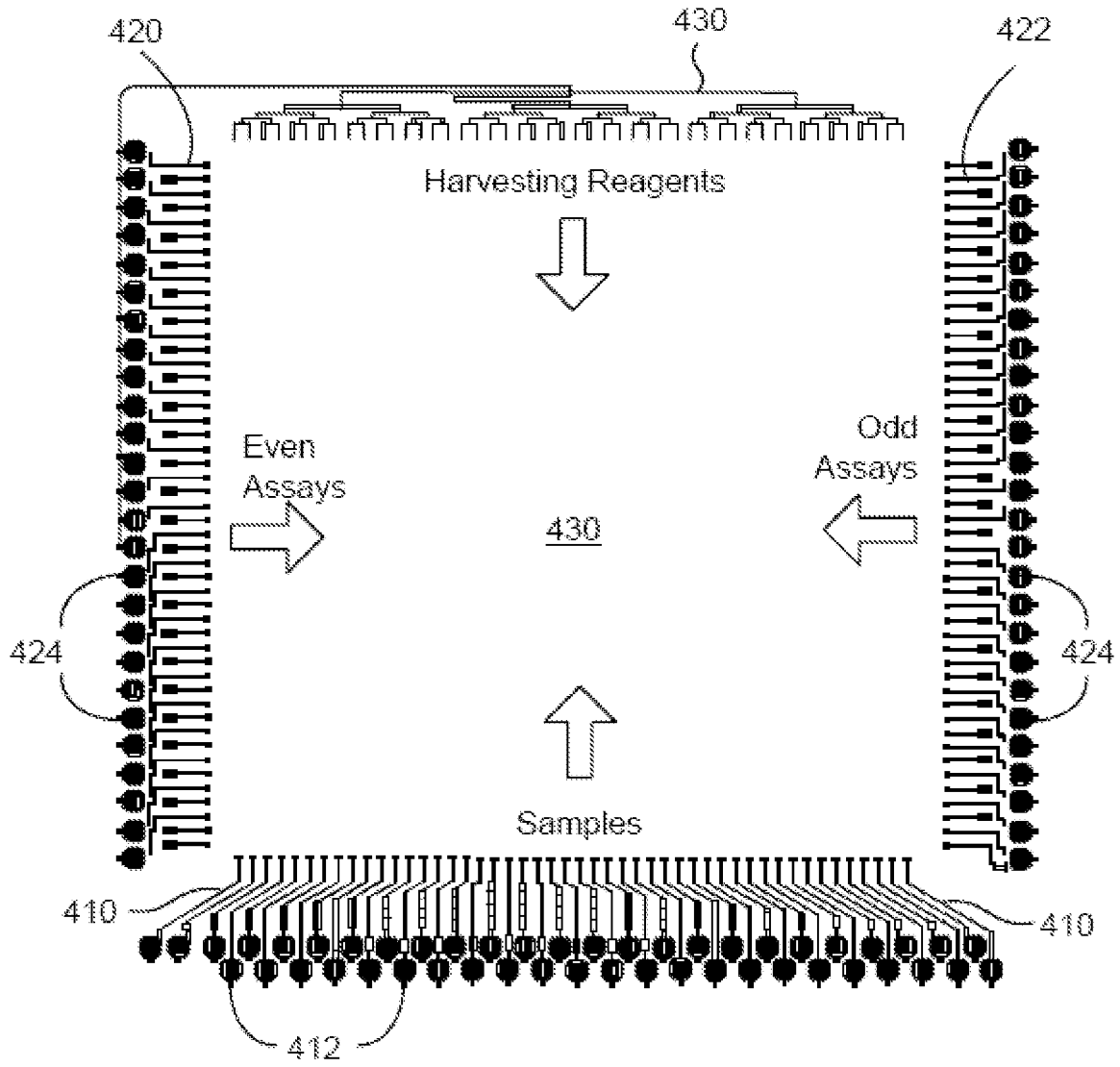

420
430
422
Harvesting Reagents
Even
Assays
Odd
Assays
430
424
424
Samples
410
410
412

Fig. 5B

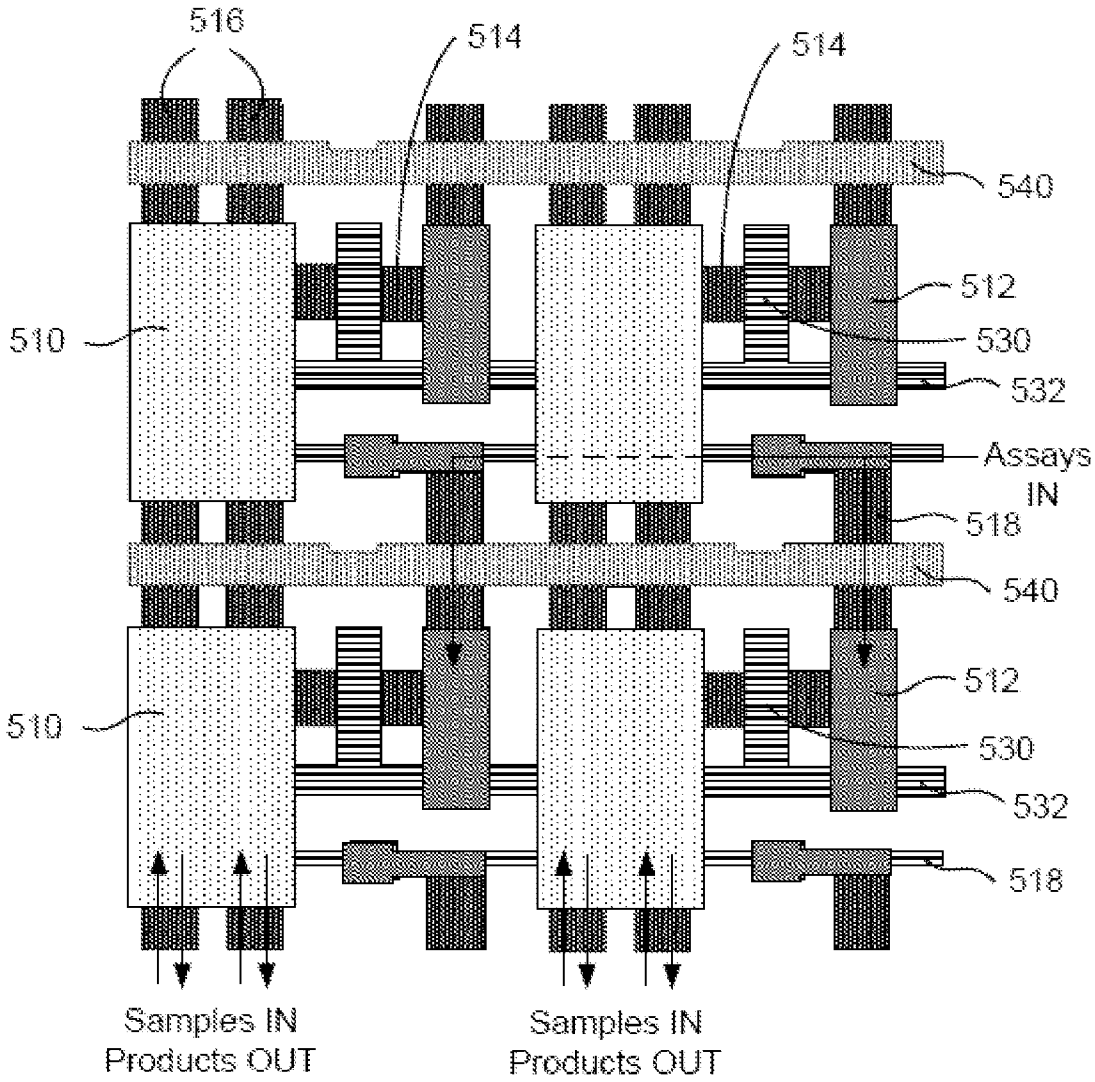

516
514
514
540
512
510
530
532
Assays IN
518
540
512
510
530
532
518
Samples IN
Products OUT
Samples IN
Products OUT

Fig. 6

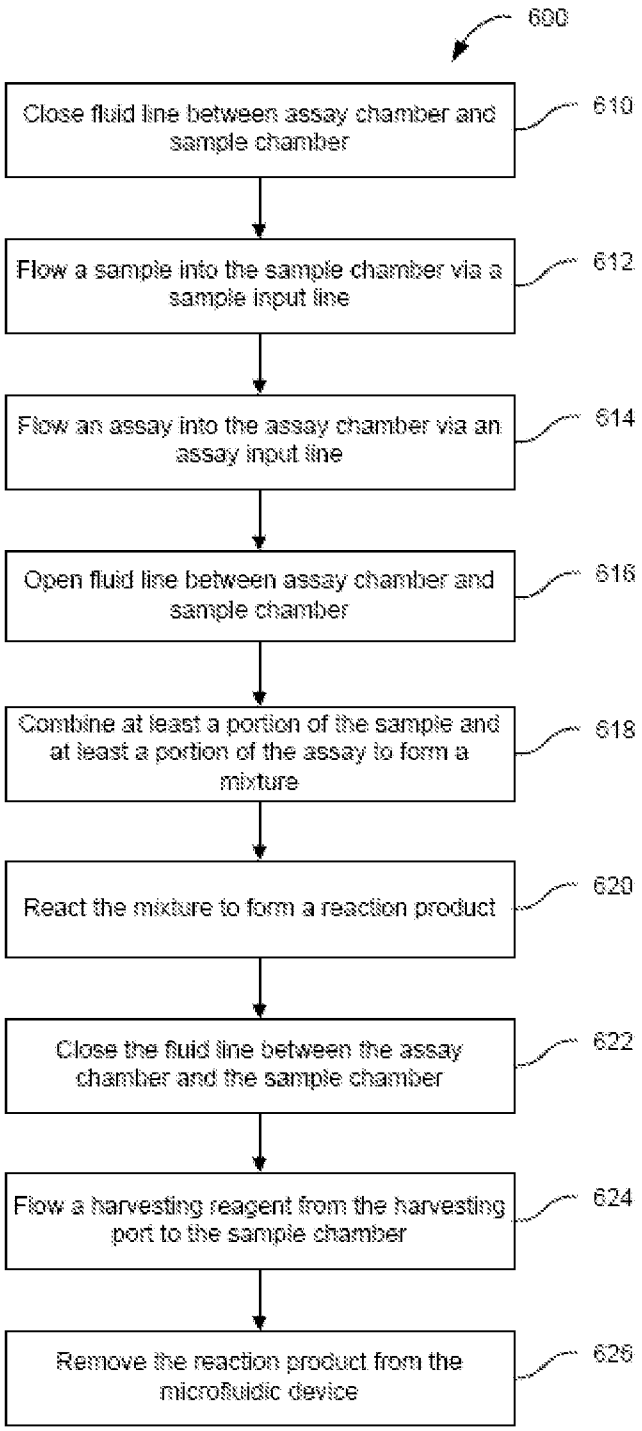

600
Close fluid line between assay chamber and sample chamber
610
Flow a sample into the sample chamber via a sample input line
612
Flow an assay into the assay chamber via an assay input line
614
Open fluid line between assay chamber and sample chamber
616
Combine at least a portion of the sample and at least a portion of the assay to form a mixture
618
React the mixture to form a reaction product
620
Close the fluid line between the assay chamber and the sample chamber
622
Flow a harvesting reagent from the harvesting port to the sample chamber
624
Remove the reaction product from the microfluidic device
626

Fig. 7

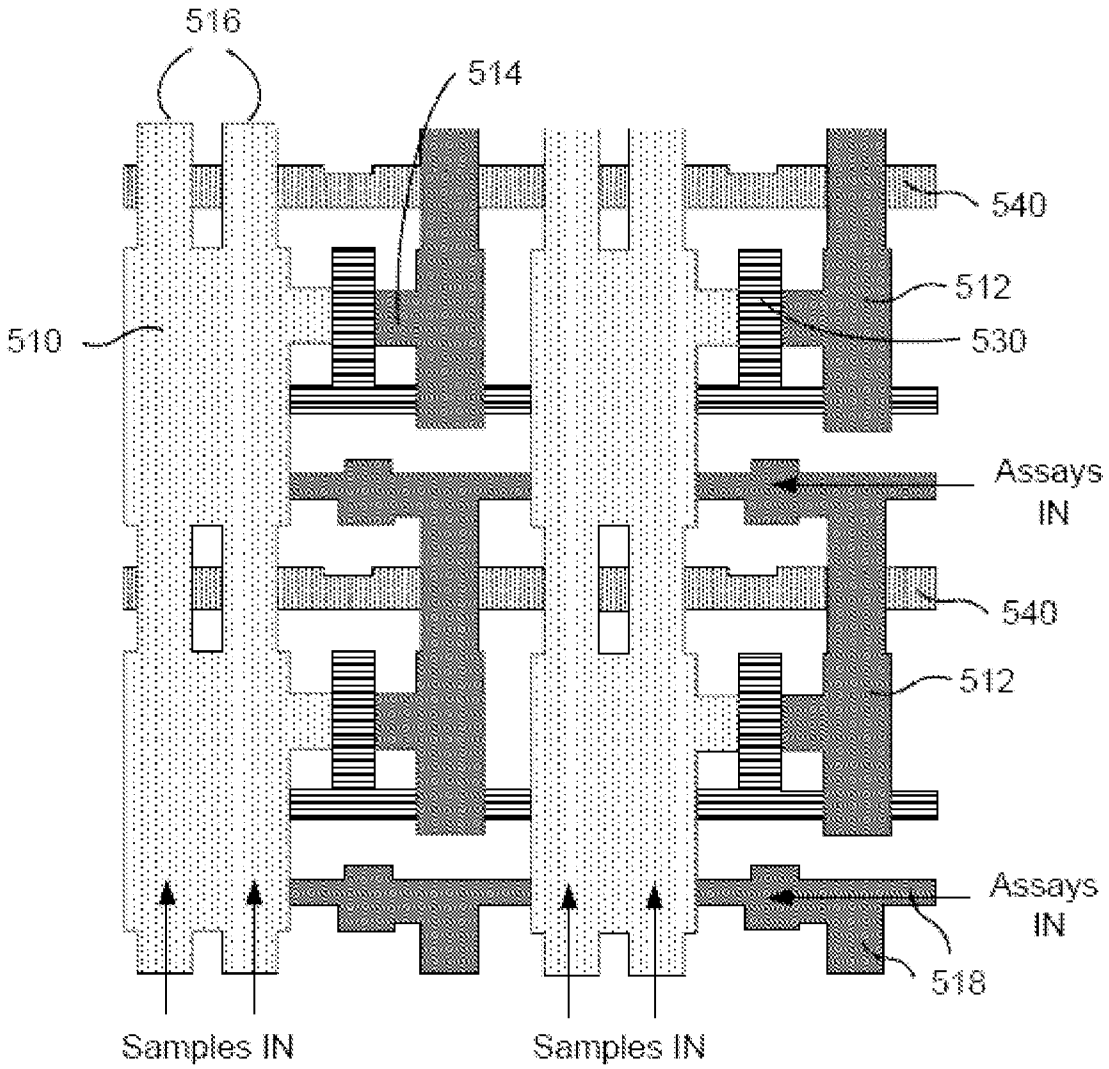

516
514
540
512
510
530
Assays IN
540
512
Assays IN
518
Samples IN
Samples IN

Fig. 8A

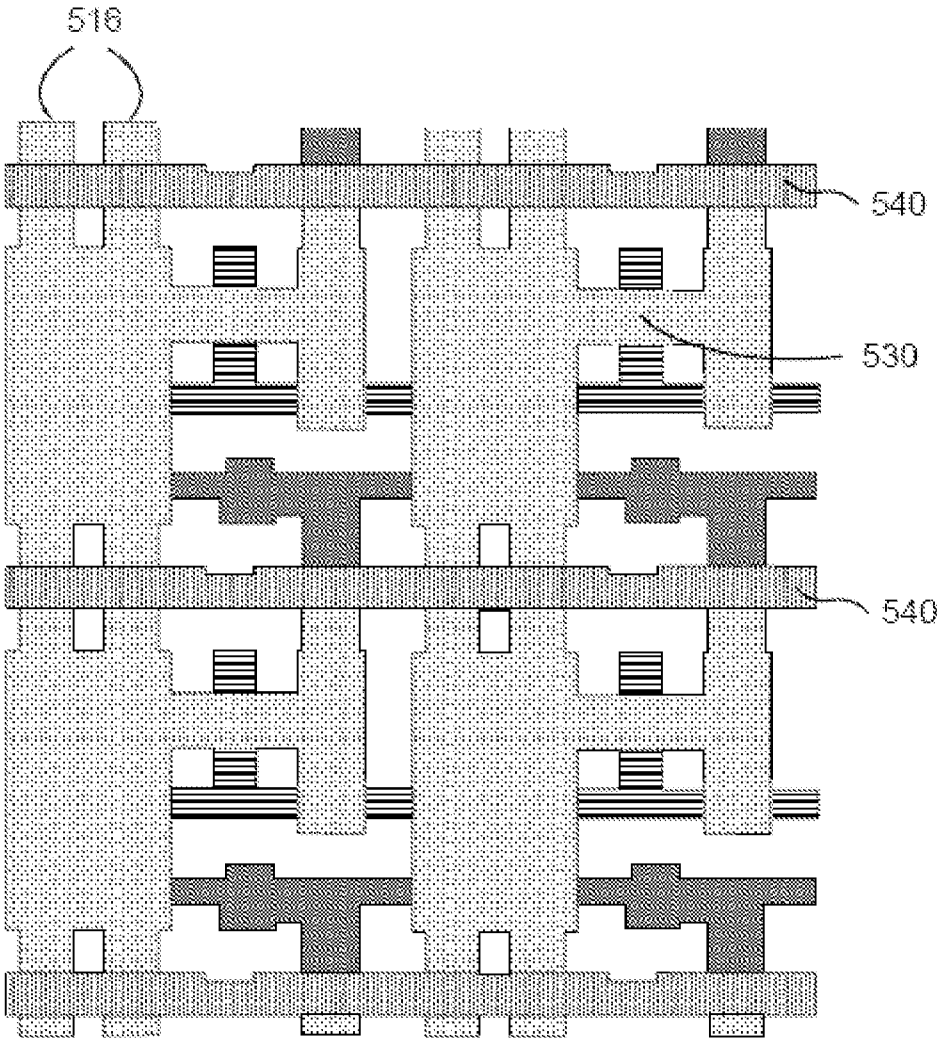


Fig. 8B

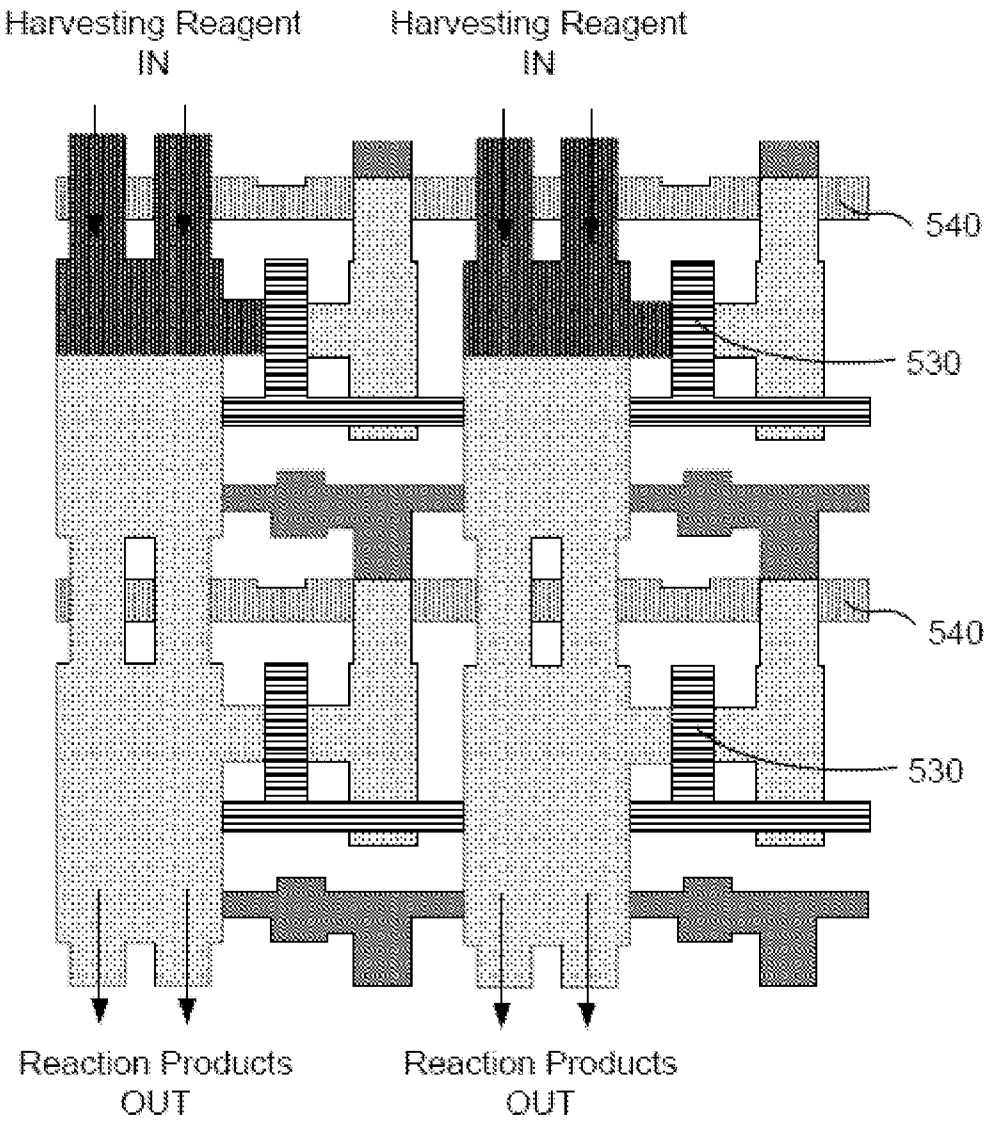

Harvesting Reagent IN
Harvesting Reagent IN
540
530
540
530
Reaction Products OUT
Reaction Products OUT

Fig. 8C

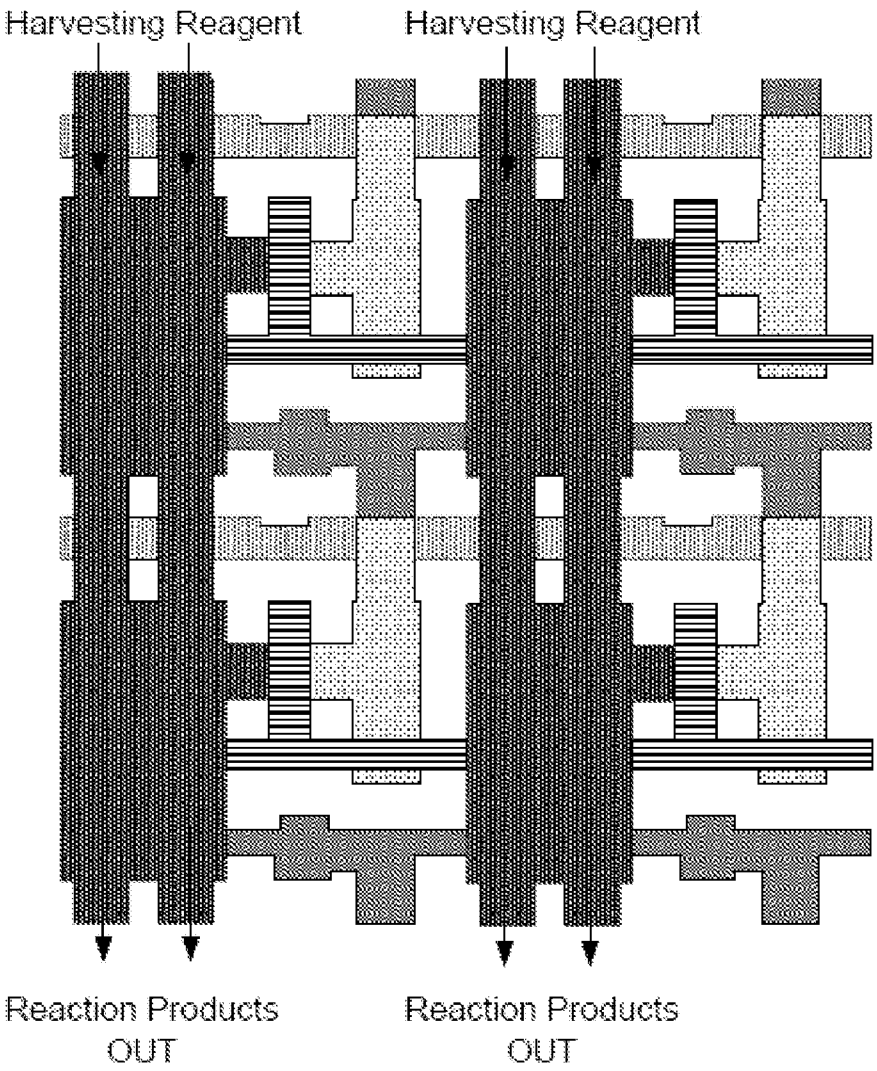

Harvesting Reagent
Harvesting Reagent
Reaction Products OUT
Reaction Products OUT

Fig. 8D

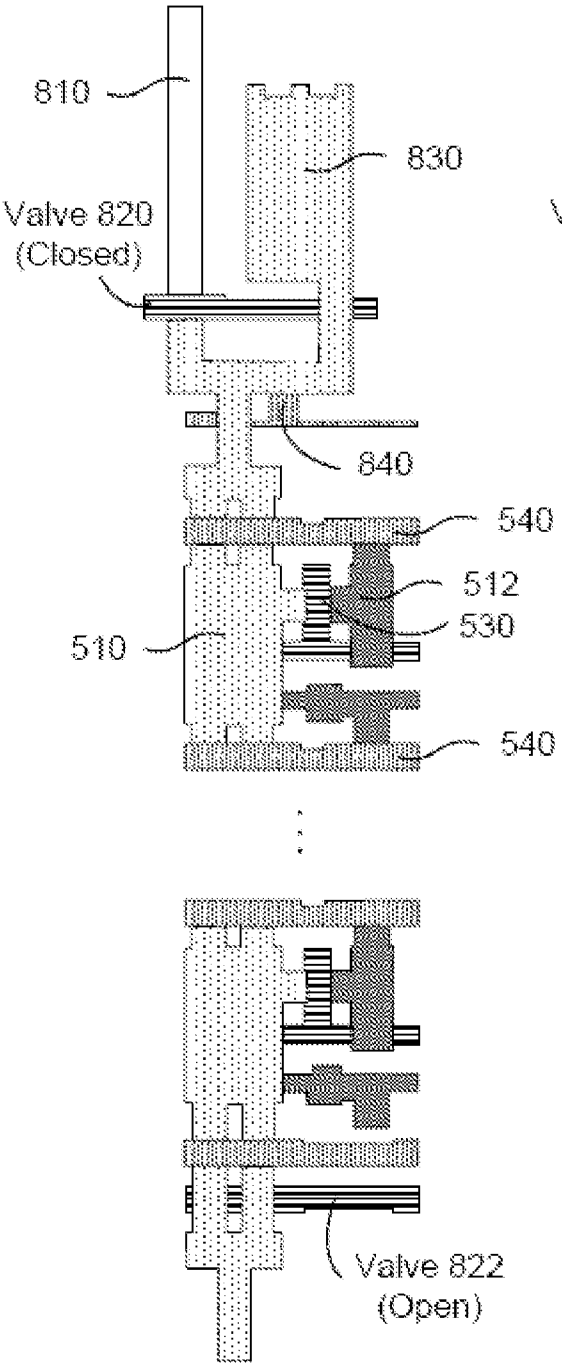

810
830
Valve 820
(Closed)
840
540
512
530
510
540
Valve 822
(Open)

Fig. 9A

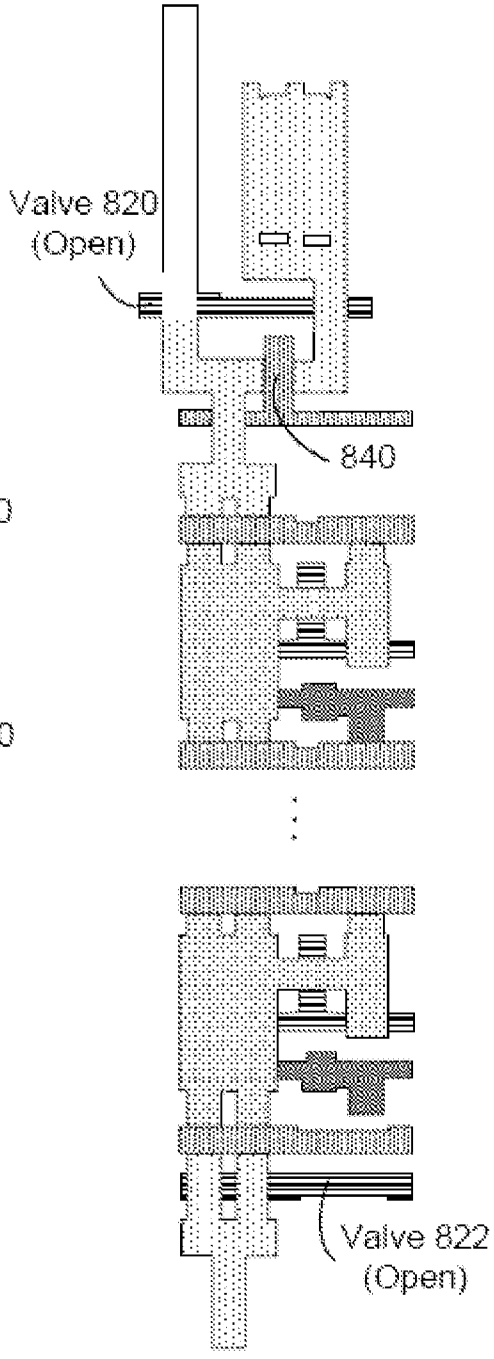

Valve 820
(Open)
840
Valve 822
(Open)

Fig. 9B

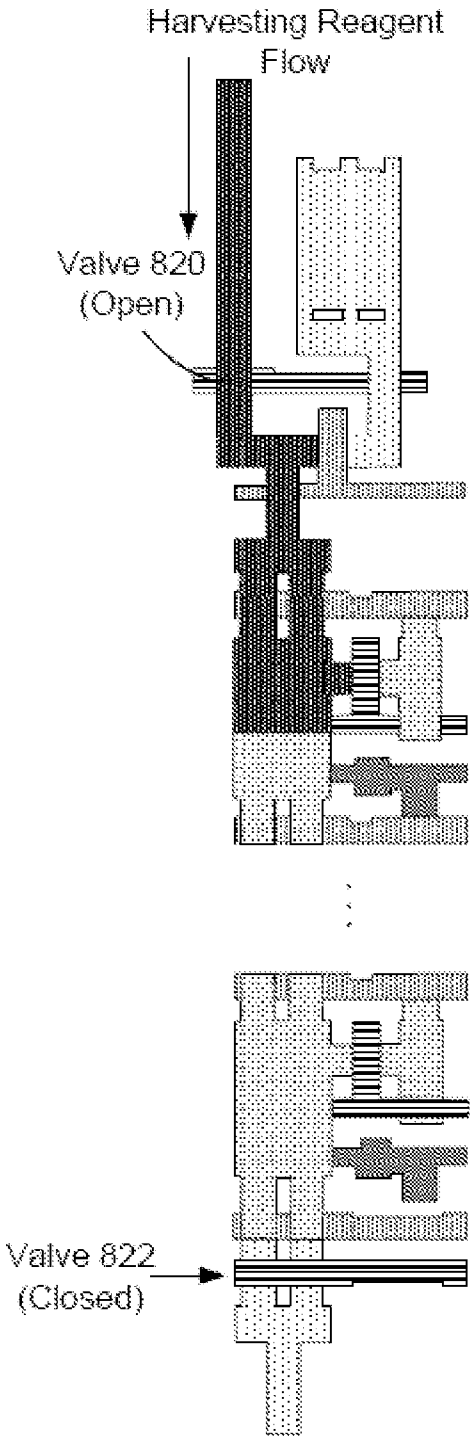

Harvesting Reagent Flow
Valve 820 (Open)
Valve 822 (Closed)

Fig. 9C

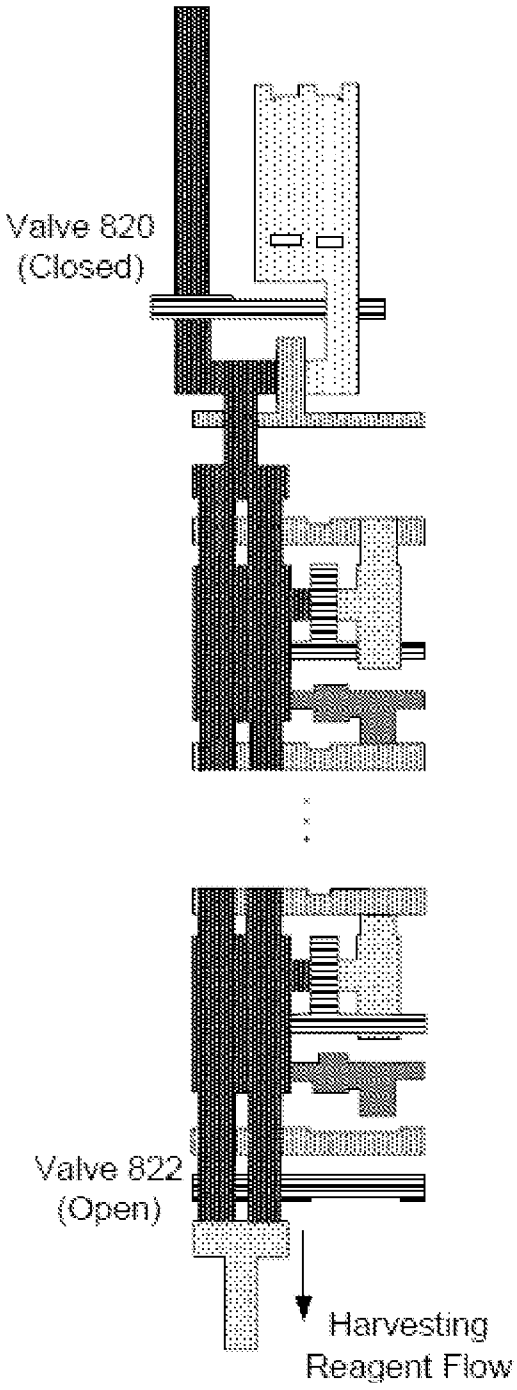

Valve 820 (Closed)
Valve 822 (Open)
Harvesting Reagent Flow

Fig. 9D

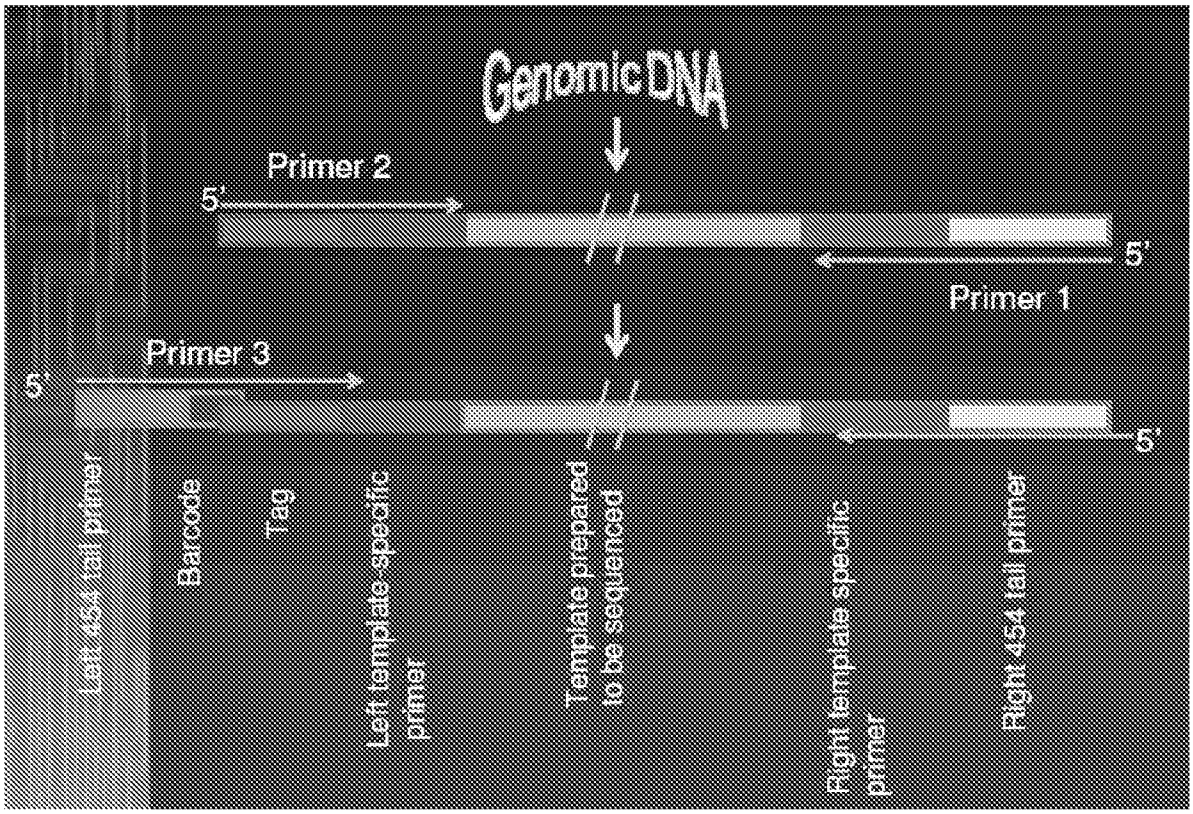

Genomic DNA
Primer 2
5'
5'
Primer 1
Primer 3
5'
5'
Left 454 tail primer
Barcode
Tag
Left template-specific primer
Template prepared to be sequenced
Right template specific primer
Right 454 tail primer

Fig. 10

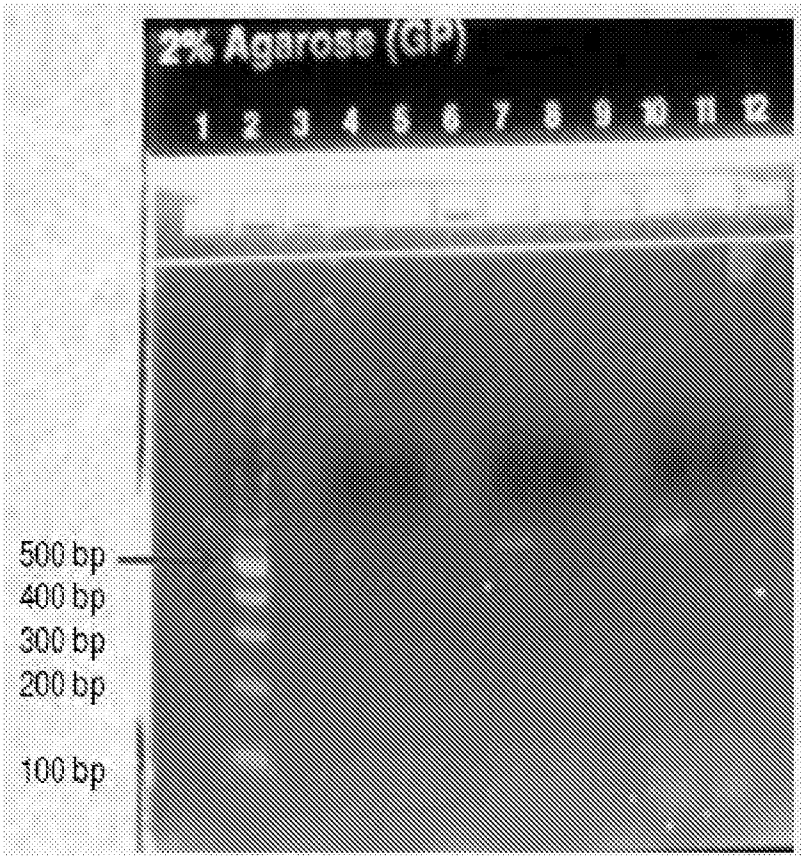

2% Agarose (dP)
1 2 3 4 5 6 7 8 9 10 11 12
500 bp
400 bp
300 bp
200 bp
100 bp

Fig. 11

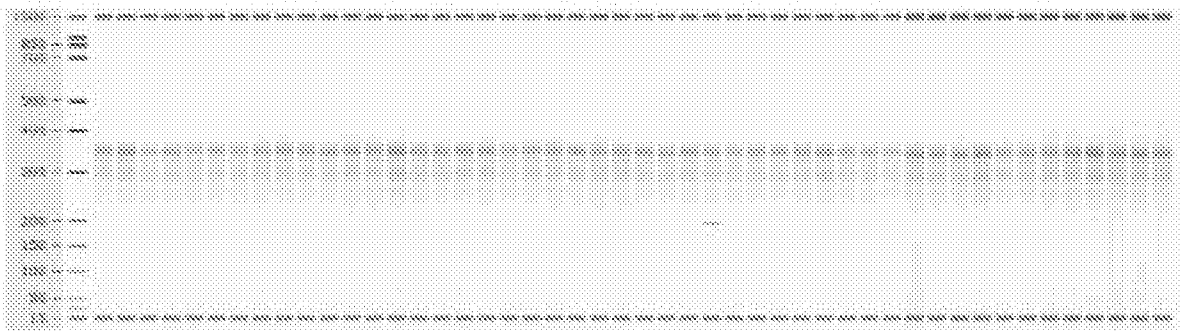
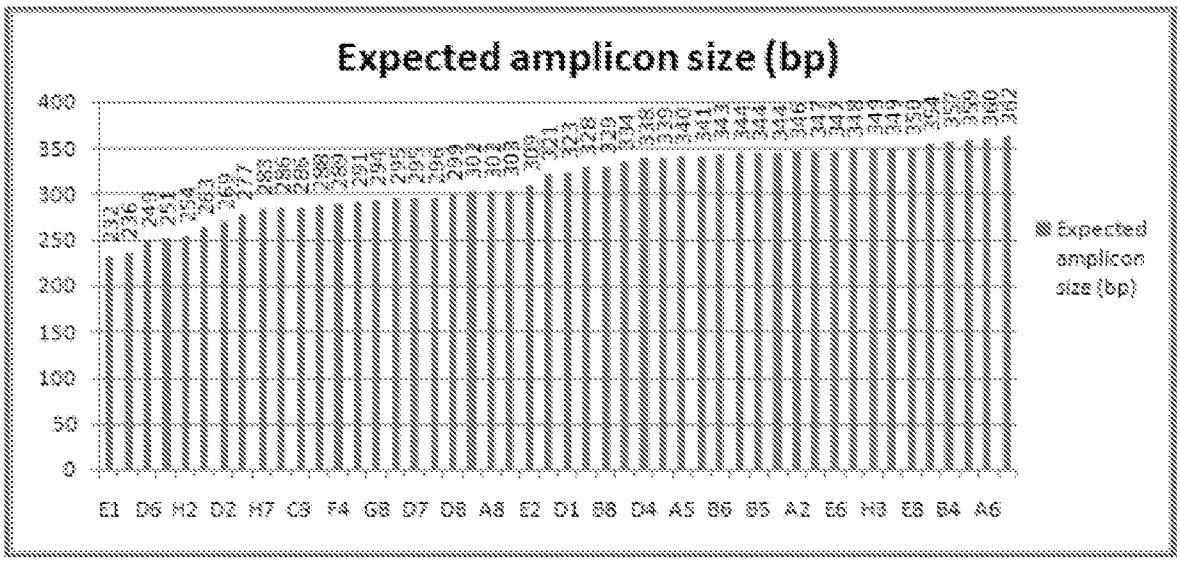


Fig. 12

A


Expected amplicon size (bp)
400
350
300
250
200
150
100
50
0
232
236
243
251
258
263
269
277
283
286
286
289
289
291
294
295
296
299
299
302
303
305
309
307
321
323
328
329
334
336
336
340
341
343
344
344
346
346
347
348
348
349
349
350
354
357
359
360
362
E1 D6 H2 D2 H7 C9 F4 G8 D7 D8 A8 E2 D1 B8 D4 A5 B6 B5 A2 E6 H3 E8 B4 A6
Expected amplicon size (bp)

B

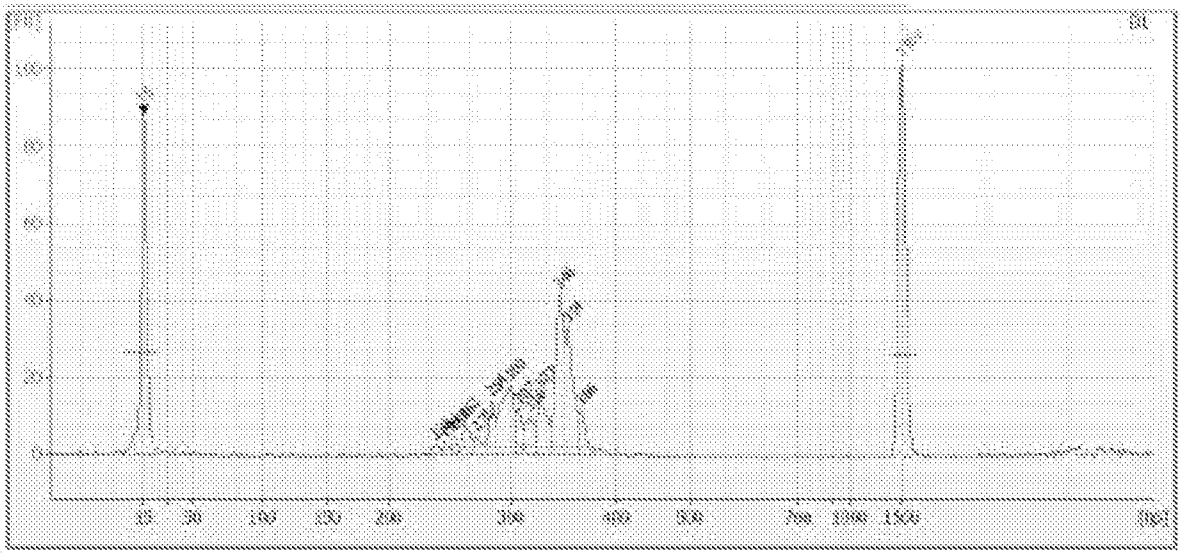
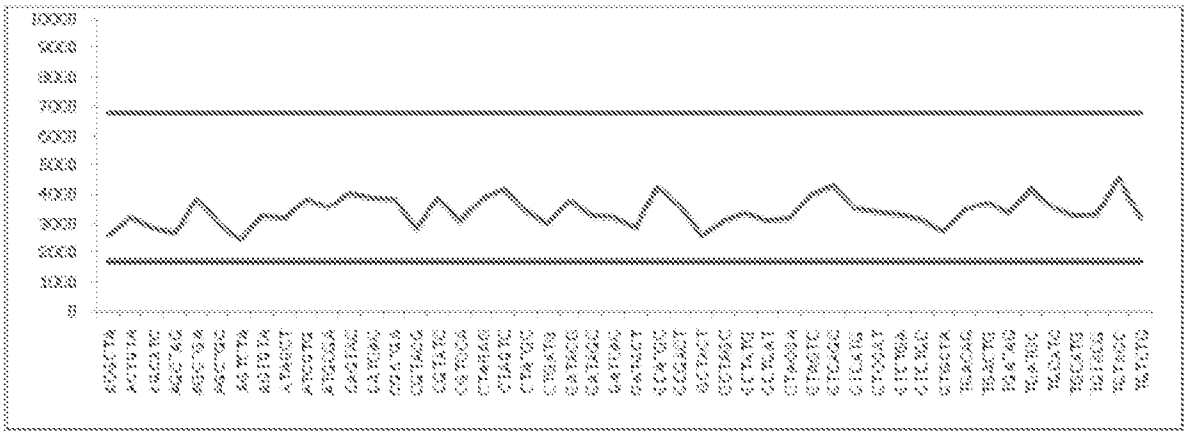


Fig. 13A-B

A



B

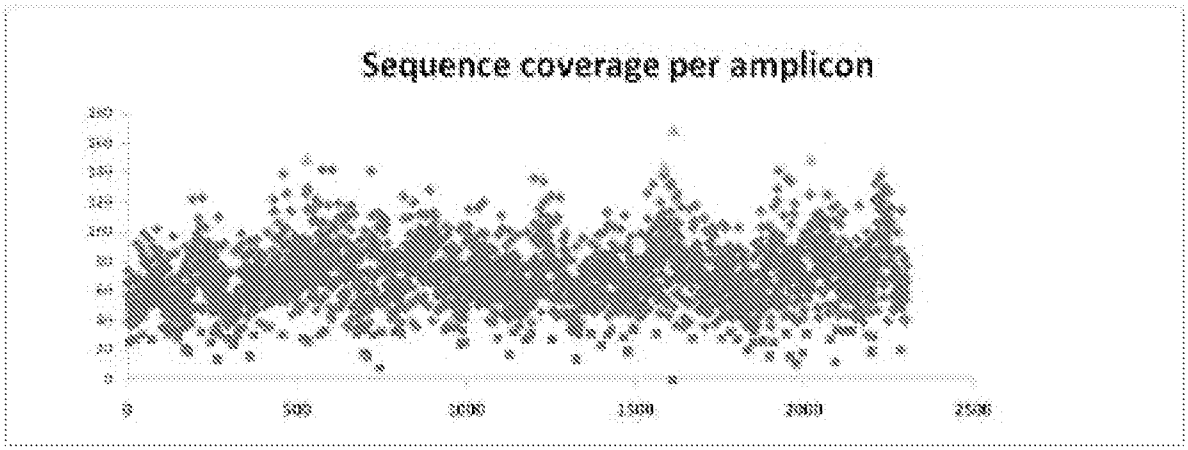


Fig. 14A-B

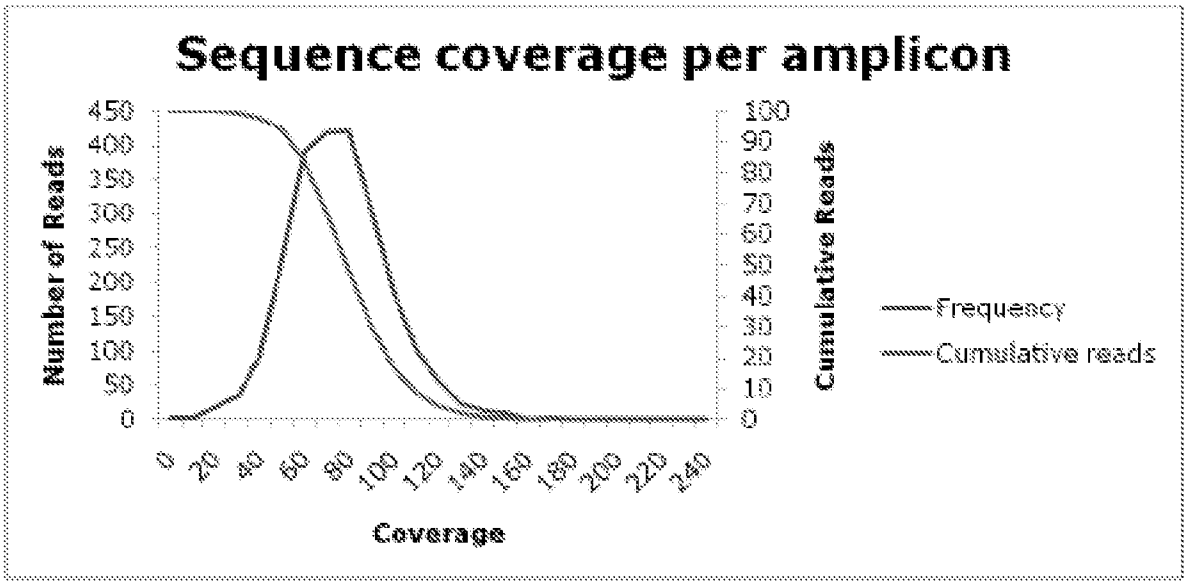
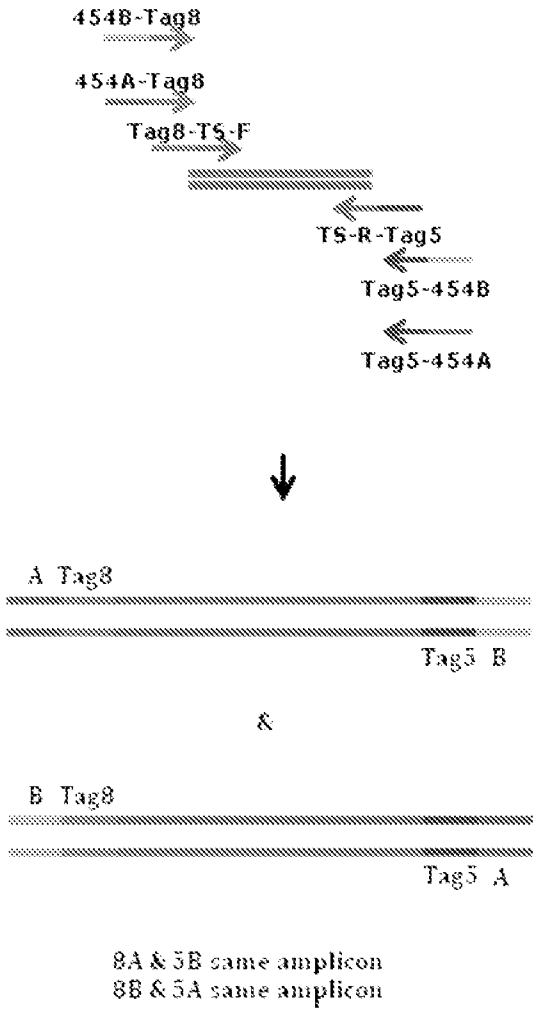

Sequence coverage per amplicon
Number of Reads
Cumulative Reads
Coverage
Frequency
Cumulative reads
450
400
350
300
250
200
150
100
50
0
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
200
220
240

Fig. 14C

A


454B-Tag8
454A-Tag8
Tag8-TS-F
TS-R-Tag5
Tag5-454B
Tag5-454A
A Tag8
Tag5 B
&
B Tag8
Tag5 A
8A & 5B same amplicon
8B & 5A same amplicon

B

Fig. 15A-B

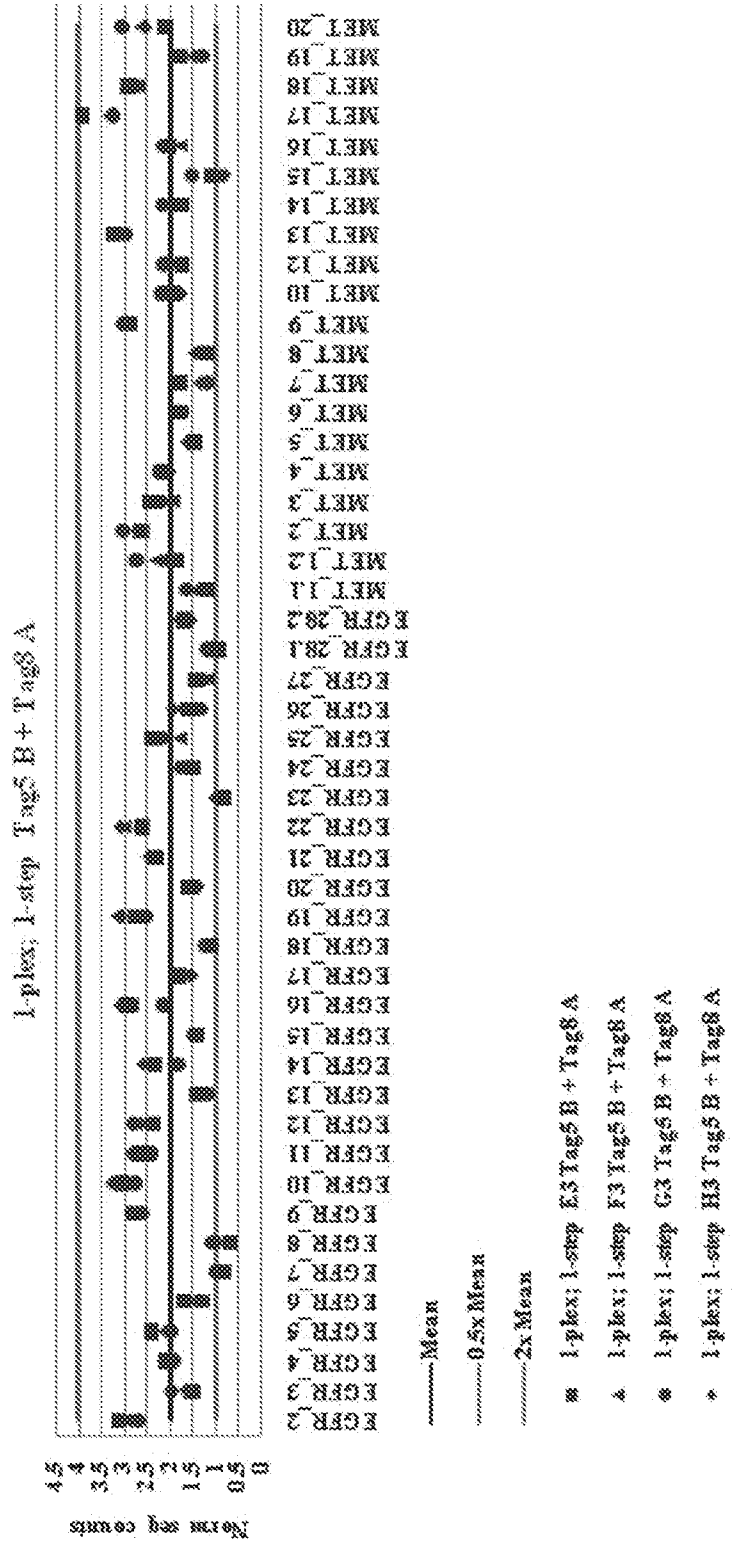

1-plex; 1-step Tag5 B + Tag8 A
Norm req counts
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
EGFR_2
EGFR_3
EGFR_4
EGFR_5
EGFR_6
EGFR_7
EGFR_8
EGFR_9
EGFR_10
EGFR_11
EGFR_12
EGFR_13
EGFR_14
EGFR_15
EGFR_16
EGFR_17
EGFR_18
EGFR_19
EGFR_20
EGFR_21
EGFR_22
EGFR_23
EGFR_24
EGFR_25
EGFR_26
EGFR_27
EGFR_28.1
EGFR_28.2
MET_1.1
MET_1.2
MET_2
MET_3
MET_4
MET_5
MET_6
MET_7
MET_8
MET_9
MET_10
MET_12
MET_13
MET_14
MET_15
MET_16
MET_17
MET_18
MET_19
MET_20
Mean
0.5x Mean
2x Mean
1-plex; 1-step E3 Tag5 B + Tag8 A
1-plex; 1-step E3 Tag5 B + Tag8 A
1-plex; 1-step G3 Tag5 B + Tag8 A
1-plex; 1-step H3 Tag5 B + Tag8 A

Fig. 16A

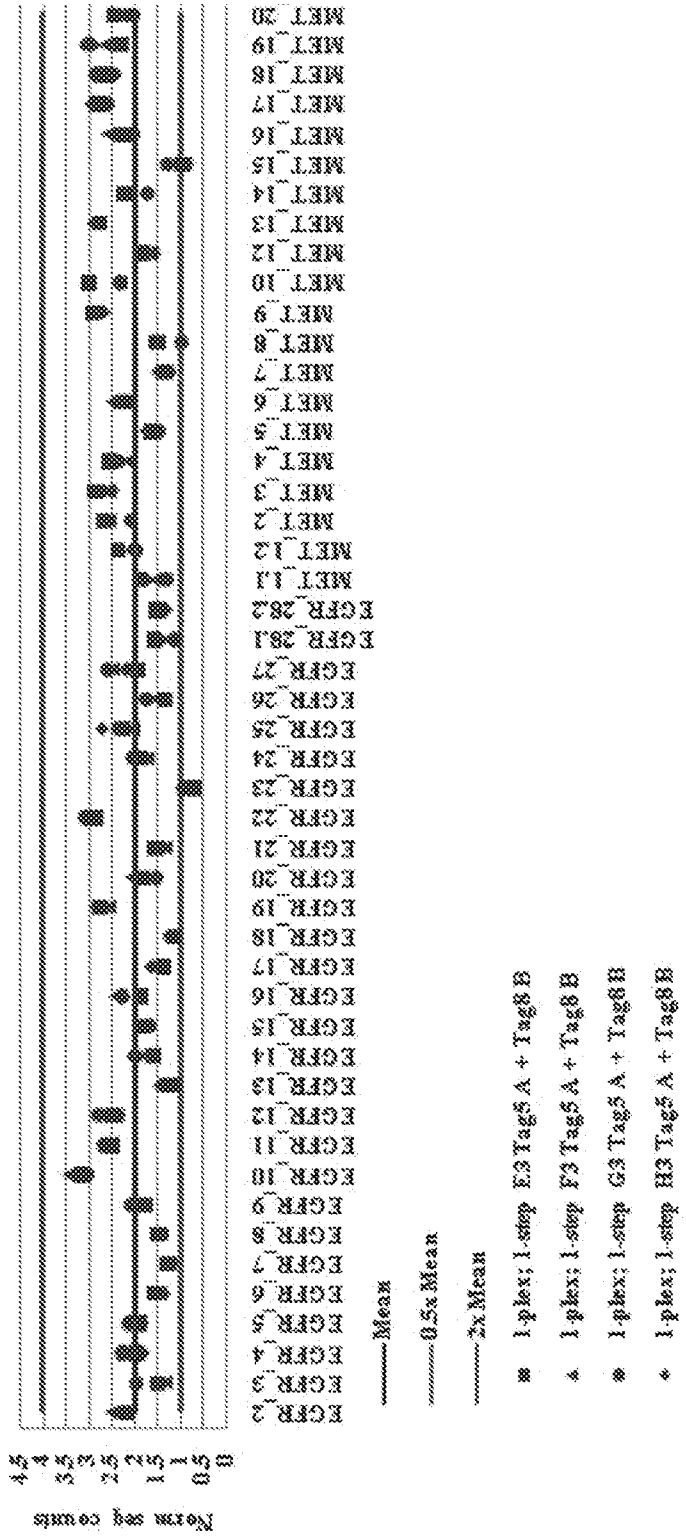

1-plex; 1-step Tag5 A + Tag8 B
Norm seq counts
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
EGFR_2
EGFR_3
EGFR_4
EGFR_5
EGFR_6
EGFR_7
EGFR_8
EGFR_9
EGFR_10
EGFR_11
EGFR_12
EGFR_13
EGFR_14
EGFR_15
EGFR_16
EGFR_17
EGFR_18
EGFR_19
EGFR_20
EGFR_21
EGFR_22
EGFR_23
EGFR_24
EGFR_25
EGFR_26
EGFR_27
EGFR_28.1
EGFR_28.2
MET_1.1
MET_1.2
MET_2
MET_3
MET_4
MET_5
MET_6
MET_7
MET_8
MET_9
MET_10
MET_12
MET_13
MET_14
MET_15
MET_16
MET_17
MET_18
MET_19
MET_20
Mean
0.5x Mean
2x Mean
1-plex; 1-step E3 Tag5 A + Tag8 B
1-plex; 1-step F3 Tag5 A + Tag8 B
1-plex; 1-step G3 Tag5 A + Tag8 B
1-plex; 1-step H3 Tag5 A + Tag8 B

Fig. 16B

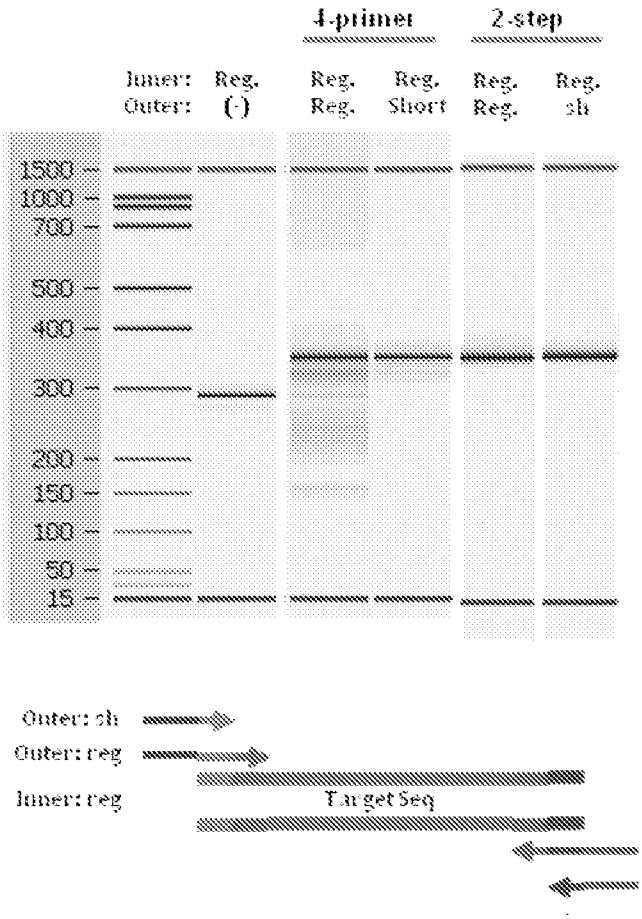

4-primer
2-step
Inner: Reg.
Outer: (-)
Reg. Reg.
Reg. Short
Reg. Reg.
Reg. sh
1500
1000
700
500
400
300
200
150
100
50
15
Outer: sh
Outer: reg
Inner: reg
Target Seq

Fig. 17

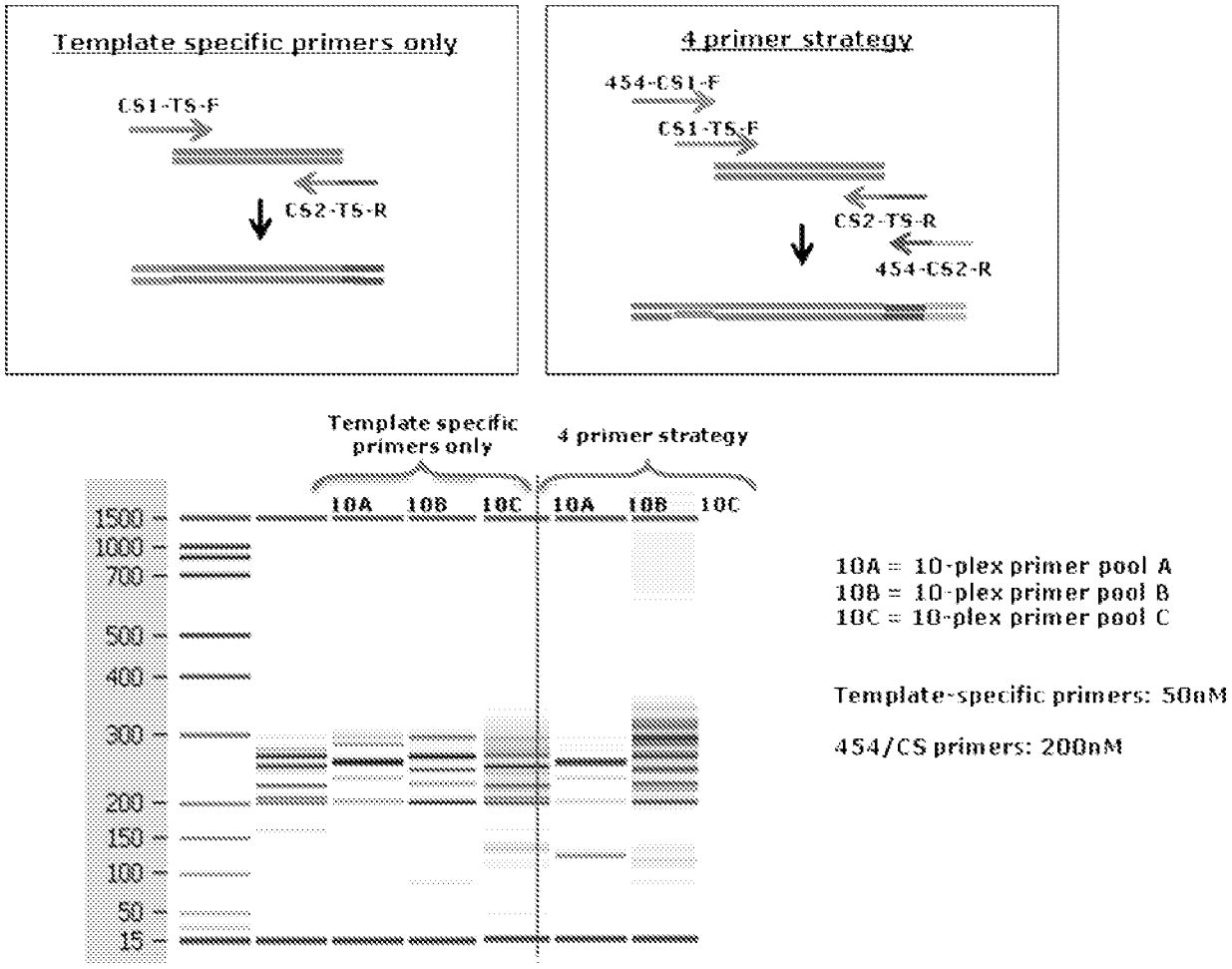

Template specific primers only
CS1-TS-F
CS2-TS-R
4 primer strategy
454-CS1-F
CS1-TS-F
CS2-TS-R
454-CS2-R
Template specific primers only
4 primer strategy
10A
10B
10C
10A
10B
10C
1500
1000
700
500
400
300
200
150
100
50
15
10A = 10-plex primer pool A
10B = 10-plex primer pool B
10C = 10-plex primer pool C
Template-specific primers: 50nM
454/CS primers: 200nM

Fig. 18

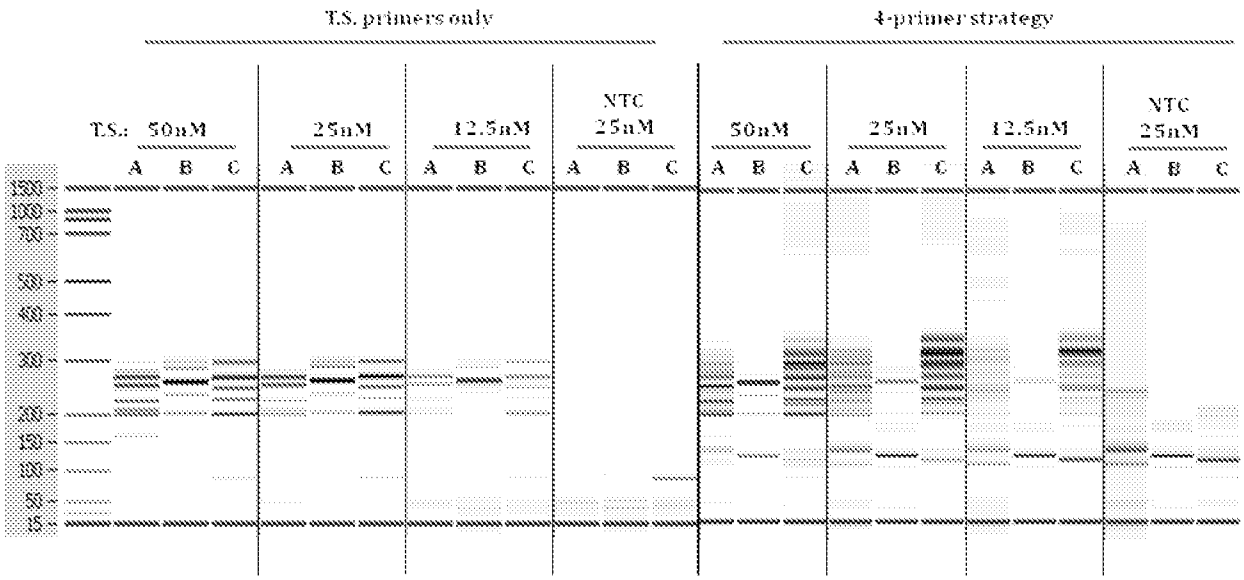

T.S. primers only
4-primer strategy
T.S.: 50nM
25nM
12.5nM
NTC 25nM
50nM
25nM
12.5nM
NTC 25nM
A B C
150
100
75
60
50
40
30
25
20
15
10
5

Fig. 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/29854

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2010.01)
USPC - 435/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC- 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/91.2

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest (PGPB,USPT,EPAB,JPAB): MAY ANDREW; CHEN PEILIN; WANG JUN; KAPER FIONA; ANDERSON MEGAN; BARCODE IDENTIFICATION, "ID", SEQUENCE, NUCLEOTIDE, PCR

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0053719 A1 (Lo et al.) 26 February 2009 (26.02.2009) para [0008], [0012], [0024], [0019]	11-12, 16-18, 24, 26-33, 38-56
Y	US 4,683,202 A (Mullis) 28 July 1987 (28.07.1987) col 2, ln 26-40, 51-59; col 4, ln 51-58; fig 4-1; col 14, ln 8-13	1-18, 24, 26-56
Y	US 2007/0020640 A1 (McCloskey et al.) 25 January 2007 (25.01.2007) para [0008], [0002], [0084]	1-16, 34-37
Y	US 2008/0108063 A1 (Lucero et al.) 8 May 2008 (08.05.2008) para [0009]	28, 46
Y	US 5,066,584 A (Gyllensten et al.) 19 November 1991 (19.11.1991) col 3, ln 68 to col 4, ln 2	1-16, 34-37

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
9 August 2010 (09.08.2010)	30 AUG 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/29854

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 19-23 and 25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
Group I: Claims 1-18, 24, 26-56 are directed to a method of amplifying a plurality of target nucleic acids in a plurality of samples.
Group II: Claims 57-81 are directed to a microfluidic device a method of using said device.
Group III: Claims 82-95 are directed to a method of preparing reaction products.

The inventions listed as Groups I - III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is a method of amplifying a plurality of target nucleic acids in a plurality of samples. The special technical feature of Group I is not present in Group II or Group III.

The special technical feature of Group II is a microfluidic device. The special technical feature of Group II is not present in Group I or Group III.

********** See Supplemental Box to continue **********

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-18, 24, and 26-56

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/29854

********** See Supplemental Box **********

In continuation of Box III: Observations where unity of invention is lacking:

The special technical feature of Group III is a method of preparing reaction products. The special technical feature of Group III is not present in Group I or Group II.

Accordingly, the inventions listed as Groups I - III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.