



(51) International Patent Classification:

C40B 20/00 (2006.01) *C40B 30/04* (2006.01)
C40B 50/06 (2006.01) *C12P 19/34* (2006.01)
C40B 40/06 (2006.01) *C12Q 1/68* (2006.01)

(21) International Application Number:

PCT/US2012/038894

(22) International Filing Date:

21 May 2012 (21.05.2012)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/519,348 20 May 2011 (20.05.2011) US

(71) Applicant (for all designated States except US): **FLUIDIGM CORPORATION** [US/US]; 7000 Shoreline Court, Suite 100, South San Francisco, California 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ANDERSON, Megan** [US/US]; 2400 M Street NW, Apt 439, Washington, District of Columbia 20037 (US). **CHEN, Peilin** [US/US]; 1023 Landmark Circle, Richmond, California 94806 (US). **FOWLER, Brian** [US/US]; 3362 Los Prados Street, San Mateo, California 94403 (US). **JONES, Robert C.** [US/US]; 274 Merritt Road, Los Altos, California

94022 (US). **KAPER, Fiona** [NL/US]; 1808 Parkwood Drive, San Mateo, California 94403 (US). **LEBOFSKY, Ronald** [CA/CA]; 79 Elsfield Road, Etobicoke, Ontario M8Y 3R7 (CA). **MAY, Andrew** [GB/US]; 564 29th Avenue, San Francisco, California 94121 (US).

(74) Agents: **HALIDAY, Emily, M.** et al.; Weaver Austin Villeneuve & Sampson LLP, P.O. Box 70250, Oakland, California 94612-0250 (US).

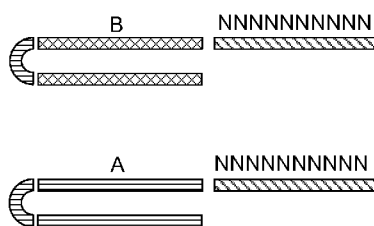
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG,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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

[Continued on next page]

(54) Title: NUCLEIC ACID ENCODING REACTIONS

Degenerate-tailed hairpin sequencing linkers



A- Sequencer specific tag1

B- Sequencer specific tag2

Rest. Site: Optional restriction site/specific enzyme cutting site
(dUTP, phosphothiorate possible)

NNNNNNNNNN- Degenerate primer region-10mer, 20mer, 3' end protected

FIG. 1A

(57) Abstract: Described herein are methods useful for incorporating one or more adaptors and/or nucleotide tag(s) and/or barcode nucleotide sequence(s) one, or typically more, target nucleotide sequences. In particular embodiments, nucleic acid fragments having adaptors, e.g., suitable for use in high-throughput DNA sequencing are generated. In other embodiments, information about a reaction mixture is encoded into a reaction product. Also described herein are methods and kits useful for amplifying one or more target nucleic acids in preparation for applications such as bidirectional nucleic acid sequencing. In particular embodiments, methods of the invention entail additionally carrying out bidirectional DNA sequencing. Also described herein are methods for encoding and detecting and/or quantifying alleles by primer extension.



WO 2012/162267 A2



TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

NUCLEIC ACID ENCODING REACTIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application no. 61/519,348, filed May 20, 2011, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the incorporation of nucleic acid sequences into target nucleic acids, e.g., the addition of one or more adaptors and/or nucleotide tag(s) and/or barcode nucleotide sequence(s) to target nucleotide sequences. The methods described herein are useful, e.g., in the areas of high-throughput assays for detection and/or sequencing of particular target nucleic acids.

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. For many applications, it is desirable to detect and/or analyze many target nucleic acids in multiple samples, e.g., multiple individual cells within a population, simultaneously.

SUMMARY OF THE INVENTION

[0004] In certain embodiments, the invention provides a method of adding adaptor molecules to each end of a plurality of target nucleic acids that include sticky ends. The method entails annealing adaptor molecules to the sticky ends of double-stranded target nucleic acid molecules to produce annealed adaptor-target nucleic acid molecules, wherein the adaptor molecules are:

(i) hairpin structures each including:

an adaptor nucleotide sequence, which is linked to
a nucleotide linker, which is linked to

a nucleotide sequence that is capable of annealing to the adaptor nucleotide sequence and is linked to

a degenerate tail sequence; or

(ii) double-stranded or single-stranded molecules each including:

5 a first adaptor nucleotide sequence, which is linked to

a nucleotide linker, which is linked to

a second adaptor nucleotide sequence; and

a degenerate tail sequence on each strand, wherein double-stranded molecules each include two degenerate tail sequences as sticky end(s). After annealing, the

10 method entails filling any gaps in the resulting annealed adaptor-target nucleic acid

molecules, and ligating any adjacent nucleotide sequences in the annealed adaptor-target nucleic acid molecules to produce adaptor-modified target nucleic acid molecules. In

related embodiments, the invention provides a plurality of adaptor molecules, wherein the adaptor molecules are the hairpin structures of (i) above or the double-stranded or single-

15 stranded molecules of (ii) above. Also contemplated is a kit, which, in various

embodiments, can include the plurality of adaptor molecules in combination with a DNase enzyme, an exonuclease, an endonuclease, a polymerase, a ligase, or any combination thereof.

[0005] In other embodiments, the invention provides a method for tagging a

20 plurality of target nucleic acids with nucleotide sequences. The method entails preparing a first reaction mixture for each target nucleic acid, the first reaction mixture including a pair of inner primers and a pair of outer primers, wherein:

(i) the inner primers include:

25 a forward, inner primer including a first nucleotide tag, a first barcode nucleotide sequence, and a target-specific portion; and

a reverse, inner primer including a target-specific portion, a first barcode nucleotide sequence, and a second nucleotide tag; and

(ii) the outer primers include:

a forward, outer primer including a second barcode nucleotide sequence and a first nucleotide tag-specific portion; and

a reverse, outer primer including a second nucleotide tag-specific portion and a second barcode nucleotide sequence, wherein the outer primers are in excess of the inner primers. Each first reaction mixture is subjected to a reaction to produce a plurality of tagged target nucleotide sequences, each including 5'-second barcode nucleotide sequence-first nucleotide tag sequence-first barcode nucleotide sequence-target nucleotide sequence-first barcode nucleotide sequence-second nucleotide tag sequence-second barcode nucleotide sequence-3'. In related embodiments, the invention provides a kit that includes a polymerase in combination with the inner primers of (i) above and the outer primers of (ii) above, wherein the outer primers are in excess of the inner primers.

[0006] In certain embodiments, the invention provides a method for tagging a plurality of target nucleic acids with nucleotide sequences. The method entails preparing a first reaction mixture for each target nucleic acid, the first reaction mixture including a pair of inner primers, a pair of stuffer primers, and a pair of outer primers, wherein:

(i) the inner primers include:

a forward, inner primer including a first nucleotide tag and a target-specific portion; and

a reverse, inner primer including a target-specific portion and a second nucleotide tag;

(ii) the stuffer primers include:

a forward, stuffer primer including a third nucleotide tag, a first barcode nucleotide sequence, and a first nucleotide tag-specific portion; and

a reverse, stuffer primer including a second nucleotide tag-specific portion, a first barcode nucleotide sequence, a fourth nucleotide tag; and

(iii) the outer primers include:

a forward, outer primer including a second barcode nucleotide sequence and a third nucleotide tag-specific portion; and

a reverse, outer primer including a fourth nucleotide tag-specific portion and a second barcode nucleotide sequence, wherein the outer primers are in excess of the stuffer primers, which are in excess of the inner primers. Each first reaction mixture is subjected to a reaction to produce a plurality of tagged target nucleotide sequences, each including 5'-second barcode nucleotide sequence-third nucleotide tag sequence-first barcode nucleotide sequence-first nucleotide tag sequence-target nucleotide sequence-second nucleotide tag sequence-first barcode nucleotide sequence-fourth nucleotide tag sequence-second barcode nucleotide sequence-3'. In related embodiments, the invention provides a kit that includes a polymerase in combination with the inner primers of (i) above, the stuffer primers of (ii) above, and the outer primers of (iii) above, wherein the outer primers are in excess of the stuffer primers, which are in excess of the inner primers.

[0007] In particular embodiments, the invention provides a method for combinatorial tagging of a plurality of target nucleotide sequences. The method employs a plurality of tagged target nucleotide sequences derived from target nucleic acids, each tagged target nucleotide sequence including an endonuclease site and a first barcode nucleotide sequence, wherein tagged target nucleotide sequences in the plurality include the same endonuclease site, but N different first barcode nucleotide sequences, wherein N is an integer greater than 1. The method entails cutting the plurality of tagged target nucleotide sequences with an endonuclease specific for the endonuclease site to produce a plurality of sticky-ended, tagged target nucleotide sequences. The method further entails ligating a plurality of adaptors including a second barcode nucleotide sequence and complementary sticky ends to the plurality of sticky-ended, tagged target nucleotide sequences in a first reaction mixture, wherein the plurality of adaptors include M different second barcode nucleotide sequences, wherein M is an integer greater than 1. This ligation produces a plurality of combinatorially tagged target nucleotide sequences, each including first and second barcode nucleotide sequences, wherein the plurality includes $N \times M$ different first and second barcode combinations. In related embodiments, the invention provides a plurality of adaptors including:

a plurality of first adaptors, each including the same endonuclease site, N different barcode nucleotide sequences, wherein N is an integer greater than 1, a first primer binding site and a sticky end;

a second adaptor including a second primer binding site and a sticky end; and

a plurality of third adaptors including a second barcode nucleotide sequence and sticky ends complementary to those produced upon cutting the first adaptors at the endonuclease site, wherein the plurality of third adaptors include M different second
5 barcode nucleotide sequences, wherein M is an integer greater than 1. Also contemplated is a kit including the plurality of first adaptors, the second adaptor, and the plurality of third adaptors, in combination with an endonuclease specific for the endonuclease site in the first adaptors and/or a ligase.

[0008] In other embodiments, the invention provides a method for combinatorial
10 tagging of a plurality of target nucleotide sequences, wherein the method entails annealing a plurality of barcode primers to a plurality of tagged target nucleotide sequences derived from target nucleic acids. Each tagged target nucleotide sequence includes a nucleotide tag at one end and a first barcode nucleotide sequence, wherein tagged target nucleotide
15 nucleotide sequences in the plurality include the same nucleotide tag, but N different first barcode nucleotide sequences, wherein N is an integer greater than one. Each barcode primer includes:

a first tag-specific portion linked to;

a second barcode nucleotide sequence linked to;

a second tag-specific portion, wherein the barcode primers in the plurality
20 each include the same first and second tag-specific portions, but M different second barcode nucleotide sequences, wherein M is an integer greater than one. The method further entails amplifying the tagged target nucleotide sequences in a first reaction mixture to produce a plurality of combinatorially tagged target nucleotide sequences, each including first and
25 second barcode nucleotide sequences, wherein the plurality includes $N \times M$ different first and second barcode combinations. In related embodiments, the invention provides a kit including one or more nucleotide tags(s), which can be used for producing tagged target nucleotide sequences, together with the plurality of barcode primers above.

[0009] In certain embodiments, the invention provides an assay method for detecting a plurality of target nucleic acids that entails preparing M first reaction mixtures

that will be pooled prior to assay, wherein M is an integer greater than 1. Each first reaction mixture includes:

sample nucleic acid(s);

a first, forward primer including a target-specific portion;

5 a first, reverse primer including a target-specific portion, wherein the first, forward primer or the first, reverse primer additionally includes a barcode nucleotide sequence, and wherein each barcode nucleotide sequence in each of the M reaction mixtures is different. Each first reaction mixture is subjected to a first reaction to produce a plurality of barcoded target nucleotide sequences, each including a target nucleotide sequence linked
10 to a barcode nucleotide sequence. The method further entails, for each of the M first reaction mixtures, pooling the barcoded target nucleotide sequences to form an assay pool. The assay pool, or one or more aliquots thereof, is subjected to a second reaction using unique pairs of second primers, wherein each second primer pair includes:

15 a second, forward or a reverse primer that anneals to a target nucleotide sequence; and

 a second, reverse or a forward primer, respectively, that anneals to a barcode nucleotide sequence. The method then entails determining, for each unique, second primer pair, whether a reaction product is present in the assay pool, or aliquot thereof, whereby the presence of a reaction product indicates the presence of a particular target nucleic acid in a
20 particular first reaction mixture.

[0010] A variation of this assay method for detecting a plurality of target nucleic acids entails, in particular embodiments, preparing M first reaction mixtures that will be pooled prior to assay, wherein M is an integer greater than 1, and each first reaction mixture includes:

25 sample nucleic acid(s)

a first, forward primer including a target-specific portion;

a first, reverse primer including a target-specific portion, wherein the first, forward primer or the first, reverse primer additionally includes a nucleotide tag; and

at least one barcode primer including a barcode nucleotide sequence and a nucleotide tag-specific portion, wherein the barcode primer is in excess of the first, forward and/or first, reverse primer(s), and wherein each barcode nucleotide sequence in each of the M reaction mixtures is different. Each first reaction mixture is subjected to a first reaction to produce a plurality of barcoded target nucleotide sequences, each including a target nucleotide sequence linked to a nucleotide tag, which is linked to a barcode nucleotide sequence. The method further entails, for each of the M first reaction mixtures, pooling the barcoded target nucleotide sequences to form an assay pool. The assay pool, or one or more aliquots thereof, is subjected to a second reaction using unique pairs of second primers, wherein each second primer pair includes:

a second, forward or a reverse primer that anneals to a target nucleotide sequence; and

a second, reverse or a forward primer, respectively, that anneals to a barcode nucleotide sequence. The method then entails determining, for each unique, second primer pair, whether a reaction product is present in the assay pool, or aliquot thereof, whereby the presence of a reaction product indicates the presence of a particular target nucleic acid in a particular first reaction mixture.

[0011] In certain embodiments, the invention provides methods and kits useful for amplifying one or more target nucleic acids in preparation for applications such as bidirectional nucleic acid sequencing. In some embodiments, methods of the invention entail additionally carrying out bidirectional DNA sequencing.

[0012] In particular bidirectional embodiments, these methods entail amplifying, tagging, and barcoding a plurality of target nucleic acids in a plurality of samples. Nucleotide tag sequences can include primer binding sites that can be used to facilitate amplification and/or DNA sequencing. Barcode nucleotide sequences can encode information about amplification products, such as the identity of the sample from which the amplification product was derived.

[0013] In certain bidirectional embodiments, a method for amplifying a target nucleic acid entails amplifying a target nucleic acid using:

a set of inner primers, wherein the set includes:

an inner, forward primer including a target-specific portion and a first primer binding site;

an inner, reverse primer including a target-specific portion and a second primer binding site, wherein the first and second primer binding sites are different;

5 a first set of outer primers, wherein the set includes:

a first outer, forward primer including a portion specific for the first primer binding site; and

a first outer, reverse primer including a barcode nucleotide sequence and a portion specific for the second primer binding site;

10 a second set of outer primers, wherein the set includes:

a second outer, forward primer including a barcode nucleotide sequence and a portion specific for the first primer binding site; and

a second outer, reverse primer including a portion specific for the second primer binding site. This amplification produces two target amplicons, wherein:

15 a first target amplicon includes 5'-first primer binding site-target nucleotide sequence-second primer binding site-barcode nucleotide sequence-3'; and

a second target amplicon includes 5'-barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-3'. In variations of these embodiments, the barcode nucleotide sequence in each target amplicon is the same, and each target amplicon includes only one barcode nucleotide sequence.

[0014] In some bidirectional embodiments, the first and second primer binding sites are binding sites for DNA sequencing primers. The outer primers can, optionally, each additionally include an additional nucleotide sequence, wherein:

25 the first outer, forward primer includes a first additional nucleotide sequence, and the first outer, reverse primer includes a second additional nucleotide sequence; and

the second outer, forward primer includes the second additional nucleotide sequence, and the second outer, reverse primer includes the first additional nucleotide sequence; and the first and second additional nucleotide sequences are different. In such embodiments, the amplification produces two target amplicons, wherein:

a first target amplicon includes: 5'-first additional nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-barcode nucleotide sequence-second additional nucleotide sequence-3'; and

a second target amplicon includes: 5'-second additional nucleotide sequence-barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-first additional nucleotide sequence 3'. In particular embodiments, the first and/or second additional nucleotide sequence includes a primer binding site. In an illustrative embodiment, the first set of outer primers includes PE1-CS1 and PE2-BC-CS2, and the second set of outer primers includes PE1-CS2 and PE2-BC-CS1 (Table 1, Example 9).

[0015] In certain bidirectional embodiments, the amplification is carried out in a single amplification reaction. In other embodiments, the amplification includes employing the inner primers in a first amplification reaction and employing the outer primers in a second amplification reaction, wherein the second amplification reaction is separate from the first. In a variation of this, latter embodiment, the second amplification reaction includes two separate amplification reactions, wherein one employs the first set of outer primers and the other employs the second set of outer primers. The target amplicons produced in the two separate second amplification reactions can, optionally, be pooled.

[0016] In any of the above-described bidirectional embodiments, the method can include amplifying a plurality of target nucleic acids. The plurality of target nucleic acids can be, for example, genomic DNA, cDNA, fragmented DNA, DNA reverse-transcribed from RNA, a DNA library, or nucleic acids is extracted or amplified from a cell, a bodily fluid or a tissue sample. In specific embodiments, the plurality of target nucleic acids is amplified from a formalin-fixed, paraffin-embedded tissue sample.

[0017] Any of the above-described bidirectional methods can additionally include sequencing the target amplicons. For example, when the target amplicons produced as described above include additional nucleotide sequences, the method can include an additional amplification using primers that bind to the first and second additional nucleotide sequences to produce templates for DNA sequencing. In specific embodiments, one or both of the primers that bind to the first and second additional nucleotide sequences are immobilized on a substrate. In particular embodiments, the amplification to produce DNA

sequencing templates can be carried out by isothermal nucleic acid amplification. In certain embodiments, the method includes performing DNA sequencing using the templates and primers that bind to the first and second primer binding sites and prime sequencing of the target nucleotide sequence(s); these primers are preferably present in substantially equal amounts. In some embodiments, the method includes performing DNA sequencing using the templates and primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequences(s); these primers are preferably present in substantially equal amounts. In specific embodiments, the method includes performing DNA sequencing using the templates and primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequences(s), wherein the primers are reverse complements of the primers that prime sequencing of the target nucleotide sequences. In illustrative embodiments, the primers employed to prime sequencing of the target nucleotide sequence(s) and barcode nucleotide sequence(s) include CS1, CS2, CS1rc, and CS2rc (Table 2, Example 9).

[0018] In any of the above-described bidirectional embodiments, the barcode nucleotide sequence can be selected so as to avoid substantial annealing to the target nucleic acids. In certain embodiments, the barcode nucleotide sequence identifies a particular sample.

[0019] When bidirectional DNA sequencing is carried out according to the above-described methods, in some embodiments, at least 50 percent of the sequences determined from DNA sequencing are present at greater than 50 percent of the average number of copies of sequences and less than 2-fold the average number of copies of sequences. In certain embodiments, at least 70 percent of the sequences determined from DNA sequencing are present at greater than 50 percent of the average number of copies of sequences and less than 2-fold the average number of copies of sequences. In specific embodiments, at least 90 percent of the sequences determined from DNA sequencing are present at greater than 50 percent of the average number of copies of sequences and less than 2-fold the average number of copies of sequences.

[0020] In any of the above-described bidirectional embodiments, the average length of the target amplicons is less than 200 bases. In various embodiments, the first amplification (i.e., the amplification to produce target amplicons) is carried out in a volume

in the range of about 1 picoliter to about 50 nanoliters or about 5 picoliters to about 25 nanoliters. In particular embodiments, the first amplification (i.e., the amplification to produce target amplicons) reaction(s) is/are formed in, or distributed into, separate compartments of a microfluidic device prior to amplification. The microfluidic device can be, for example, one that is fabricated, at least in part, from an elastomeric material. In certain embodiments, the first amplification (i.e., the amplification to produce target amplicons) reaction(s) is/are carried out in (a) fluid droplet(s).

[0021] Another aspect of the invention includes a kit useful for carrying out the bidirectional embodiments discussed above. In certain embodiments, the kit includes:

10 a first set of outer primers, wherein the set includes:

a first outer, forward primer including a portion specific for a first primer binding site; and

a first outer, reverse primer including a barcode nucleotide sequence and a portion specific for a second primer binding site, wherein the first and second primer binding sites are different;

a second set of outer primers, wherein the set includes:

a second outer, forward primer including a barcode nucleotide sequence and a portion specific for the first primer binding site; and

a second outer, reverse primer including a portion specific for the second primer binding site. In particular embodiments, the first and second primer binding sites are binding sites for DNA sequencing primers. In specific embodiments, the outer primers each additionally include an additional nucleotide sequence, wherein:

the first outer, forward primer includes a first additional nucleotide sequence, and the first outer, reverse primer includes a second additional nucleotide sequence; and

25 the second outer, forward primer includes the second additional nucleotide sequence, and the second outer, reverse primer includes the first additional nucleotide sequence, and the first and second additional nucleotide sequences are different. In an illustrative embodiment, the first set of outer primers includes PE1-CS1 and PE2-BC-CS2, and the second set of outer primers includes PE1-CS2 and PE2-BC-CS1 (Table 1,

Example 9). In certain embodiments, the kit additionally includes a set of inner primers, wherein the set includes:

an inner, forward primer including a target-specific portion and the first primer binding site; and

5 an inner, reverse primer including a target-specific portion and the second primer binding site. In some embodiments, the kit includes a plurality of sets of inner primers, each specific for a different target nucleic acid.

[0022] Any of the above described kits useful for carrying out bidirectional
embodiments can additionally include DNA sequencing primers that bind to the first and
10 second primer binding sites and prime sequencing of the target nucleotide sequence(s)
and/or additionally include DNA sequencing primers that bind to the first and second primer
binding sites and prime sequencing of the barcode nucleotide sequence(s). In specific
embodiments, the primers that bind to the first and second primer binding sites and prime
sequencing of the barcode nucleotide sequences(s) are reverse complements of the primers
15 that prime sequencing of the target nucleotide sequences. For example, the primers
employed to prime sequencing of the target nucleotide sequence(s) and barcode nucleotide
sequence(s) include CS1, CS2, CS1rc, and CS2rc (Table 2, Example 9).

[0023] The invention further provides, in some embodiments, a method for
detecting, and/or quantifying the relative amounts of, at least two different target nucleic
20 acids in a nucleic acid sample. The method entails, producing first and second tagged target
nucleotide sequences from first and second target nucleic acids in the sample,

the first tagged target nucleotide sequence including a first nucleotide tag;
and

the second tagged target nucleotide sequence including a second nucleotide
25 tag, wherein the first and second nucleotide tags are different. The tagged target nucleotide
sequences are subjected to a first primer extension reaction using a first primer that anneals
to the first nucleotide tag, and a second primer extension reaction using a second primer that
anneals to the second nucleotide tag. The method further entails detecting and/or
quantifying a signal that indicates extension of the first primer, and a signal that indicates

extension of the second primer, wherein the a signal for a given primer indicates the presence, and/or relative amount of, the corresponding target nucleic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1A-1D: Illustration of hairpin adaptor molecules to produce adaptor-modified target nucleic acid molecules, e.g. a library suitable for use in high-throughput DNA sequencing. (A) Hairpin adaptor molecules each including: an adaptor nucleotide sequence, which is linked to a nucleotide linker, which is linked to a nucleotide sequence that is capable of annealing to the adaptor nucleotide sequence and is linked to a degenerate tail sequence; N = an nucleotide; an optional specific enzyme cutting site can be included in the nucleotide linker. (B) Target nucleic acid molecule preparation can include fragmentation and digestion of 5' ends to produce 3' sticky ends. (C) Annealing, gap-filling, and ligation is carried out. (D) The resultant DNA is conveniently linearized using an enzyme that cuts within the linker.

[0025] Figure 2A-2D: Illustration of double-stranded adaptor molecules to produce adaptor-modified target nucleic acid molecules, e.g. a library suitable for use in high-throughput DNA sequencing. (A) Double-stranded adaptor molecules each including: a first adaptor nucleotide sequence, which is linked to a nucleotide linker, which is linked to a second adaptor nucleotide sequence; and a degenerate tail sequence on each strand, wherein double-stranded molecules each include two degenerate tail sequences as sticky end(s); N = an nucleotide; an optional specific enzyme cutting site can be included in the nucleotide linker. (B) Target nucleic acid molecule preparation can include fragmentation and digestion of 5' ends to produce 3' sticky ends. (C) Annealing, gap-filling, and ligation is carried out. (D) The resultant circular DNA is conveniently linearized using an enzyme that cuts within the linker.

[0026] Figure 3: A four-primer, combinatorial barcoding method can be employed to put a combination of two barcodes on either end of each amplicon. Inner primers include target-specific portions ("TS-F" in the forward primer and "TS-R" in the reverse primer), a barcode nucleotide sequence ("bc2"), and different nucleotide tags. Outer primers include tag-specific portions ("CS1" and "CS2"), a different barcode nucleotide sequence ("bc1"), primer binding sites for sequencing primers ("A" and "B").

[0027] Figure 4: A six-primer, combinatorial barcoding method can be employed to put a combination of two barcodes on either end of each amplicon. Inner primers include target-specific portions (“TS-F” in the forward primer and “TS-R” in the reverse primer) and different nucleotide tags. Stuffer primers include tag-specific portions (“CS1” and “CS2”), a barcode nucleotide sequence (“bc2”), and two additional different nucleotide tags. Outer primers include portions specific for the two additional nucleotide tags (“CS3” and “CS4”), a different barcode nucleotide sequence (“bc1”), and primer binding sites for sequencing primers (“A” and “B”).

[0028] Figure 5A-5B: A combinatorial ligation-based tagging method employing tagged target nucleotide sequences (A) to produce combinatorially tagged target nucleotide sequences. PE1, PE1 = Illumina sequencing flowcell binding sequences; Seq1, Seq2 = sequencing priming sites; BC1, BC2 = barcode sequences. See Example 2.

[0029] Figure 6: Combinatorial insertional mutagenesis-based tagging for sequencing (e.g., Illumina sequencing). A barcode is inserted into Transposon tag sequence. TagA and TagB need to be long enough to prime sequencing. BC2 should contain a 4-base barcode plus 3 degenerate primers at 5' end (e.g. NNNAGTC). Transposon end sequence = 5'-AGATGTGTATAAGAGACAG-3' (SEQ ID NO:1). PE1, PE1 = Illumina sequencing flowcell binding sequences; BC1, BC2 = barcode sequences.

[0030] Figure 7A-C: Barcoding and pooling of reaction mixtures for subsequent analysis: production of barcoded target nucleotide sequences. (A) In an illustrative embodiment, cells are loaded in limiting dilution in an ACCESS ARRAY™ IFC (“Integrated Fluidic Circuit,” also referred to herein as a “chip”). Primer sets are loaded as shown, with each chamber in the chip receiving a complete set of 96 forward primers (F1-96) and 96 reverse primers (R1-96) for amplifying 96 targets. The reverse primer is tagged with a tag that can anneal to a barcode primer. Each chamber in a row of the chip receives a different barcode primer. (B) As described in Example 5, reverse transcription and preamplification is carried out in the chip to generate barcoded target nucleotide sequences using a 3-primer method. Any given chamber will have amplified all genes, and all amplicons will have been tagged with a single barcode. The reaction products are exported by pool (90 degrees to different primer sets, i.e. by sample). (C) For detection, a DYNAMIC ARRAY™ IFC can be loaded as shown, with the forward primer (e.g., F1) used to amplify

a particular target nucleic acid and the barcode primer (e.g., BC1) used to amplify this sequence in a particular chamber in a particular pool (e.g. pool 1).

[0031] Figure 8A-C: Barcoding and pooling of reaction mixtures for subsequent analysis: exemplary strategies for amplification/detection of barcoded target nucleotide sequences. (A) An illustrative embodiment employs LCR to detect barcoded target nucleotide sequences having the structure: 5'-forward primer sequence-target nucleotide sequence-reverse primer sequence-barcode nucleotide sequence-3'. In this case, one primer can anneal to the reverse primer sequence, and the other primer can anneal to the adjacent barcode nucleotide sequence, which is followed by ligation and repeated cycles of annealing and ligation. (B) Detection can be carried out in real time using a flap endonuclease-ligase chain reaction. This reaction employs a labeled probe and an unlabeled probe, wherein the simultaneous hybridization of the probes to a reaction product results in the formation of a flap at the 5' end of the labeled probe, and cleavage of the flap produces a signal. As shown, cleavage of the flap can separate a fluorophore from a quencher to generate a signal. (C) An alternative real time detection method that is useful, e.g., for detecting amplicons produced by LCR from barcoded target nucleotide sequences having the structure: 5'-forward primer sequence-target nucleotide sequence-reverse primer sequence-barcode nucleotide sequence-3'. This method relies on using a double-stranded DNA-binding dye to detect melting temperature differences between the reactions products and the primers employed for the LCR. The melting temperature analysis includes detection at a temperature at which reaction products are substantially double-stranded and capable of producing signal in the presence of a double-stranded DNA-binding dye, but primers are substantially single-stranded and incapable of producing signal ("Temp High"). For example, to detect barcoded target nucleotide sequences having the structure: 5'-forward primer sequence-target nucleotide sequence-reverse primer sequence-barcode nucleotide sequence-3', one primer can anneal to the reverse primer sequence, and the other primer can anneal to the adjacent barcode nucleotide sequence, which is followed by ligation and repeated cycles of annealing and ligation. See Figure 8C.

[0032] Figure 9: A schematic diagram of the unit cell architecture for a microfluidic device adapted for Cell handling ("MA006"), showing on-chip processes.

[0033] Figure 10: The use of limiting dilution of a cell suspension to obtain a single cell per separate reaction volume (“chamber” of a microfluidic device or “chip”). The theoretical distribution (Poisson distribution) for various cell densities is shown.

5 [0034] Figure 11A-B: The results of cell counting in a chip using brightfield (A) to image, as compared to the theoretical distribution (B). Cell density in the chip, based on brightfield imaging, is close to, but lower than, the Poisson distribution, with this tendency exacerbated at higher cell densities.

[0035] Figure 12A-B: Fluorescent cell “ghost” images (A) permit detection of more cells than pre-PCR brightfield imaging, so that the cell density more closely approximates
10 the Poisson distribution (B).

[0036] Figure 13: Specific methods for detecting cells in a chip that can be used include, e.g., the use of a cell membrane-permeant nucleic acid stain and/or cell-specific surface marker detection with an antibody. The results of these more specific approaches are shown for a cell density of 1E6/ml.

15 [0037] Figure 14A-B: (A) A comparison the use of pre-RT-PCR nucleic acid stain (Syto10 DNA stain) to detect cells in a chip versus post RT-PCR ghost images (Cell ghost). (B) Syto10 does not inhibit RT-PCR of GAPDH.

[0038] Figure 15: RT-PCR of GAPDH carried out in the presence of 0.5% Tween 20 or 0.5% NP40 (the latter is a cell lysis reagent). Neither inhibited RT-PCR of GAPDH
20 significantly

[0039] Figure 16: Standard curve amplification of 11 genes, carried out in the MA006 chip. These results demonstrate that the CellsDirect™ One-Step qRT-PCR kit can be used with 0.5% NP40 (for cell lysis and to prevent the depletion effect in the chip) to convert gene-specific RNA in cells into amplicons in an MA006 chip.

25 [0040] Figure 17: A four-primer, combinatorial barcoding method was employed to put a combination of two barcodes on either end of each amplicon. Inner primers include target-specific portions (“TS-F” in the forward primer and “TS-R” in the reverse primer), a barcode nucleotide sequence (“bc2”), and different nucleotide tags. Outer primers include tag-specific portions (“CS1” and “CS2”), a different barcode nucleotide sequence (“bc1”),
30 primer binding sites for sequencing primers (“A” and “B”).

[0041] Figure 18A-B: An illustration of how 4-primer barcoding can be carried out on a chip, such as the MA006. (A) Amplification is carried out on-chip with inner primers, where each row of chambers has the same pair of inner primers with the same barcode. (B) Reaction products from each column of chambers can be harvested as a pool and each pool subjected to amplification using a different pair of outer primers. This amplification produces amplicons having barcode combinations at either end of the amplicon that uniquely identify the chamber (by row and column) in which the initial amplification was carried out.

[0042] Figure 19: A comparison of the results obtained upon sequencing gene-specific amplicons from single cells (Example 5), expressed as number of reads for each gene-specific amplicon, as compared to that for total RNA. As is apparent from this figure, the representation of these RNAs is different when measured in individual cells, as compared to that observed in the total RNA.

[0043] Figure 20A-B: A capture site with a capture feature and drain. (A) A site without baffles to focus flow. (B) A site with baffles.

[0044] Figure 21: Additional capture site designs.

[0045] Figure 22A-C: Capture architectures can be designed to maximize the probability that cells will come into contact with the surface markers. For example, baffles on one or more channel walls can be used to direct beads towards capture feature.

(A) Illustrative capture feature/baffle combinations. (B) Performance of the capture feature can be adjusted by adjusting one or more variables, including angle of baffles, distance of baffles from capture site, length of baffles, size and shape of capture feature, size of drain in capture feature (if present). Baffles on the channel wall are used to direct beads towards a capture feature. (C) The capture feature is coupled to a baffle on a channel wall; individual capture feature/baffle combinations can be located on alternate walls to focus flow towards the adjacent capture feature/baffle combination.

[0046] Figure 23A-B: A strategy for using capture features to catch single, affinity-reagent-coated beads, which then display the affinity reagent (e.g., antibody) so as to capture single particles (e.g., cells). (A-1) Flow is initiated in a channel containing capture features. (A-2) Antibody-bound beads flow toward the capture features until a bead lodges in the capture feature. (A-3) The channel is then washed to remove non-captured beads.

(B-1) cells bearing a cell-surface marker to which the antibody binds are flowed into the channel containing the captured beads. (B-2) Cells bearing the marker interact with and bind to antibodies displayed by the captured bead. The display area is sized so that a bound cell will inhibit other cells from interacting with the captured bead through steric occlusion, such that only one cell binds to each captured bead. (B-3) The channel is then washed to remove non-bound cells, leaving one cell immobilized at each capture site.

[0047] Figure 24A-G: (A) A schematic of a microfluidic device designed to capture single cells at discrete locations (niches). Single cell capture allows analysis of biological events at the single cell level. (B) Flow is designed to be stronger over niches than through an overflow channel. Niches contain small gaps (~3 μm tall). When a cell enters a niche, it blocks the niche and prevents any more flow into the niche. Flow passes through to the next unoccupied niche, until it too is blocked by a cell. Every niche should capture one cell before cells pass through the overflow channel and out to waste. (C) Schematic of (A) shown with additional detail provided in (D)-(F). (D) A buffer inlet converges with a cell inlet so as to force cells to a side of a feeder channel that is closest to a series of transverse cell capture channels. (E) The resistance of the transverse cell capture channels is lower than that of a cell overflow channel to induce preferential flow of cells into niches versus into the cell overflow channel. (F) Each niche is large enough to capture just one cell. A cell in a niche raises the resistance of that particular circuit, and flow is directed to the circuits without cells. (G) An actual device of (A), with captured human umbilical vein endothelial cells (HUVEC) located in niches.

[0048] Figure 25A-B: The amplicon tagging strategy employed in Example 9. (A) Standard 4-primer amplicon tagging versus bidirectional sequencing amplicon tagging. The standard 4-primer amplicon tagging approach incorporated the paired-end Illumina sequencing primer annealing sites in Common Sequence tag 1 (CS1) and Common Sequence tag 2 (CS2). Sequencing of both the 5' end and the 3' end of each PCR product required a paired-end sequencing run. (B) Target-specific primers were appended with Common Sequence tags CS1 and CS2. The sample-specific primer pairs were comprised of common sequence tags CS1 or CS2, appended with the adaptor sequences used by the Genome Analyzer (PE1 and PE2) in both permutations. Two PCR product types were generated from the same target region: Product A allowed for sequencing of the 5' end of

the target region whereas product B allowed for sequencing of the 3' end of the target region during the same sequencing read.

[0049] Figure 26A-B: Overview of the segregated-primer PCR strategy used in Example 9. The first PCR with the target-specific primer pairs was carried out in the ACCESS ARRAY™ IFC. The harvested PCR product pools were split into two subsequent PCR reactions with sample-specific barcode primers. (A) The reaction that generated products that allowed for sequencing of the 5' end of the target region utilized the PE1_CS1 and PE2_BC_CS2 primer combination. (B) The reaction that generated products that allowed for sequencing of the 3' end of the target region utilized the PE1_CS2 and PE2_BC_CS1 primer combination.

[0050] Figure 27: Overview of the sequencing work flow used in Example 9. Both PCR product types were present on the flow cell. An equimolar mixture of CS1 and CS2 allowed for sequencing of both the 5' end and 3' end of the target regions. After stripping and rehybridization of the clusters with an equimolar mixture of CS1rc and CS2rc the barcodes were sequenced. The sequencing primers CS1 and CS2 were provided in the reagent FL1. The indexing primers CS1rc and CS2rc were provided in the reagent FL2.

[0051] Figure 28: Bioanalyzer products obtained from barcoding reactions run with barcodes from Plate 1 and Plate 2 in Example 10.

[0052] Figure 29: Alternative sequencing primers used in Example 10. Use of an equimolar mixture of all target-specific PCR primers that were used on the ACCESS ARRAY™ IFC as a sequencing primer pool circumvents sequencing through the uninformative target-specific primer region.

[0053] Figure 30: Per-base coverage of the gene EGFR for one sample in Example 10. Reads from each strand are shown in different shades.

[0054] Figure 31A-B: (A) Allele-specific PCR on target DNA is done in one reaction prior to the 454 sequencing emulsion PCR reaction. The forward primers have 454 adapters and allele-specific tags. Different tags are indicated with different shadings. This reaction produces amplicons which are ready for 454 bead emulsion PCR. (B) After emulsion PCR and loading onto the sequencer, the amplicons on individual beads in each well are either wildtype or mutant. The first 454 cycle flows a primer which binds to the

wildtype tag (pink arrow), and it flows all the dNTP's. As this primer extends, multiple nucleotides are incorporated, giving a very robust signal, but only in wells with wildtype molecules. The second cycle flows in all dNTP's and a primer to the mutant tag and produces signal only in the wells with mutant molecules.

5 **[0055]** Figure 32: Agilent Bioanalyzer results from a cross talk experiment between Fluidigm and Illumina TruSeq sequencing primers on Illumina generated libraries. The PCR reactions for each lane are as follows:

1. Illumina standard library + Fluidigm FL1 sequencing primers
2. Illumina standard library + Illumina TruSeq sequencing primers
- 10 3. Illumina standard library + Fluidigm FL1 and Illumina TruSeq sequencing primers
4. Illumina standard library + Illumina standard sequencing primers (control)
5. Illumina Multiplex library + Fluidigm FL1 sequencing primers
6. Illumina Multiplex library + Illumina TruSeq sequencing primers
7. Illumina Multiplex library + Fluidigm FL1 and Illumina TruSeq sequencing primers
- 15 8. Illumina Multiplex library + Illumina Multiplex sequencing primers (control)
9. Illumina Small RNA library + Fluidigm FL1 sequencing primers
10. Illumina Small RNA library + Illumina TruSeq sequencing primers
11. Illumina Small RNA library + Fluidigm FL1 and Illumina TruSeq sequencing primers
- 20 12. Illumina Small RNA library + Illumina Small RNA sequencing primers (control)

[0056] Figure 33: Agilent Bioanalyzer results from a cross talk experiment between Fluidigm and Illumina TruSeq sequencing primers on an ACCESS ARRAY™ IFC generated library. The PCR reactions for each lane are as follows:

- 25 1. Fluidigm ACCESS ARRAY™ IFC library + Fluidigm FL1 sequencing primers
2. Fluidigm ACCESS ARRAY™ IFC library + Illumina TruSeq sequencing primers
3. Fluidigm ACCESS ARRAY™ IFC library + Fluidigm FL1 and Illumina TruSeq sequencing primers

DETAILED DESCRIPTION

30 **[0057]** For a variety of applications, it is necessary or desirable to incorporate nucleic acid sequences into target nucleic acids derived, e.g., from a sample, such as a biological sample. The sequences incorporated can, in certain embodiments, facilitate further analysis of the target nucleic acids. Accordingly, described herein are methods useful for incorporating one or more adaptors and/or nucleotide tag(s) and/or barcode

35 nucleotide sequence(s) one, or typically more, target nucleotide sequences. In particular embodiments, nucleic acid fragments having adaptors, e.g., suitable for use in high-

throughput DNA sequencing are generated. In other embodiments, information about a reaction mixture is encoded into a reaction product. For example, if a nucleic acid amplification is carried out in the separate reaction volumes, it may be desirable to recover the contents for subsequent analysis, e.g., by PCR and/or nucleic acid sequencing. The contents of the separate reaction volumes may be analyzed separately and the results associated with the original reaction volumes. Alternatively, the particle/reaction volume identity can be encoded in the reaction product, e.g., as discussed below with respect to multi-primer nucleic acid amplification methods. Furthermore, these two strategies can be combined so that sets of separate reaction volumes are encoded, such that each reaction volume within the set is uniquely identifiable, and then pooled, with each pool then being analyzed separately.

[0058] In certain embodiments, the present invention provides amplification methods in which a barcode nucleotide sequence and additional nucleotide sequences that facilitate DNA sequencing are added to target nucleotide sequences. The barcode nucleotide sequence can encode information, such as, e.g., sample origin, about the target nucleotide sequence to which it is attached. The added sequences can, for example, serve as binding sites for DNA sequencing primers. 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.

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 to sequences that directly abut one another. As those of skill in the art appreciate, two nucleotide sequences that are to be ligated together will generally 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.

5 [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.

10 [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] A double-stranded nucleic acid that is not double-stranded along the entire length of both strands has a 5' or 3' extension that is referred to herein as a “sticky end” or as a “tail sequence.” The term “sticky end” is often used to refer to a relatively short 5' or
15 3' extension, such as that produced by a restriction enzyme, whereas the term “tail sequence” is often used to refer to longer 5' or 3' extensions.

[0065] The term “degenerate sequence,” as used herein denotes a sequence in a plurality of molecules, wherein a plurality of different nucleotide sequences are present. For example, all possible sequences for the degenerate sequence may be present.

20 [0066] The term “degenerate tail sequence” is used to describe a tail sequence in a plurality of molecules, wherein the tail sequences have a plurality of different nucleotide sequences; e.g., all possible different nucleotide sequences (1 per tail) may be present in the plurality of molecules.

[0067] The term nucleic acid also encompasses any chemical modification thereof,
25 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
30 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.

5 [0068] 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
10 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.

15 [0069] 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
20 processes.

[0070] The order of elements within a nucleic acid molecule is typically described herein from 5' to 3'. In the case of a double-stranded molecule, the "top" strand is typically shown from 5' to 3', according to convention, and the order of elements is described herein with reference to the top strand.

25 [0071] The term "target nucleic acids" is used herein to refer to particular nucleic acids to be detected in the methods of the invention.

[0072] 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
30 upon reverse transcription of an RNA target nucleic acid.

[0073] 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
5 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. A first nucleotide sequence is said to be the “complement” of a second sequence if
10 the first nucleotide sequence is complementary to the second nucleotide sequence. A first nucleotide sequence is said to be the “reverse complement” of a second sequence, if the first nucleotide sequence is complementary to a sequence that is the reverse (i.e., the order of the nucleotides is reversed) of the second sequence.

[0074] “Specific hybridization” refers to the binding of a nucleic acid to a target
15 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.

[0075] In particular embodiments, hybridizations are carried out under stringent
20 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
25 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
30 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.

[0076] 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.

[0077] The term “adaptor” is used to refer to a nucleic acid that, in use, becomes appended to one or both ends of a nucleic acid. An adaptor may be single-stranded, double-stranded, or may include single- and double-stranded portions.

[0078] 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.

[0079] 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.

[0080] 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.

[0081] 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.

[0082] In embodiments in which two primer pairs are used, e.g., in an amplification reaction, the primer pairs may be denoted “inner” and “outer” primer pairs to indicate their relative position; i.e., “inner” primers are incorporated into the reaction product (e.g., an amplicon) at positions in between the positions at which the outer primers are incorporated.

[0083] In embodiments in which three primer pairs are used, e.g., in an amplification reaction, the term “stuffer primer” can be used to refer to a primer that has a position in between inner and outer primers; i.e., the “stuffer” primer is incorporated into

the reaction product (e.g., an amplicon) at positions intermediate between the inner and outer primers.

5 [0084] A primer pair is said to be “unique” if it can be employed to specifically produce (e.g., amplify) a particular reaction product (e.g., amplicon) in a given reaction (e.g., amplification) mixture.

10 [0085] 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).

20 [0086] 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.

30 [0087] 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.

5 **[0088]** The term “affinity tag” is used herein to refer to a portion of a molecule that is specifically bound by a binding partner. This portion can, but need not be, a nucleotide sequence. The specific binding can be used to facilitate affinity purification of affinity tagged molecules.

10 **[0089]** The term “transposon end” refers to an oligonucleotide that is capable of being appended to a nucleic acid by a transposase enzyme.

15 **[0090]** 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.

20 **[0091]** 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
25 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.
30 Patent Publication No. 2005/0260640, which is hereby incorporated by reference in its entirety, and in particular for ligation reactions.

[0092] As used herein an “encoding reaction” can produce a “tagged target nucleotide sequence,” which includes a nucleotide tag linked to a target nucleotide sequence.

5 [0093] 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.

[0094] 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.

10 [0095] 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),
15 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,
20 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.,
25 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
30

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-

- 5 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.,
10 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.

[0096] In some embodiments, amplification comprises at least one cycle of the
15 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.

20 [0097] 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.”

[0098] The term “substantially” as used herein with reference to a parameter means that the parameter is sufficient to provide a useful result. Thus, “substantially
25 complementary,” as applied to nucleic acid sequences generally means sufficiently complementary to work in the described context. Typically, substantially complementary means sufficiently complementary to hybridize under the conditions employed. In some embodiments described herein, reaction products must be differentiated from unreacted primers. In this context, the statement that the “reaction products are substantially double-
30 stranded,” taken with the statement that the “primers are substantially single-stranded,” means that there is a sufficient difference between the amount of double-stranded reaction

products and the single-stranded primer, that the presence and/or amount of the reaction products can be determined.

[0099] 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
5 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.

[0100] The term “universal detection probe” is used herein to refer to any probe that
10 identifies the presence of an amplification product, regardless of the identity of the target nucleotide sequence present in the product.

[0101] 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
15 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
20 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
25 amplification products can be detected in a given amplification mixture or aliquot thereof.

[0102] 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.

30 [0103] 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.

[0104] The term “stain”, as used herein, generally refers to any organic or inorganic molecule that binds to a component of a reaction or assay mixture to facilitate detection of that component.

[0105] 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.

[0106] 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.

[0107] 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.

[0108] As use herein, the term “variation” is used to refer to any difference. A variation can refer to a difference between individuals or populations. A variation encompasses a difference from a common or normal situation. Thus, a “copy number variation” or “mutation” can refer to a difference from a common or normal copy number or nucleotide sequence. An “expression level variation” or “splice variant” can refer to an expression level or RNA or protein that differs from the common or normal expression level or RNA or protein for a particular, cell or tissue, developmental stage, condition, etc.

[0109] 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.

[0110] 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.

[0111] As used herein with respect to reactions, reaction mixtures, reaction volumes, etc., the term “separate” refers to reactions, reaction mixtures, reaction volumes, etc., where reactions are carried out in isolation from other reactions. Separate reactions, reaction mixtures, reaction volumes, etc. include those carried out in droplets (See, e.g., U.S. Patent No., 7,294,503, issued November 13, 2007 to Quake et al., entitled “Microfabricated crossflow devices and methods,” which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets; U.S. Patent Publication No. 20100022414, published January 28, 2010, by Link et al., entitled “Droplet libraries,” which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets;

and U.S. Patent Publication No. 20110000560, published January 6, 2011, by Miller et al., entitled "Manipulation of Microfluidic Droplets," which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets.), which may, but need not, be in an emulsion, as well as those wherein
5 reactions, reaction mixtures, reaction volumes, etc. are separated by mechanical barriers, e.g., separate vessels, separate wells of a microtiter plate, or separate compartments of a matrix-type microfluidic device.

Production of Adaptor-Modified Target Nucleic Acid Molecules

[0112] In certain embodiments, the invention relates to a method of adding adaptor
10 molecules to each end of a plurality of target nucleic acids that include sticky ends. These embodiments are useful, for example, in fragment generation for high-throughput DNA sequencing. The adaptors can be selected to facilitate sequencing using the DNA sequencing platform of choice.

[0113] In particular embodiments, such a method entails annealing adaptor
15 molecules to the sticky ends of double-stranded target nucleic acid molecules to produce annealed adaptor-target nucleic acid molecules. The target nucleic acid molecules that include sticky ends can be produced by any convenient method. In certain embodiments, DNA molecules are fragmented, e.g., by any of enzymatic digestion, nebulization, sonication, and the like. For example, DNA molecules can be fragmented by digestion with
20 a DNase enzyme, such as DNase I, terminated by heat treatment. Fragmentation that does not produce sticky ends can be followed by digesting the fragmented DNA molecules with an enzyme to produce sticky ends. In particular embodiments, the sticky ends of double-stranded target nucleic acid molecules are 3' extensions. A strand-specific endonuclease that does not have polymerase activity under the conditions employed in the digestion can
25 be used to produce sticky ends. In an illustrative embodiment, sticky ends are produced by digesting 5' ends with Exonuclease III in the absence of dNTPs.

[0114] In a first embodiment, the adaptor molecules are hairpin structures each including: an adaptor nucleotide sequence, which is linked to a nucleotide linker, which is linked to a nucleotide sequence that is capable of annealing to the adaptor nucleotide
30 sequence and is linked to a degenerate tail sequence. See Figure 1A. This embodiment employs two types of adaptor molecules, wherein the each type includes an adaptor

nucleotide sequence that is different from the other type (i.e., a first adaptor nucleotide sequence and a second adaptor nucleotide sequence).

[0115] In a second embodiment, the adaptor molecules are double-stranded or single-stranded molecules each including: a first adaptor nucleotide sequence, which is
5 linked to a nucleotide linker, which is linked to a second adaptor nucleotide sequence; and a degenerate tail sequence on each strand, wherein double-stranded molecules each include two degenerate tail sequences as sticky end(s). See Figure 2A.

[0116] In certain embodiments, for example, those in which target nucleic acid molecules are being prepared for high-throughput DNA sequencing, the first and second
10 adaptor sequences can include primer binding sites that are capable of being specifically bound by DNA sequencing primers, i.e., sequencer-specific tag 1 and sequencer specific tag 2. See Figures 1A and 2A.

[0117] In all cases, the degenerate tail sequence(s) can be at the 3' ends of the adaptor molecules. The degenerate tail sequences of the adaptor molecules are essentially
15 complementary to at least a portion of the sticky ends on target nucleic acid molecules; i.e., the adaptor molecules are capable of annealing to the target nucleic acid molecules under the conditions employed. The length of the degenerate tail sequences will typically be sufficient to facilitate this annealing, e.g., about 10 to about 20 nucleotides. In certain
20 embodiments, the degenerate tail sequences are protected at their 3' ends, e.g., with phosphothionate or dUTP to protect against exonuclease digestion.

[0118] The adaptor molecules can, optionally, include one or more additional nucleotide sequences. In certain embodiments, the nucleotide linker portion of the adaptor
molecules can include an endonuclease site, a barcode nucleotide sequence, an affinity tag, and any combination thereof. For example, the nucleotide linker can include a restriction
25 enzyme site and, optionally, at least one barcode nucleotide sequence.

[0119] In both the first and second embodiments, after annealing to target nucleic acids molecules, the method entails filling any gaps in the annealed adaptor-target nucleic acid molecules (e.g. using a DNA polymerase), and ligating any adjacent nucleotide
sequences in the annealed adaptor-target nucleic acid molecules to produce adaptor-
30 modified target nucleic acid molecules. In some embodiments, sticky end generation and ligation can be carried out in the same reaction mixture. For example an exonuclease can be

used in concert with a ligase (e.g., a thermostable ligase) and a polymerase (e.g., PHUSION®) in a single reaction mixture.

[0120] When the adaptor molecules are hairpin structures, ligation of adaptors to target nucleic acids converts the annealed adaptor-target nucleic acid molecules to single-stranded circular DNA molecules that can form a double-stranded structure as shown in Figure 1D. When the adaptor molecules are single-stranded or double-stranded molecules, ligation of adaptors to target nucleic acids converts the annealed adaptor-target nucleic acid molecules to double-stranded circular DNA molecules. When the nucleotide linker includes an endonuclease site, the method can additionally include digesting the single-stranded or double-stranded circular DNA molecules to produce linear DNA molecules. See Figures 1D and 2D. In particular, double-stranded circular DNA molecules can be digested with a restriction enzyme that cuts at a site in the nucleotide linker to produce linear DNA molecules. In particular embodiments, the linear DNA molecules include 5'-a first portion of a nucleotide linker-second adaptor nucleotide sequence-a first degenerate tail sequence-target nucleic acid molecule-a second degenerate tail sequence-first adaptor nucleotide sequence-a second portion of a nucleotide linker-3'.

[0121] In an illustrative embodiment, the method described above can be carried out by:

producing the plurality of target nucleic acid molecules that include sticky ends by:

digesting DNA molecules with DNase I to produce fragmented DNA molecules, and then heat inactivating the DNase I;

digesting the fragmented DNA molecules with a nuclease having 5' to 3' exonuclease activity (such as Exonuclease III) in the absence of deoxynucleotides to produce a plurality sticky-ended target nucleic acid molecules;

annealing the adaptors to the sticky ends of the plurality of target nucleic acid molecules, wherein the nucleotide linker of the adaptors includes an endonuclease site;

filling any gaps and ligating any adjacent nucleotide sequences in the annealed adaptor-target nucleic acid molecules in a single reaction including a polymerase and a ligase to produce circular DNA molecules; and

digesting the circular DNA molecules with an endonuclease that cuts at the endonuclease site to produce linear DNA molecules.

[0122] In particular embodiments, methods of adding adaptor molecules to each end of a plurality of target nucleic acids can include sequencing the adaptor-modified target nucleic acid molecules by any available method, such as any available high-throughput DNA sequencing technique.

Incorporation of Nucleic Acid Sequences into Target Nucleic Acids

[0123] Reactions to incorporate one or more nucleotide sequences into target nucleic acids can be carried out using two or more primers that contain one or more nucleic acid sequences in addition to portions that anneal to the target nucleic acids. One or more of these portions may contain random sequences to incorporate nucleic acid sequences into essentially all nucleic acids in the sample. Alternatively, or in addition, one or more of these portions may be specific for one or more sequences common to a plurality of, or all, nucleic acids present. In other embodiments, the primers include portions specific for one or more particular target nucleic acids. Nucleic acid sequences can be incorporated using as few as two primers. However, various embodiments employ three, four, five, or six or more primers, as discussed in more detail below. Such reactions are discussed below in terms of nucleic acid amplification; however, those of skill in the art will readily appreciate that the strategies discussed below can be employed in other types of reactions, e.g., polymerase extension and ligation.

Three-Primer Methods

[0124] In particular embodiments, the invention provides an amplification method for incorporating 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, in some embodiments, 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 (e.g.,

first and second nucleotide tags). 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, a nucleotide tag for the reverse primer. Generally, the nucleotide tags are 5' of the target-specific portions. The third
5 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
10 generally 5' of the tag-specific portion.

[0125] The barcode primer is typically present in the amplification mixture in excess of the forward and/or reverse or (inner) 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
15 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
20 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 5×10^3 -fold, at least 10^4 -fold, at least 5×10^4 -fold, at least 10^5 -fold, at least 5×10^5 -fold, at least 10^6 -fold, or higher, relative to the concentration of the forward and/or reverse
25 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
30 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 μ M, about 25 nM to about 7.5 μ M, about 50 nM to about 5 μ M, about 75 nM to about 2.5 μ M, about 100 nM to about 1 μ M, 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).

[0126] 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'.

Four-Primer Methods

[0127] 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 elements discussed above, plus an optional additional barcode e.g., 5'-(barcode nucleotide sequence)-(first nucleotide tag from the forward primer)-(target nucleotide sequence)-(second nucleotide tag from the reverse primer)-(additional barcode nucleotide sequence)-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 a barcode primer, as described above. The second outer

primer can include a second tag-specific portion and an additional barcode nucleotide sequence, i.e., it can be a second barcode primer.

[0128] 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 applies to the concentrations of the outer primers relative to inner primers in a one-step, four-primer amplification reaction.

Combinatorial Methods

[0129] 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 barcode nucleotide sequence)-(first nucleotide tag)-3', and the second barcode primer would be constructed of the elements 5'-(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.

[0130] In a further illustrative embodiment of the invention, more than four primers can be combined in a single reaction to append different combinations of barcode nucleotide sequences and nucleotide tags. For example, outer barcode primers containing the following elements: 5'-(first barcode nucleotide sequence)-(first nucleotide tag)-3', 5'-(first barcode nucleotide sequence)-(second nucleotide tag)-3', 5'-(second barcode nucleotide sequence)-(first nucleotide tag)-3', 5'-(second 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.

[0131] 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.

Bidirectional Combinatorial Methods

[0132] In an illustrative embodiment of the four-primer amplification reaction, inner and outer primers can each include a unique barcode, such that amplification produces a barcode combination at each end of the resultant amplicons. This approach is useful when the amplicons are to be sequenced because the barcode combination can be read from either end of the sequence. For example, four primers can be employed to produce molecules having the following elements: 5'-second barcode nucleotide sequence-first nucleotide tag sequence-first barcode nucleotide sequence-target nucleotide sequence-first barcode nucleotide sequence-second nucleotide tag sequence-second barcode nucleotide sequence-3'. In an illustrative four-primer embodiment, two inner primers can include:

a forward, inner primer including a first nucleotide tag, a first barcode nucleotide sequence, and a target-specific portion; and

a reverse, inner primer including a target-specific portion, a first barcode nucleotide sequence, and a second nucleotide tag. Two outer primers can include:

a forward, outer primer including a second barcode nucleotide sequence and a first nucleotide tag-specific portion; and

a reverse, outer primer including a second nucleotide tag-specific portion and a second barcode nucleotide sequence. As discussed above, if the inner and

outer primers are included in the same reaction mixture, the outer primers are preferably present in excess.

[0133] A similar combination of elements may be produced in a six-primer amplification method that employs “stuffer” primers, in addition to inner and outer primers.

5 Thus, for example, two inner primers can include:

- a forward, inner primer including a first nucleotide tag and a target-specific portion; and

- a reverse, inner primer including a target-specific portion and a second nucleotide tag. Two stuffer primers can include:

10 a forward, stuffer primer including a third nucleotide tag, a first barcode nucleotide sequence, and a first nucleotide tag-specific portion; and

- a reverse, stuffer primer including a second nucleotide tag-specific portion, a first barcode nucleotide sequence, a fourth nucleotide tag. Two outer primers can include:

15 a forward, outer primer including a second barcode nucleotide sequence and a third nucleotide tag-specific portion; and

- a reverse, outer primer including a fourth nucleotide tag-specific portion and a second barcode nucleotide sequence. Nucleic acid amplification produces an amplicon including the following elements: 5'-second barcode nucleotide sequence-third nucleotide tag sequence-first barcode nucleotide sequence-first nucleotide tag sequence-target nucleotide sequence-second nucleotide tag sequence-first barcode nucleotide sequence-fourth nucleotide tag sequence-second barcode nucleotide sequence-3'.

Amplification can be carried out in one, two, three amplification reactions. For example, all three primer pairs can be included in one reaction. Alternatively, two reactions can be

25 carried out, e.g., a first reaction including the inner and stuffer primers, and a second reaction including only the outer primers; or a first reaction including only the inner primers, followed by a second reaction including the stuffer and outer primers. Where more than one primer pair is present, the primer pair that is the “outer” pair, relative to the other pair is preferably present in excess, as discussed above. Thus, if the inner and stuffer primers are included in a reaction mixture, the stuffer primers are preferably present in excess, and if the stuffer and outer primers are included in a reaction mixture, the outer primers are preferably present in excess. When all three primer pairs are included in a

30

single reaction, the stuffer primers can be present at a concentration intermediate between that of the inner primers and the outer primers.

[0134] In certain embodiments of the above-described four-primer and six-primer amplification methods, e.g., where the molecules produced in the reaction will be subjected to DNA sequencing, the outer primers can additionally include first and second primer binding sites that are capable of being bound by DNA sequencing primers. For example, a four-primer reaction can produce tagged target nucleotide sequences including 5'-first primer binding site-second barcode nucleotide sequence-first nucleotide tag sequence-first barcode nucleotide sequence-target nucleotide sequence-first barcode nucleotide sequence-second nucleotide tag sequence-second barcode nucleotide sequence-second primer binding site-3'. This embodiment offers the advantage that the barcode combination can be determined in a sequencing read from either end of the molecule. Similarly, a six-primer reaction can produce tagged target nucleotide sequences comprising 5'-first primer binding site-second barcode nucleotide sequence-third nucleotide tag sequence-first barcode nucleotide sequence-first nucleotide tag sequence-target nucleotide sequence-second nucleotide tag sequence-first barcode nucleotide sequence-fourth nucleotide tag sequence-second barcode nucleotide sequence-second primer binding site-3'.

Combinatorial Ligation-Based Tagging

[0135] In certain embodiments, the invention includes a ligation-based method for combinatorial tagging (e.g., barcoding) of a plurality of target nucleotide sequences. The method employs a plurality of tagged target nucleotide sequences derived from target nucleic acids. Each tagged target nucleotide sequences includes an endonuclease site and a first barcode nucleotide sequence. Tagged target nucleotide sequences in the plurality include the same endonuclease site, but N different first barcode nucleotide sequences, wherein N is an integer greater than 1.

[0136] The tagged target nucleotide sequences are cut with an endonuclease specific for the endonuclease site to produce a plurality of sticky-ended, tagged target nucleotide sequences. A plurality of adaptors is then ligated, in a first reaction mixture, to the tagged target nucleotide sequences. The plurality of adaptors includes a second barcode nucleotide sequence and complementary sticky ends to the plurality of sticky-ended, tagged target nucleotide sequences. Furthermore, the plurality of adaptors includes M different second

barcode nucleotide sequences, wherein M is an integer greater than 1. The ligation produces a plurality of combinatorially tagged target nucleotide sequences, each including first and second barcode nucleotide sequences, wherein the plurality includes $N \times M$ different first and second barcode combinations.

5 **[0137]** In certain embodiments, the endonuclease site is adjacent to the first barcode nucleotide sequence in the tagged target nucleotide sequences. In variations of such embodiments, second barcode nucleotide sequence is adjacent to the complementary sticky end in the adaptors. In specific embodiments, the combinatorially tagged target nucleotide sequences, for example, include the first and second barcode nucleotide sequences separated
10 by fewer than 5 nucleotides.

[0138] In particular embodiments, e.g., when the combinatorially tagged target nucleic nucleotide sequences are intended for sequencing, the tagged target nucleotide sequences can include first and second primer binding site, which can have either of the following arrangements: 5'-endonuclease site-first barcode nucleotide sequence-first
15 primer binding site-target nucleotide sequence-second primer binding site; and 5'-first primer binding site-target nucleotide sequence-second primer binding site-first barcode nucleotide sequence-endonuclease site-3'. To facilitate sequencing, the first and second primer binding sites can be binding sites for DNA sequencing primers. In variations of such embodiments, the combinatorially tagged nucleic can include the second barcode nucleotide
20 sequence in one of the following arrangements: 5'-second barcode nucleotide sequence-first barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site; or 5'-first primer binding site-target nucleotide sequence-second primer binding site-first barcode nucleotide sequence- second barcode nucleotide sequence -
3'.

25 **[0139]** Tagged target nucleotide sequences useful in this method can be prepared by any convenient means, such as, for example, by ligating adaptors onto a plurality of target nucleic acids, wherein the adaptors include: a first adaptor including the endonuclease site, the first barcode nucleotide sequence, the first primer binding site, and a sticky end; and a second adaptor including a second primer binding site and a sticky end.

30 **[0140]** In some embodiments, it is advantageous to include one or more additional nucleotide sequences in the tagged target nucleotide sequences, e.g., to facilitate handling

and/or identification. Thus, the tagged target nucleotide sequences can include a first additional nucleotide sequence having an arrangement selected from: 5'-endonuclease site-first barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site- first additional nucleotide sequence; and/or 5'- first additional nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-first barcode nucleotide sequence-endonuclease site-3'. For example, in Illumina sequencing, flow cell binding sequences (e.g., PE1 and PE2) are incorporated at either end of a DNA template to be sequenced. In the present method, the tagged target nucleotide sequences can include one flow cell binding sequence as the first additional nucleotide sequence, and the other flow cell binding sequence can be introduced via an adaptor. See, e.g., Figure 5A-B. Thus, the present method can employ adaptors that include a second additional nucleotide sequence and have the arrangement: 5'-second additional nucleotide sequence-second barcode nucleotide sequence-complementary sticky end-3'. In this case, ligation of the adaptors to the above-described tagged target nucleotide sequences containing a first additional nucleotide sequence produces combinatorially tagged target nucleotide sequences including: 5'-second additional nucleotide sequence-second barcode nucleotide sequence-first barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-first additional nucleotide sequence; and/or 5'- second additional nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-first barcode nucleotide sequence- second barcode nucleotide sequence-first additional nucleotide sequence -3'. In variations of this embodiment, the first and/or second additional nucleotide sequences include a primer binding site.

[0141] Tagged target nucleotide sequences that contain a first additional nucleotide sequence can be prepared by any convenient means, such as, for example, by ligating adaptors onto a plurality of target nucleic acids, wherein the adaptors include: a first adaptor including the endonuclease site, the first barcode nucleotide sequence, the first primer binding site, and a sticky end; and a second adaptor including a first additional nucleotide sequence, a second primer binding site and a sticky end.

Combinatorial Insertional Mutagenesis-Based Tagging

[0142] Combinatorial tagging can also be carried out using insertional mutagenesis. In certain embodiments, combinatorial tagging of a plurality of target nucleotide sequences is carried out by annealing a plurality of barcode primers to a plurality of tagged target
5 nucleotide sequences derived from target nucleic acids, and then amplifying the tagged target nucleotide sequences in a first reaction mixture to produce a plurality of combinatorially tagged target nucleotide sequences, each including first and second barcode nucleotide sequences, wherein the plurality includes $N \times M$ different first and second barcode combinations.

10 [0143] In particular embodiments, each tagged target nucleotide sequence includes a nucleotide tag at one end and a first barcode nucleotide sequence, wherein tagged target nucleotide sequences in the plurality include the same nucleotide tag, but N different first barcode nucleotide sequences, wherein N is an integer greater than one. In variations of such embodiments, the first barcode nucleotide sequence is separated from the nucleotide
15 tag by the target nucleotide sequence. Each barcode primer includes: a first tag-specific portion linked to a second barcode nucleotide sequence, which is itself linked to a second tag-specific portion, wherein the barcode primers in the plurality each include the same first and second tag-specific portions, but M different second barcode nucleotide sequences, wherein M is an integer greater than one. The first tag-specific portion of the barcode
20 primer anneals to a 5' portion of the nucleotide tag, and the second tag-specific portion of the barcode primer anneals to an adjacent 3' portion of the nucleotide tag, and the second barcode nucleotide sequence does not anneal to the nucleotide tag, forming a loop between the annealed first and second tag-specific portions.

[0144] In particular embodiments, useful e.g. in DNA sequencing, the tagged
25 nucleotide sequences additionally include a primer binding site between the target nucleotide sequence and the first barcode nucleotide sequence. In variations of such embodiments, the first and second tag-specific portions of the barcode primer are sufficiently long to serve as primer binding sites. To facilitate sequencing one or more, or preferably all, of these binding sites are binding sites for DNA sequencing primers. In such
30 embodiments, the combinatorially tagged target nucleotide sequences can include 5'-first

tag-specific portion-second barcode nucleotide sequence-second tag-specific portion-target nucleotide sequence-primer binding site-first barcode nucleotide sequence-3'.

[0145] In some embodiments, it is advantageous to include one or more additional nucleotide sequences in the tagged target nucleotide sequences, e.g., to facilitate handling and/or identification. Thus, the tagged target nucleotide sequences can include a first additional nucleotide sequence having the arrangement: 5'-nucleotide tag-target nucleotide sequence-primer binding site-first barcode nucleotide sequence-first additional nucleotide sequence-3'. For example, in Illumina sequencing, flow cell binding sequences (e.g., PE1 and PE2) are incorporated at either end of a DNA template to be sequenced. In the present method, the tagged target nucleotide sequences can include one flow cell binding sequence as the first additional nucleotide sequence, and the other flow cell binding sequence can be introduced via the barcode primers. See, e.g., Figure 6. Thus, the present method can employ bar code primers that include a second additional nucleotide sequence and have the arrangement: 5'-second additional nucleotide sequence-first tag-specific portion-second barcode nucleotide sequence-second tag-specific portion-3'. In this case, amplification produces combinatorially tagged target nucleotide sequences that include 5'- second additional nucleotide sequence-first tag-specific portion-second barcode nucleotide sequence-second tag-specific portion-target nucleotide sequence-primer binding site-first barcode nucleotide sequence-first additional nucleotide sequence-3'. In variations of this embodiment, the first and/or second additional nucleotide sequences include a primer binding site.

[0146] The target nucleotide sequences can be tagged by any convenient means, including the primer-based methods described herein. In certain embodiments, the nucleotide tag includes a transposon end, which is incorporated into the tagged target nucleotide sequences using a transposase.

Reactions to Incorporate Nucleic Acid Sequences

[0147] Any method can be employed to incorporate nucleic acids sequences into target nucleic acids. In illustrative embodiments, PCR is employed. When using three or more primers, the amplification is generally carried out for at least three cycles to incorporate 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
5 falling within a range having any of these values as endpoints).

[0148] 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
10 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.

15 Applications

[0149] 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
20 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
25 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
30 different tag-specific portions. In either case, the amplification produces a set of T amplicons from each sample that bear sample-specific barcodes.

[0150] 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.

[0151] In certain embodiments, target amplicons produced in any of the methods described herein 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 compartment (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. In certain embodiments, the amount of target amplicons produced in the amplification mixtures can be quantified during amplification, e.g., by quantitative real-time PCR, or after.

[0152] In embodiments that are useful in single-particle analysis, combinatorial barcoding can be used to encode the identity of a reaction volume, and thus particle, that was the source of an amplification product. In specific embodiments, nucleic acid amplification is carried out using at least two barcode sequences, and the combination of barcode sequences encodes the identity of the reaction volume that was the source of the reaction product (termed “combinatorial barcoding”). These embodiments are conveniently employed when the separate reaction volumes are in separate compartments of a matrix-type microfluidic device, e.g., like those available from Fluidigm Corp. (South San Francisco, CA) and described below (see “Microfluidic Devices”). Each separate compartment can contain a combination of barcode nucleotide sequences that identifies the row and column of the compartment in which the encoding reaction was carried out. If the reaction volumes are recovered and subjected to further analysis that includes detection of the barcode combination (e.g., by DNA sequencing), the results can be associated with a particular compartment and, thereby, with a particular particle in the compartment. Such

embodiments are particularly useful when separate reaction volumes are combined during or after the recovery process, such that reaction products from a plurality of separate reaction volumes are combined (“pooled”). In a matrix-type microfluidic device, for example, reaction products from all compartments in a row, all compartments in a column, or all compartments in the device could be pooled. If all compartments in a row are pooled, each column within a row preferably has a unique barcode combination. If all compartments in a column are pooled, each row within a column has a unique barcode combination. If all compartments with a device are pooled, every compartment within the device has a unique barcode combination.

10 **Barcoding and Pooling of Reaction Mixtures for Subsequent Analysis**

[0153] In other embodiments, a barcoding and pooling strategy is used to detect a plurality of target nucleic acids in individual reaction mixtures, which can, for example, contain individual particles, such as cells. This strategy is described for single-cell analysis of gene expression in Example 7, below.

15 [0154] In one embodiment, the method entails preparing M first reaction mixtures that will be pooled prior to assay, wherein M is an integer greater than 1. Each reaction mixture includes sample nucleic acid(s); a first, forward primer comprising a target-specific portion; and a first, reverse primer comprising a target-specific portion. The first, forward primer or the first, reverse primer can additionally include a barcode nucleotide sequence, wherein each barcode nucleotide sequence in each of the M reaction mixtures is different. Alternatively, the first, forward primer or the first, reverse primer additionally includes a nucleotide tag, and each reaction mixture additionally includes at least one barcode primer including a barcode nucleotide sequence and a nucleotide tag-specific portion, wherein each barcode nucleotide sequence in each of the M reaction mixtures is different. In this
25 embodiment, the barcode primer is generally in excess of the first, forward and/or first, reverse primer(s). Each first reaction mixture is subjected to a first reaction to produce a plurality of barcoded target nucleotide sequences, each comprising a target nucleotide sequence linked to a barcode nucleotide sequence. The barcoded target nucleotide sequences for each of the M first reaction mixtures are pooled to form an assay pool.
30 Within this assay pool, a particular target nucleotide sequence from a particular reaction mixture is uniquely identified by a particular barcode nucleotide sequence. The assay pool,

or one or more aliquots thereof, is subjected to a second reaction using unique pairs of second primers, wherein each second primer pair includes a second, forward or a reverse primer that anneals to a target nucleotide sequence; and a second, reverse or a forward primer, respectively, that anneals to a barcode nucleotide sequence. The method includes
5 determining whether a reaction product is present in the assay pool, or aliquot thereof for each unique, second primer pair. For each unique, second primer pair, the presence of a reaction product indicates the presence of a particular target nucleic acid in a particular first reaction mixture.

[0155] In certain embodiments, the method entails preparing $M \times N$ first reaction
10 mixtures, wherein N is an integer greater than 1, and each first reaction mixture includes a pair of first, forward and reverse primers that is specific for a different target nucleic acid. After the first reaction, N assay pools are prepared, each including M first reaction mixtures, wherein each barcoded target nucleotide sequence in an assay pool includes a different barcode nucleotide sequence. The second reaction is carried out in each of the N assay
15 pools, with each assay pool being separate from every other assay pool.

[0156] For the first reaction, any reaction capable of producing target nucleotide sequences linked to a barcode nucleotide sequences can be carried out. Convenient first reactions include amplification and ligation.

[0157] The second reaction can be any reaction that relies on primer-based detection
20 of barcoded target nucleotide sequences. Methods that include amplification and/or ligation steps, including any of those described herein and/or known in the art can be used. For example, the presence of reaction products can be detected using polymerase chain reaction (PCR) or ligase chain reaction (LCR). In some embodiments, real-time detection is employed.

[0158] An illustrative second reaction can employ LCR to detect barcoded target
25 nucleotide sequences having the structure: 5'-forward primer sequence-target nucleotide sequence-reverse primer sequence-barcode nucleotide sequence-3'. In this case, one primer can anneal to the reverse primer sequence, and the other primer can anneal to the adjacent barcode nucleotide sequence, which is followed by ligation and repeated cycles of annealing
30 and ligation. The reverse primer sequence provides target information, and the barcode

nucleotide sequence identifies the pool (which could, for example, represent a pool of all target amplified in a particular sample). See Figure 8A.

[0159] An illustrative second reaction can include real time detection, e.g., using a flap endonuclease-ligase chain reaction. This reaction employs a labeled probe and an unlabeled probe, wherein the simultaneous hybridization of the probes to a reaction product results in the formation of a flap at the 5' end of the labeled probe, and cleavage of the flap produces a signal. For example, cleavage of the flap can separate a fluorophore from a quencher to generate a signal. An illustrative embodiment can be employed the detect reaction products having the structure: 5'-forward primer sequence-target nucleotide sequence-reverse primer sequence-barcode nucleotide sequence-3'. In this case, the reaction can employ an unlabeled probe that anneals to the reverse primer sequence and a labeled probe that anneals to the adjacent barcode nucleotide sequence. Annealing of the 3' end of the unlabeled probe prevents annealing of the 5' end of the labeled probe, forming a flap. This 5' flap portion can be labeled with a fluorophore, and the portion that anneals to the barcode nucleotide sequence can bear a quencher, so that cleavage of the flap by an enzyme such as 5' flap endonuclease releases the flap, whereby the quencher can no longer quench the fluorophore. See Figure 8B.

[0160] An alternative real time detection method that is useful, e.g., for detecting amplicons produced by LCR, relies on using a double-stranded DNA-binding dye to detect melting temperature differences between the reactions products and the primers employed for the LCR. The melting temperature analysis includes detection at a temperature at which reaction products are substantially double-stranded and capable of producing signal in the presence of a double-stranded DNA-binding dye, but primers are substantially single-stranded and incapable of producing signal. For example, to detect barcoded target nucleotide sequences having the structure: 5'-forward primer sequence-target nucleotide sequence-reverse primer sequence-barcode nucleotide sequence-3', one primer can anneal to the reverse primer sequence, and the other primer can anneal to the adjacent barcode nucleotide sequence, which is followed by ligation and repeated cycles of annealing and ligation. See Figure 8C. The length of the ligated primer sequences, e.g., R_1 plus BC_1 and its complement, is sufficiently longer than the length of R_1 or BC_1 and their complements so that at high temperature, the ligated primer sequences are substantially double-stranded (i.e. producing a signal), whereas the unligated primer sequences are substantially single-

stranded (i.e., no producing a signal). In various embodiments, at least about 10, 20, 30, 40, 50, 60, 70, 80, or 90 percent of the unligated primers are single-stranded. In each of these embodiments, the percentage of ligated primers that are double-stranded can be at least about 10, 20, 30, 40, 50, 60, 70, 80, or 90 percent.

5 **[0161]** In certain embodiments, the first reaction mixtures are prepared in separate compartments of a microfluidic device, the separate compartments being arranged as an array defined by rows and columns, e.g., like those available from Fluidigm Corp. (South San Francisco, CA) and described below (see “Microfluidic Devices”). For example, a matrix-type microfluidic device that is adapted to permit recovery of the contents of
10 reaction compartments (see below) can be employed for the first reaction. This approach is particularly convenient for preparing N assay pools, each including M first reaction mixtures. More specifically, the first reactions are carried out in separate compartments of a microfluidic device, wherein the separate compartments are arranged as an array defined by rows and columns. Each of the N assay pools is obtained by pooling the first
15 reaction mixtures in a row or a column of the device. The barcode nucleotide sequence in each barcoded target nucleotide sequence, taken with the identity of the assay pool, identifies the row and column of the compartment that was the source of the barcoded target nucleotide sequence. In particular embodiments, the second reaction mixtures are prepared in separate compartments of a microfluidic device, having separate compartments arranged
20 as an array defined by rows and columns. For example, the first reaction mixtures can be prepared in separate compartments of a first microfluidic device to incorporate the barcode nucleotide sequences (e.g., Fluidigm Corporation’s ACCESS ARRAY™ IFC (Integrated Fluidic Circuit) or MA006 IFC), and the second reaction mixtures can be prepared in separate compartments of a second, different microfluidic device, e.g., to facilitate detection (e.g.,
25 one of Fluidigm Corporation’s DYNAMIC ARRAY™ IFCs, using PCR or RT-PCR, with a double-stranded DNA binding dye, such as EvaGreen for detection).

[0162] In particular embodiments, at least one of the first and/or second reactions is performed in individual particles, such as cells. Particle capture and assay can be carried out as described below or as known in the art. Fluidigm Corporation’s MA006 IFC is well-
30 suited for this purpose. The particles may be substantially intact when subjected to the first and/or second reactions, provided the necessary reagents will come into contact with the target nucleic acids of interest. Alternatively, the particles may be disrupted prior to the first

or second reaction to facilitate barcoding and/or subsequent analysis. In some embodiments, the particles are treated with an agent that elicits biological response prior to performing the plurality of first reactions.

Subsequent Analysis

5 **[0163]** Any of the above-described methods of incorporating nucleic acid sequences into target nucleic acids (including the barcoding and pooling method described above) can be include any of a number of analytical steps, such as determining the amount of at least one target nucleic acid in the first reaction mixtures or determining the copy number(s) of one or more DNA molecule(s) in the first reaction mixtures. In certain embodiments in
10 which tagged or barcoded target nucleotide sequences are produced by PCR, e.g., those in which copy number determinations are being made, it is advantageous to conduct fewer than 20 cycles of PCR to preserve the relative copy numbers of different target nucleotide sequences.

15 **[0164]** Any of the above-described methods can include determining the genotype(s) at one or more loci in the first reaction mixtures and/or determining a haplotype for a plurality of loci in the first reaction mixtures. Haplotype determinations can, for example, be carried out by condensing chromosomes and distributing chromosomes into first reaction mixtures to produce a plurality of first reaction mixtures that include a single chromosome. This distribution can be carried out, e.g., as described below with respect to
20 single particle analysis (in this case, the “particle” under analysis is a chromosome). A plurality of loci in the first reaction mixtures, and therefore necessarily on the same chromosome, can be sequenced to provide a haplotype for those loci.

25 **[0165]** In any of the above-described methods, e.g., where RT-PCR is carried out, the expression of levels of one or more RNA molecule(s) in the first reaction mixtures can be determined. As for DNA copy number determinations, it is advantageous to conduct fewer than 20 cycles of PCR to preserve the relative copy numbers of differences.

[0166] Regardless of whether the target nucleic acids in the first reaction mixtures are DNA or RNA, subsequent analysis can include determining the sequence of the target nucleotide sequences generated therefrom.

[0167] In some embodiments, the methods described herein include performing a plurality of reactions in each first reaction mixture, wherein one of the plurality of reactions includes amplification to produce a tagged or barcoded target nucleotide sequence, analyzing the results of the plurality of reactions, and associating the results of the analysis with each first reaction mixture. This association can be facilitated by the tagging or barcoding of target nucleotide sequences as alluded to above. For example, combinatorial barcoding can be used to encode information about the source reaction mixture. Alternatively, a combination of primer sequence and barcode can encode this information as discussed above with respect the barcoding and pooling method.

10 **Bidirectional Nucleic Acid Sequencing**

[0168] In particular embodiments, the invention provides methods for preparing nucleic acids for bidirectional DNA sequencing, which facilitates the sequencing of both ends of amplification products in a single read sequencing run. Such methods are illustrated in Example 9.

15 [0169] The DNA to be sequenced can be any type of DNA. In particular embodiments, the DNA is genomic DNA or cDNA from an organism. In some embodiments, the DNA can be fragmented DNA. The DNA to be sequenced can be a representation of the RNA in a sample, where the DNA is obtained, e.g., by reverse transcription or amplification of RNA. In certain embodiments, the DNA can be a DNA
20 library.

[0170] To prepare nucleic acids for bidirectional DNA sequencing according to the methods described herein, each target nucleic acid to be sequenced is amplified using a set of inner primers, wherein the set includes:

an inner, forward primer including a target-specific portion and a first
25 primer binding site;

an inner, reverse primer including a target-specific portion and a second primer binding site, wherein the first and second primer binding sites are different. These first and second primer binding sites serve the dual function of acting as nucleotide tags that facilitate the addition of further nucleotide sequences (as described below) and, in
30 certain embodiments, as primer binding sites to which DNA sequencing primers can anneal.

In the specific embodiment of Example 9, the first and second primer binding sites are designated as “CS1” and “CS2” for “Common Sequence tag 1” and “Common Sequence tag 2.” In this embodiment, the target-specific portions of the inner primers are designated “TS-F” for “Target-Specific Forward” and “TS-R” for “Target-Specific Reverse.”

5 [0171] Upon amplification, the target nucleotide sequences become tagged with first and second primer binding sites. These tagged target nucleotide sequences are annealed to two sets of outer primers that anneal to the first and second primer binding sites. The two sets of outer primers include:

a first set of outer primers, wherein the set includes:

10 a first outer, forward primer including a portion specific for the first primer binding site; and

a first outer, reverse primer including a barcode nucleotide sequence and a portion specific for the second primer binding site;

a second set of outer primers, wherein the set includes:

15 a second outer, forward primer including a barcode nucleotide sequence and a portion specific for the first primer binding site; and

a second outer, reverse primer including a portion specific for the second primer binding site. Amplification then produces two target amplicons, namely:

20 a first target amplicon that includes 5'-first primer binding site-target nucleotide sequence-second primer binding site-barcode nucleotide sequence-3'; and

a second target amplicon that includes 5'-barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-3'.

In particular embodiments, the barcode nucleotide sequence in each of the two target amplicons is the same, and each target amplicon includes only one barcode nucleotide

25 sequence. In some embodiments, where more than one target nucleic acid is amplified, each pair of target amplicons produced can have the same barcode sequence, but different pairs can have different barcode sequences. In this case, the barcode sequences would differ between different target amplicons produced from different target nucleic acids. As discussed above, sets of different target nucleic acids, e.g., from a particular biological
30 sample can be barcoded with the same set-specific sequence (i.e., one that differs between sets). In a specific embodiment, the set-specific barcode can be a sample-specific barcode, i.e., one that identifies the sample from which the target amplicons were derived.

[0172] In certain embodiments, the outer primers each additionally include an additional nucleotide sequence, wherein:

the first outer, forward primer includes a first additional nucleotide sequence, and the first outer, reverse primer includes a second additional nucleotide sequence; and

the second outer, forward primer includes the second additional nucleotide sequence, and the second outer, reverse primer includes the first additional nucleotide sequence, and the first and second additional nucleotide sequences are different. In such embodiments, the outer primer amplification produces two target amplicons, namely:

a first target amplicon that includes 5'-first additional nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-barcode nucleotide sequence-second additional nucleotide sequence-3'; and

a second target amplicon that includes 5'-second additional nucleotide sequence-barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-first additional nucleotide sequence 3'. (Those of skill in the art understand that amplicons described in this manner herein are described in terms of one strand and that the complementary strand will have the 5' to 3' order of these nucleotide sequences reversed.)

[0173] The first and/or second additional nucleotide sequences can also include a primer binding site. An illustrative primer configuration of this type described in Example 9, wherein the additional nucleotide sequences are designated "PE-1" and "PE-2." These sequences are adaptor sequences used by the Genome Analyzer (commercially available from Illumina, Inc., San Diego, CA). The barcode nucleotide sequence is designated "BC." Outer primer amplification using these primers produces two target amplicons, namely:

a first target amplicon that includes 5'-PE1-CS1-target nucleotide sequence-CS2-BC-PE2-3'; and

a second target amplicon that includes 5'-PE2-BC-CS1-target nucleotide sequence-CS2-PE1-3'. In a specific, illustrative embodiment, the first set of outer primers, PE1-CS1 and PE2-BC-CS2, and the second set of outer primers, PE1-CS2 and PE2-BC-CS1, have the nucleotide sequences shown in Table 1 in Example 9.

[0174] The inner and outer primer amplifications can be carried out in a single amplification reaction. Alternatively, the inner primer amplification can be carried out in a first amplification reaction, and the outer primer amplification can be carried out in a second, amplification reaction that is separate from the first. In certain embodiments, the second amplification reaction can be carried out in two separate second amplification reactions: one that employs the first set of outer primers and another employs the second set of outer primers. See Example 9, Figure 2. In such embodiments, the target amplicons produced in each separate second amplification reaction can be pooled for further analysis, such as DNA sequencing.

[0175] In many embodiments, the methods described above will be carried out on a plurality of target nucleic acids, such as, e.g., a DNA library. In this case, the methods can be used to produce a pool of target amplicons that includes two types of amplicons (described above and illustrated in Example 9, Figure 2, as “A” and “B”) for each target nucleic acid. One type of target amplicon (“A”) facilitates sequencing of the 5’ end of the target nucleic acid, and the other type of target amplicon (“B”) facilitates sequencing of the 3’ end of the target nucleic acid. In addition, each target amplicon includes a barcode sequence, which, in certain embodiments, is the same in each of the two types of target amplicons. The barcode nucleotide sequence can encode information about the target nucleotide sequence, such as the identity of the reaction that produced it and/or the identity of the sample from which the target nucleic acid was derived. As described in more detail below, the target nucleotide sequence and the barcode nucleotide sequence in each target amplicon can readily be determined using any suitable available DNA sequencing method. In particular embodiments, the DNA sequencing method is a high-throughput sequencing method, such as the bridge amplification (cluster generation) and sequencing method commercialized by Illumina, Inc., San Diego, CA. In certain embodiments, e.g., those employing bridge amplification and sequencing, the average length of the target amplicons is less than 200 bases, less than 150 bases, or less than 100 bases.

[0176] In bridge amplification and sequencing, target amplicons, e.g., produced as described herein are hybridized to a lawn of immobilized primer pairs via the first and second additional nucleotide sequences (e.g., PE1 and PE2). One immobilized primer in each primer pair is cleavable. First strand synthesis is carried out to produce double-stranded molecules. These are denatured, and the original hybridized target amplicon strand

that served as the template for first strand synthesis is washed away, leaving immobilized first strands. These can flip over and hybridize to a suitable adjacent primer, forming a bridge. Second strand synthesis is carried out to produce double-stranded bridges. These are denatured, and each bridge yields two immobilized single-stranded molecules that can
5 once again hybridize to suitable immobilized primers. Isothermal bridge amplification is carried out to produce multiple double-stranded bridges. Double-stranded bridges are denatured, and “reverse” strands are cleaved and washed away, leaving clusters of immobilized “forward” strands available as a template for DNA sequencing.

[0177] When target amplicons produced as described herein are subjected to bridge
10 amplification and sequencing, primers that anneal to the first and second primer binding sites (e.g., CS1 and CS2) can be employed to sequence either the target nucleotide sequence or the barcode nucleotide sequence, both of which are present in the immobilized template produced from the amplicon. In certain embodiments, a pair of primers suitable for sequencing the target nucleotide sequence is contacted with the immobilized templates
15 under conditions suitable for annealing, followed by DNA sequencing. After these sequences have been read, the sequencing products can be denatured and washed away. The immobilized templates can then be contacted with a pair of primers suitable for sequencing the barcode nucleotide sequence under conditions suitable for annealing, followed by DNA sequencing. The order of these sequencing reactions is non-critical and
20 can be reversed (i.e., the barcode nucleotide sequences can be sequenced first, followed by sequencing of the target nucleotide sequences). See Example 9, Figure 3. In certain embodiments, primers that prime sequencing of the barcode nucleotide sequence are reverse complements of the primers that prime sequencing of the target nucleotide sequences. In a specific, illustrative embodiment, the primers employed to prime sequencing of the target
25 nucleotide sequence(s) and barcode nucleotide sequence(s) are CS1, CS2, CS1rc, and CS2rc (Table 2, Example 9).

[0178] Conveniently, both types of target amplicons are subjected to bridge
amplification and sequencing in the same reaction(s) to allow for simultaneous sequencing of the templates from each type of target amplicon. See Example 9, Figure 3. This allows
30 for the simultaneous sequencing of each target nucleotide sequence from the 5' end (e.g., by sequencing templates from amplicon type A in Example 9, Figure 3) and from the 3' end (e.g., by sequencing templates from amplicon type B in Example 9, Figure 3). In particular

embodiments, the primers that bind to the first and second primer binding sites and prime sequencing of the target nucleotide sequence(s) are present in substantially equal concentrations so as to produce both 5' and 3' DNA sequence information from each target nucleotide sequence. Similarly, in certain embodiments, the primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequence(s) are present in substantially equal concentrations so as to produce barcode sequences from each template type (i.e., derived from amplicon type A or amplicon type B in Example 9, Figure 3).

[0179] When the inner amplification is performed as a separate reaction, especially when amplifying a plurality of target nucleic acids, it may be convenient to perform individual reactions (e.g., with 1, 2, 3, 4, 5 or more target nucleic acids amplified per reaction) in separate compartments of a microfluidic device, such as any of those described herein or known in the art. As discussed below, suitable microfluidic devices can be fabricated, at least in part, from an elastomeric material.

[0180] In particular embodiments, the inner or (inner and outer) amplification(s) is/are carried out in a microfluidic device designed to facilitate recovery of amplification products after the amplification reaction has been carried out, such as the ACCESS ARRAY™ IFC described herein (See Figures 2-9) and available from Fluidigm, Inc., South San Francisco, CA. In illustrative devices of this type, dilation pumping can be 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.

[0181] Those of skill in the art will be aware of other devices and strategies that can be employed to perform the inner (or inner and outer) amplification(s) described herein on a plurality of different target nucleic acids, each in separate reactions. For example, droplet-based amplification is well-suited to performing this inner amplification. See, e.g., U.S. Patent No., 7,294,503, issued November 13, 2007 to Quake et al., entitled "Microfabricated crossflow devices and methods," which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets; U.S. Patent Publication No. 20100022414, published January 28, 2010, by Link et al., entitled "Droplet libraries," which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets; and U.S. Patent Publication No. 20110000560, published January 6, 2011, by Miller et al., entitled "Manipulation of Microfluidic Droplets," which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets. In particular embodiments, inner amplification is carried out in fluid droplets in an emulsion.

Encoding and Detecting/Quantifying Alleles by Primer Extension

[0182] Nucleic acid encoding can be employed in a method for detecting and estimating the fraction of particular target nucleic acids (e.g., rare mutations) in a nucleic acid sample. This method entails producing first and second tagged target nucleotide sequences from first and second target nucleic acids in the sample. For example, the method can be carried out by using allele-specific amplification to introduce allele-specific nucleotide tags into the resultant tagged target nucleotide sequences. The tagged target nucleotide sequences are then subjected to primer extension reactions using primers specific for each nucleotide tag. The method entails detecting and/or quantifying a signal that indicates extension of the first primer and a signal that indicates extension of the second primer. The signal for a given primer indicates the presence, and/or relative amount, of the corresponding target nucleic acid. This method can be conveniently carried out on a high-throughput (e.g., next-generation) DNA sequencing platform to detect, e.g., known

mutations in a sample by detecting the presence of tags, rather than by determining the DNA sequence of each molecule. The advantages of this method are speed, sensitivity, and precision. The large number of clonal molecules examined in next-generation sequencing allows reliable detection of very rare sequences (e.g., less than 1 in 10^6 sequences).

5 Furthermore, the fraction of target sequence(s) (e.g., mutations) can be determined more precisely than with PCR, as next-generation sequencing platforms are available with very high numbers of reads.

[0183] To facilitate primer extension on a DNA sequencing platform, adaptors for, e.g., high-throughput DNA sequencing can be introduced into the first and second tagged
10 target nucleotide sequences. In particular embodiments, the adaptors are introduced at each end of the tagged target nucleotide sequence molecule. These adaptors can conveniently be introduced, together with the nucleotide tags, in one reaction.

[0184] Nucleotide tags and/or DNA sequencing adaptors can be introduced into the target nucleotide sequences using any suitable method, such as, e.g., amplification or
15 ligation. For example, first and second tagged target nucleotide sequences can be produced by amplifying first and second target nucleic acids with first and second primer pairs, respectively. At least one primer in the first primer pair comprises a first nucleotide tag and at least one primer in the second primer pair comprises a second nucleotide tag. When
20 introducing DNA sequencing adaptors in the same reaction, one primer in each primer pair comprises 5'-(DNA sequencing adaptor)-(nucleotide tag)-(target-specific portion)-3' and the other primer in each primer pair comprises 5'-(DNA sequencing adaptor)-(target-specific portion)-3'.

[0185] Many high-throughput DNA sequencing techniques include an amplification step prior to DNA sequencing. Accordingly, in some embodiments, the tagged target
25 nucleotide sequences are further amplified prior to primer extension on a DNA sequencing platform. For example, emulsion amplification or bridge amplification can be carried out. 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.
30 emPCR is used in the methods by Marguilis et al. (commercialized by 454 Life Sciences, Branford, CT), Shendure and Porreca et al. (referred to herein as "454 sequencing;" also

known as “polony sequencing”) and SOLiD sequencing, (Life Technologies, 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.

[0186] 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. “Pyrosequencing” 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. 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 detecting fluorescence at each position in real time, by repeated removal of the blocking group to allow polymerization of another nucleotide.

[0187] In one embodiment of the detection-by-primer extension method, which can conveniently be carried out on the 454 sequencing platform, the first and second primer extension reactions are carried out sequentially in at least two cycles of primer extension. In particular, a first cycle of primer extension is carried out using the first primer that anneals to the first nucleotide tag, and a second cycle of primer extension is carried out using the second primer that anneals to the second nucleotide tag. All deoxynucleoside triphosphates (dNTPs) are provided in each cycle of primer extension. The incorporation of any dNTP into a DNA molecule produces a detectable signal. The signal detected in the first cycle indicates the presence of the first target nucleic acid in the nucleic acid sample,

whereas the signal detected in the second cycle indicates the presence of the second target nucleic acid in the nucleic acid sample. Thus, each target nucleic acid (e.g., mutation) can be detected with only a single cycle of the sequencing platform.

5 [0188] Because the signal detected is proportional to the number of copies of target nucleic acid, the signal can also be used to estimate the amount of the target nucleic acid in the sample. In particular, the signal can be used to determine the amounts of the two or more target nucleic acids relative to one another.

10 [0189] In an illustrative embodiment that uses the 454 sequencing platform to detect wild-type and mutant target nucleic acids, allele-specific PCR reactions are prepared with specific tags for wild-type and each mutant to be detected. As shown in Fig. 31, the forward primers have 454 adapters and allele-specific tags (indicated with different shadings). The adapters are 5' of the tags, which are 5' of the allele-specific portion of the primer. The reverse primer includes a 454 adaptor 5' of the target-specific portion. As shown in Fig. 31, only one reverse primer is needed to detect a single nucleotide polymorphism. In this example, the two allele-specific PCR reactions are carried out in a single PCR reaction, although this is not a requirement of the method. The PCR reaction produces tagged target nucleotide sequences that are ready for 454 bead emulsion PCR. The emulsion PCR step can be omitted, for example, by annealing the tagged target nucleotide sequences directly to beads preloaded with allele-specific oligonucleotides (i.e.,
20 each individual bead bears only one type of oligonucleotide). In either case, an individual bead will bear only one type of tagged target nucleotide. The beads are loaded onto a 454 sequencer. The first 454 cycle flows a primer that binds, e.g., to the wild-type tag and all four dNTPs. As this primer extends, multiple nucleotides are incorporated, giving a very robust signal, but only in wells containing wild-type beads. The second 454 cycle flows a
25 primer that binds to the mutant tag and all four dNTPs, giving a signal only in wells containing mutant beads.

30 [0190] In another embodiment of the detection-by-primer extension method, which can conveniently be carried out on the SOLiD sequencing platform, the first and second primer extension reactions are carried out by oligonucleotide ligation and detection. In this embodiment, the ligation of a labeled di-base oligonucleotide to the first and/or second primer(s) produces a detectable signal, and the total signal detected for a particular primer

indicates the presence, and/or relative amount of, the corresponding target nucleic acid in the nucleic acid sample. In a variation of this embodiment, the ligation of a labeled di-base oligonucleotide to the first primer produces the same detectable signal as the ligation of a labeled di-base oligonucleotide to the second primer, and the first and second primer extension reactions are carried out separately, e.g., in simultaneous or sequential cycles. In another variation, the ligation of a labeled di-base oligonucleotide to the first primer produces a different detectable signal than the ligation of a labeled di-base oligonucleotide to the second primer. The use of different signals allows the first and second primer extension reactions to be carried out simultaneously, in one reaction mixture. Any type of detectable signal can be employed in the method, but a fluorescent signal is typically employed, e.g., for SOLiD sequencing.

[0191] Tagged target nucleotide sequences containing, e.g., allele-specific tags and suitable DNA sequencing adaptors are prepared for primer extension on a SOLiD sequencing platform as described above. Emulsion PCR can be carried out, although this step is not strictly necessary. As described above with respect to 454 sequencing, any method that produces clonal populations of tagged target nucleotide sequences attached to beads may be employed to produce tagged target nucleotide sequences suitable for primer extension on a SOLiD sequencing platform.

[0192] In yet another embodiment of the detection-by-primer extension method, which can conveniently be carried out on the Illumina sequencing platform, the first and second primer extension reactions include sequencing-by-synthesis. In this embodiment, each deoxynucleoside triphosphate is labeled with a distinct, base-specific label, and the incorporation of a deoxynucleoside triphosphate into a DNA molecule produces a base-specific detectable signal. The total signal detected for a particular primer indicates the presence and/or relative amount of the corresponding target nucleic acid in the nucleic acid sample. In a variation of this embodiment, the extension of the first primer produces the same detectable signal as the extension of the second primer, and the first and second primer extension reactions are carried out separately, e.g., in simultaneous or sequential cycles. In another variation, the extension of the first primer produces a different detectable signal than the extension of the second primer. The use of different signals allows the first and second primer extension reactions to be carried out simultaneously, in one reaction mixture. Any type of detectable signal can be employed in the method, but a fluorescent

signal is typically employed, e.g., for Illumina sequencing. Tagged target nucleotide sequences containing allele-specific tags and suitable DNA sequencing adaptors can be prepared for primer extension on an Illumina sequencing platform as described above. For primer extension on an Illumina sequencing platform, the tagged target nucleotide sequences are typically further amplified by bridge PCR prior to DNA sequencing.

[0193] In the specific detection-by-primer extension embodiments described above, as well as in some other implementations of the method, amplification produces clonal populations of tagged target nucleotide sequences that are, or become, located at discrete reaction sites. The number of reaction sites including the first nucleotide tag relative to the number of reaction sites including the second nucleotide tag indicates the amount of the first target nucleic acid relative to the second target nucleic acid in the sample. In particular embodiments of this type, the method can entail detecting and comparing the total signal from all reaction sites including the first nucleotide tag with the total signal from all reaction sites including the second nucleotide tag. Alternatively or in addition, the method can entail detecting and comparing the number of reaction sites including the first nucleotide tag with the number of reaction sites including the second nucleotide tag. In either case, the comparison can include any conventional means of comparing two values, such as, e.g., determining a ratio.

[0194] The selection of suitable, distinguishable nucleotide tags for use in the method is within the level of skill in the art. In certain embodiments, the first nucleotide tag can include a homopolymer of a first nucleotide (e.g., poly-A), whereas the second nucleotide tag can include a homopolymer of second, different nucleotide (e.g., poly-G).

[0195] Although the detection-by-primer extension method is described above with respect to the analysis of two target nucleic acids, the method encompasses the analysis of three or more target nucleic acids, each of which is tagged with a distinct nucleotide tag. The resultant tagged target nucleotide sequences are subjected to three or more primer extension reactions, each using a primer that anneals to a distinct nucleotide tag, and a signal is detected and/or quantified for the extension of each primer. In particular embodiments, two or more tagged target nucleotide sequences include different barcodes, which as described above, can encode information, e.g., sample or reaction mixture, about the tagged target nucleotide sequence.

[0196] The above detection-by-primer extension method can, if desired, be carried out in multiplex. In certain embodiments, for example, multiple samples can be analyzed together in one or more primer extension reactions by incorporating one or more barcodes into the nucleotide tags, wherein the barcodes encode sample identity. Primers may be employed that are both allele- and barcode-specific for the primer extension reaction or, alternatively, the barcode may preferably be adjacent to the nucleotide tag to which the primer anneals, and the primer extension reaction can be a DNA sequencing reaction, which need only detect the sequence of the barcode. In the former embodiment, primer extension would indicate the presence of an allele from a particular sample, whereas in the latter embodiment, primer extension would indicate the presence of the allele, and the barcode nucleotide sequence would identify the sample.

Single-Particle Analysis Applications

Incorporation of Nucleic Acid Sequences Into Single Particles

[0197] In certain embodiments, the above-described methods of incorporating nucleic acid sequences into target nucleic acids (including the barcoding and pooling method described above) are used in the context of assaying single particles in a population of particles. In general, nucleic acid sequences are introduced into target nucleic acids that are associated with, or contained in, a particle. Thus, the first reactions described above are carried out in reaction volumes that contain individual particles. The ability to associate the results of single-particle analysis with each particle assayed can be exploited where, for example, two or more parameters are associated with a phenotype. The two or more parameters measured can be different types of parameters, e.g., RNA expression level and nucleotide sequence. Further applications of the single-cell analysis methods described herein are described below.

[0198] Single-particle analysis entails capturing particles of a population in separate reaction volumes to produce a plurality of separate reaction volumes containing only one particle each. Particle-containing separate reaction volumes can be formed in droplets, in emulsions, in vessels, in wells of a microtiter plate, or in compartments of a matrix-type microfluidic device. In illustrative embodiments, the separate reaction volumes are present within individual compartments of a microfluidic device, such as, for example, any of those

described herein. See also, U.S. Patent Publication No. 2004/0229349, published November 18, 2004, Daridon et al., which is incorporated herein by reference in its entirety and, in particular, for its description of micro-fluidic particle analysis systems.

[0199] In certain embodiments, a parameter is assayed by performing a reaction, such as nucleic acid amplification, in each separate reaction volume to produce one or more reaction products, which is/are analyzed to obtain the results that are then associated with the particle and entered into the data set. The particles may be captured in separate reaction volumes before being contacted with one or more reagent(s) for performing one or more reactions. Alternatively, or in addition, the particles may be contacted with one or more of such reagent(s), and the reaction mixture may be distributed into separate reaction volumes. In various embodiments, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more reactions are performed in each separate reaction volume. The analysis of the reaction products can be carried out in the separate reaction volumes. In some embodiments, however, it is advantageous to recover the contents of the separate reaction volumes for subsequent analysis or other purposes. For example, if a nucleic acid amplification is carried out in the separate reaction volumes, it may be desirable to recover the contents for subsequent analysis, e.g., by PCR and/or nucleic acid sequencing. The contents of the separate reaction volumes may be analyzed separately and the results associated with the particles present in the original reaction volumes. Alternatively, the particle/reaction volume identity can be encoded in the reaction product, e.g., as discussed above with respect to multi-primer nucleic acid amplification methods. Furthermore, these two strategies can be combined so that sets of separate reaction volumes are encoded, such that each reaction volume within the set is uniquely identifiable, and then pooled, with each pool then being analyzed separately, as illustrated by the barcoding and pooling method described above.

Particles

[0200] The methods described herein can be used to analyze any type of particle, e.g., by carrying out any of the above-described reactions on nucleic acids from one or more individual particles. In certain embodiments, a particle generally includes any object that is small enough to be suspended in a fluid, but large enough to be distinguishable from the fluid. Particles may be microscopic or near-microscopic and may have diameters of about 0.005 to 100 μm , 0.1 to 50 μm , or about 0.5 to 30 μm . Alternatively, or in addition,

particles may have masses of about 10^{-20} to 10^{-5} grams, 10^{-16} to 10^{-7} grams, or 10^{-14} to 10^{-8} grams. In certain embodiments, the particle is a particle from a biological source (“a biological particle”). Biological particles include, for example, molecules such as nucleic acids, proteins, carbohydrates, lipids, and combinations or aggregates thereof (e.g.,

lipoproteins), as well as larger entities, such as viruses, chromosomes, cellular vesicles and organelles, and cells. Particles that can be analyzed as described herein also include those that have an insoluble component, e.g., a bead, to which molecules to be analyzed are attached.

[0201] In illustrative embodiments, the particles are cells. Cells suitable for use as particles in the methods described herein generally include any self-replicating, membrane-bounded biological entity or any non-replicating, membrane-bounded descendant thereof. Non-replicating descendants may be senescent cells, terminally differentiated cells, cell chimeras, serum-starved cells, infected cells, non-replicating mutants, anucleate cells, etc. Cells used in the methods described herein may have any origin, genetic background, state of health, state of fixation, membrane permeability, pretreatment, and/or population purity, among other characteristics. Suitable cells may be eukaryotic, prokaryotic, archaeon, etc., and may be from animals, plants, fungi, protists, bacteria, and/or the like. In illustrative embodiments, human cells are analyzed. Cells may be from any stage of organismal development, e.g., in the case of mammalian cells (e.g., human cells), embryonic, fetal, or adult cells may be analyzed. In certain embodiments, the cells are stem cells. Cells may be wild-type; natural, chemical, or viral mutants; engineered mutants (such as transgenics); and/or the like. In addition, cells may be growing, quiescent, senescent, transformed, and/or immortalized, among other states. Furthermore, cells may be a monoculture, generally derived as a clonal population from a single cell or a small set of very similar cells; may be presorted by any suitable mechanism, such as affinity binding, FACS, drug selection, etc.; and/or may be a mixed or heterogeneous population of distinct cell types.

[0202] Particles that include membranes (e.g., cells or cellular vesicles or organelles), cell walls, or any other type of barrier separating one or more interior components from the exterior space may be intact or disrupted, partially (e.g., permeabilized) or fully (e.g., to release interior components). Where the particles are cells, fixed and/or unfixed cells may be used. Living or dead, fixed or unfixed cells may have

intact membranes, and/or be permeabilized/disrupted membranes to allow uptake of ions, stains, dyes, labels, ligands, etc., and/or be lysed to allow release of cell contents.

[0203] One advantage of the methods described herein is that they can be used to analyze virtually any number of particles, including numbers well below the millions of particles required for other methods. In various embodiments, the number of particles analyzed can be about 10, about 50, about 100, about 500, about 1000, about 2000, about 3000, about 4000, about 5000, about 6000, about 7,000, about 8000, about 9,000, about 10,000, about 15,000, about 20,000, about 25,000, about 30,000, about 35,000, about 40,000, about 45,000, about 50,000, about 75,000, or about 100,000. In specific embodiments, the number of particles analyzed can fall within a range bounded by any two values listed above.

Particle Capture

[0204] Particles may be captured in separate reaction volumes by any means known in the art or described herein. In certain embodiments, a capture feature retains one or more cells at a capture site within separate reaction volume. In preferred embodiments, the capture feature preferentially retains only a single cell at the capture site. In certain preferred embodiments, each capture site is located within a separate compartment of the microfluidic device. The term “separate compartment” is used herein to refer to a compartment that is at least temporarily separate from other compartments within a microfluidic device, such that the compartments can contain separate reaction volumes. Temporary separation can be achieved, e.g., with the use of valves, as in the case of microfluidic devices available from Fluidigm, Inc. (South San Francisco, CA). The degree of separation must be such that assays/reactions can be carried out separately within the compartments. As used herein, the term “capture feature” includes single or plural mechanisms, operating in series and/or in parallel. Capture features may act to overcome the positioning force exerted by fluid flow. Suitable capture features may be based on physical barriers coupled with flow (termed “mechanical capture”), chemical interactions (termed “affinity-based capture”), vacuum forces, fluid flow in a loop, gravity, centrifugal forces, magnetic forces, electrical forces (e.g., electrophoretic or electroosmotic forces), and/or optically generated forces, among others.

[0205] Capture features may be selective or nonselective. Selective mechanisms may be fractionally selective, that is, retaining less than all (a subset of) inputted particles. Fractionally selective mechanisms may rely at least in part on stochastic focusing features (see below). Alternatively, or in addition, selective mechanisms may be particle-dependent, that is, retaining particles based on one or more properties of the inputted particle, such as size, surface chemistry, density, magnetic character, electrical charge, optical property (such as refractive index), and/or the like.

Mechanical Capture

[0206] Mechanical capture may be based at least partially on particle contact with any suitable physical barrier(s) disposed, e.g., in a microfluidic device. Such particle-barrier contact generally restricts longitudinal particle movement along the direction of fluid flow, producing flow-assisted retention. Flow-assisted particle-barrier contact also may restrict side-to-side/orthogonal (transverse) movement. Suitable physical barriers may be formed by protrusions that extend inward from any portion of a channel or other passage (that is, walls, roof, and/or floor). For example, the protrusions may be fixed and/or movable, including columns, posts, blocks, bumps, walls, and/or partially/completely closed valves, among others. Some physical barriers, such as valves, may be movable or regulatable. Alternatively, or in addition, a physical barrier may be defined by a recess(es) (e.g., niches), formed in a channel or other passage, or by a fluid-permeable membrane. Other physical barriers may be formed based on the cross-sectional dimensions of passages. For example, size-selective channels may retain particles that are too large to enter the channels. (Size-selective channels also may be referred to as filter channels, microchannels, or particle-restrictive or particle-selective channels.) Examples 6 and 8 provide illustrative mechanical capture embodiments.

Affinity-Based Capture

[0207] Affinity-based capture may retain particles based on one or more chemical interaction(s), i.e., wherein a binding partner binds a particle component. The chemical interactions may be covalent and/or noncovalent interactions, including ionic, electrostatic, hydrophobic, van der Waals, and/or metal coordination interactions, among others.

Chemical interactions may retain particles selectively and/or non-selectively. Selective and

non-selective retention may be based on specific and/or non-specific chemical interactions between particles and surfaces, e.g., in a microfluidic device.

[0208] Specific chemical mechanisms may use specific binding partners (SBPs), for example, with first and second SBPs disposed on particles and device surfaces, respectively.

5 Exemplary SBPs may include biotin/avidin, antibody/antigen, lectin/carbohydrate, etc. SBPs may be disposed locally within microfluidic devices before, during and/or after formation of the devices. For example, surfaces of a substrate and/or a fluid layer component may be locally modified by adhesion/attachment of a SBP member before the substrate and fluid layer component are joined. Alternatively, or in addition, an SBP may
10 be locally associated with a portion of a microfluidic device after the device has been formed, for example, by local chemical reaction of the SBP member with the device (such as one catalyzed by local illumination with light). See also Example 7, which describes an embodiment in which beads bearing an SBP member are mechanically caught at capture sites to display the SBP member for affinity-based capture of particles (i.e., cells).

15 [0209] Non-specific chemical mechanisms may rely on local differences in the surface chemistry of microfluidic devices. Such local differences may be created before, during and/or after microfluidic device formation, as described above. The local differences may result from localized chemical reactions, for example, to create hydrophobic or hydrophilic regions, and/or localized binding of materials. The bound materials may
20 include poly-L-lysine, poly-D-lysine, polyethylenimine, albumin, gelatin, collagen, laminin, fibronectin, entactin, vitronectin, fibrillin, elastin, heparin, keratan sulfate, heparan sulfate, chondroitin sulfate, hyaluronic acid, and/or extracellular matrix extracts/mixtures, among others.

Other Capture Features

25 [0210] Other capture features may be used alternatively, or in addition to, affinity-based or mechanical capture. Some or all of these mechanisms, and/or the mechanisms described above, may rely at least partially on friction between particles and microfluidic device channels or passages to assist retention.

[0211] Capture features may be based on vacuum forces, fluid flow, and/or gravity.
30 Vacuum-based capture features may exert forces that pull particles into tighter contact with

passage surfaces, for example, using a force directed outwardly from a channel.

Application of a vacuum, and/or particle retention, may be assisted by an aperture/orifice in the wall of a channel or other passage. By contrast, fluid flow-based capture features may produce fluid flow paths, such as loops, that retain particles. These fluid flow paths may be formed by a closed channel-circuit having no outlet (e.g., by valve closure and active pumping), and/or by an eddy, such as that produced by generally circular fluid-flow within a recess. Gravity-based capture features may hold particles against the bottom surfaces of passages, thus combining with friction to restrict particle movement. Gravity-based retention may be facilitated by recesses and/or reduced fluid flow rates.

[0212] Capture features may be based on centrifugal forces, magnetic forces, and/or optically generated forces. Capture features based on centrifugal force may retain particles by pushing the particle against passage surfaces, typically by exerting a force on the particles that is generally orthogonal to fluid flow. Such forces may be exerted by centrifugation of a microfluidic device and/or by particle movement within a fluid flow path. Magnetic force-based capture features may retain particles using magnetic fields, generated external and/or internal to a microfluidic device. The magnetic field may interact with ferromagnetic and/or paramagnetic portions of particles. For example, beads may be formed at least partially of ferromagnetic materials, or cells may include surface-bound or internalized ferromagnetic particles. Electrical force-based capture features may retain charged particles and/or populations using electrical fields. By contrast, capture features that operate based on optically generated forces may use light to retain particles. Such mechanisms may operate based on the principal of optical tweezers, among others.

[0213] Another form of capture feature is a blind-fill channel, where a channel has a inlet, but no outlet, either fixedly or transiently. For example, when the microfluidic device is made from a gas permeable material, such as PDMS, gas present in a dead-end channel can escape, or be forced out of the channel through the gas permeable material when urged out by the inflow of liquid through the inlet. This is a preferred example of blind-filling. Blind-filling can be used with a channel or compartment that has an inlet, and an outlet that is gated or valved by a valve. In this example, blind filling of a gas-filled channel or compartment occurs when the outlet valve is closed while filling the channel or compartment through the inlet. If the inlet also has a valve, that valve can then be closed after the blind fill is complete, and the outlet can then be opened to expose the channel or

compartment contents to another channel or compartment. If a third inlet is in communication with the channel or compartment, that third inlet can introduce another fluid, gas or liquid, into the channel or compartment to expel the blind-filled liquid to be expelled from the channel or compartment in a measured amount.

5 **Focusing Features**

[0214] Particle capture can be enhanced in microfluidic devices with the use of a one or more focusing feature(s) to focus particle flow to each capture site. Focusing features may be categorized without limitation in various ways, for example, to reflect their origins and/or operational principles, including direct and/or indirect, fluid-mediated and/or
10 non-fluid-mediated, external and/or internal, and so on. These categories are not mutually exclusive. Thus, a given focusing feature may position a particle in two or more ways; for example, electric fields may position a particle directly (e.g., via electrophoresis) and indirectly (e.g., via electroosmosis).

[0215] The focusing features may act to define particle position longitudinally and/or transversely. The term “longitudinal position” denotes position parallel to or along
15 the long axis of a microfluidic channel and/or a fluid flow stream within the channel. In contrast, the term “transverse position” denotes position orthogonal to the long axis of a channel and/or an associated main fluid flow stream. Both longitudinal and transverse positions may be defined locally, by equating “long axis” with “tangent” in curved
20 channels. Focusing features may act to move particles along a path at any angle, relative to the long axis of a channel and/or flow stream, between longitudinal and transverse flow.

[0216] The focusing features may be used alone and/or in combination. If used in combination, the features may be used serially (i.e., sequentially) and/or in parallel (i.e., simultaneously). For example, an indirect mechanism such as fluid flow may be used for
25 rough positioning, and a direct mechanism such as optical tweezers may be used for final positioning.

[0217] Direct focusing features generally include any mechanism in which a force acts directly on a particle(s) to position the particle(s) within a microfluidic network. Direct focusing features may be based on any suitable mechanism, including optical, electrical,
30 magnetic, and/or gravity-based forces, among others. Optical focusing features use light to

mediate or at least facilitate positioning of particles. Suitable optical focusing features include “optical tweezers,” which use an appropriately focused and movable light source to impart a positioning force on particles. Electrical focusing features use electricity to position particles. Suitable electrical mechanisms include “electrokinetics,” that is, the application of voltage and/or current across some or all of a microfluidic network, which may, as mentioned above, move charged particles directly (e.g., via electrophoresis) and/or indirectly, through movement of ions in fluid (e.g., via electroosmosis). Magnetic focusing features use magnetism to position particles based on magnetic interactions. Suitable magnetic mechanisms involve applying a magnetic field in or around a fluid network, to position particles via their association with ferromagnetic and/or paramagnetic materials in, on, or about the particles. Gravity-based focusing features use the force of gravity to position particles, for example, to contact adherent cells with a substrate at positions of cell culture.

[0218] Indirect focusing features generally include any mechanism in which a force acts indirectly on a particle(s), for example, via fluid, to move the particle(s) within a microfluidic network, longitudinally and/or transversely. Longitudinal indirect focusing features generally may be created and/or regulated by fluid flow along channels and/or other passages. Accordingly, longitudinal focusing features may be facilitated and/or regulated by valves and/or pumps that regulate flow rate and/or path. In some cases, longitudinal focusing features may be facilitated and/or regulated by electroosmotic focusing features. Alternatively, or in addition, longitudinal focusing features may be input-based, that is, facilitated and/or regulated by input mechanisms, such as pressure or gravity-based mechanisms, including a pressure head created by unequal heights of fluid columns.

[0219] Transverse indirect focusing features generally may be created and/or regulated by fluid flow streams at channel junctions, laterally disposed regions of reduced fluid flow, channel bends, and/or physical barriers (i.e., baffles). Channel junctions may be unifying sites or dividing sites, based on the number of channels that carry fluid to the sites relative to the number that carry fluid away from the sites. Physical barriers may have any suitable design to direct particle flow toward capture sites. For example, a baffle may extend outward from any channel surface, e.g., at an angle to direct particle flow toward a capture site. Baffle length, angle with the channel surface, and distance from the capture site can be adjusted to enhance particle flow toward the capture site. Baffles may be formed

by protrusions that extend inward from any portion of a channel or other passage (that is, walls, roof, and/or floor). For example, the protrusions may be fixed and/or movable, including columns, posts, blocks, bumps, walls, and/or partially/completely closed valves, among others. Some physical barriers, such as valves, may be movable or regulatable.

5 **[0220]** In some embodiments, multiple baffles may be employed for each capture site. For example, a baffle extending outward, at an angle, from each lateral wall of a channel can be employed to direct particle flow toward a capture site that is centrally located in the channel. See Figure 22A-B. Where mechanical capture is employed, baffles may be spaced apart from the physical barrier(s) in the capture sites. Alternatively, or
10 additionally, baffles may contact or be an integral part of the physical barrier(s) in the capture sites. See Figure 22A and 14C. For example, a baffle extending outward, at an angle, from a channel wall can contact or be an integral part of a concave capture feature (e.g., physical barrier(s)). It will be appreciated a “concave” capture feature is concave on the side of the capture feature that generally faces the direction of fluid flow. The baffle
15 directs particle flow away from the channel wall and toward the concave capture feature, facilitating particle capture. The next capture site along the path of flow can have a similar baffle-concave capture feature configuration, with the baffle extending from the same wall of the channel. However, it is advantageous, in some embodiments, for the next baffle-concave capture feature to extend from the opposite channel wall. This alternating
20 configuration acts to focus flow from one baffle to the next, whereby flow along each baffle enhances particle flow into each concave capture feature. See Figure 22C.

[0221] Transverse indirect focusing features may be based on laminar flow, stochastic partitioning, and/or centrifugal force, among other mechanisms. Transverse positioning of particles and/or reagents in a microfluidic device may be mediated at least in
25 part by a laminar flow-based mechanism. Laminar flow-based mechanisms generally include any focusing feature in which the position of an input flow stream within a channel is determined by the presence, absence, and/or relative position(s) of additional flow streams within the channel. Such laminar flow-based mechanisms may be defined by a channel junction(s) that is a unifying site, at which inlet flow streams from two, three, or
30 more channels, flowing toward the junction, unify to form a smaller number of outlet flow streams, preferably one, flowing away from the junction. Due to the laminar flow properties of flow streams on a microfluidic scale, the unifying site may maintain the

relative distribution of inlet flow streams after they unify as laminar outlet flow streams. Accordingly, particles and/or reagents may remain localized to any selected one or more of the laminar flow streams, based on which inlet channels carry particles and/or reagents, thus positioning the particles and/or reagents transversely. See, e.g., Figure 24D.

5 **[0222]** The relative size (or flow rate) and position of each inlet flow stream may determine both position and relative width of flow streams that carry particles and/or reagents. For example, an inlet flow stream for particles/reagents that is relatively small (narrow), flanked by two larger (wider) flow streams, may occupy a narrow central position in a single outlet channel. By contrast, an inlet flow stream for particles/reagents that is
10 relatively large (wide), flanked by a comparably sized flow stream and a smaller (narrower) flow stream, may occupy a wider position that is biased transversely toward the smaller flow stream. In either case, the laminar flow-based mechanism may be called a focusing mechanism, because the particles/reagents are “focused” to a subset of the cross-sectional area of outlet channels. Laminar flow-based mechanisms may be used to individually
15 address particles and/or reagents to plural distinct capture sites.

[0223] A laminar flow-based mechanism may be a variable mechanism to vary the transverse position of particles/reagents. As described above, the relative contribution of each inlet flow stream may determine the transverse position of particles/reagents flow streams. Altered flow of any inlet flow stream may vary its contribution to the outlet flow
20 stream(s), shifting particles/reagents flow streams accordingly. In an extreme case, referred to as a perfusion mechanism, a reagent (or particle) flow stream may be moved transversely, either in contact with, or spaced from, retained particles (reagents), based on presence or absence of flow from an adjacent inlet flow stream. Such a mechanism also may be used to effect variable or regulated transverse positioning of particles, for example, to direct
25 particles to capture sites having different transverse positions.

[0224] Transverse positioning of particles and/or reagents in a microfluidic device may be mediated at least in part by a stochastic (or portioned flow) focusing feature. Stochastic transverse focusing features generally include any focusing feature in which an at least partially randomly selected subset of inputted particles or reagent is distributed
30 laterally away from a main flow stream to a region of reduced fluid flow within a channel (or, potentially, to a distinct channel). The region of reduced flow may promote particle

retention, treatment, detection, minimize particle damage, and/or promote particle contact with a substrate. Stochastic focusing features may be determined by dividing flow sites and/or locally widened channels, among others.

[0225] Dividing flow sites may effect stochastic positioning by forming regions of reduced fluid flow rate. Dividing flow sites generally include any channel junction at which inlet flow streams from one (preferably) or more inlet channels are divided into a greater number of outlet channels, including two, three, or more, channels. Such dividing sites may deliver a subset of particles, which may be selected stochastically and/or based on a property of the particles (such as mass), to a region of reduced flow rate or quasi-stagnant flow formed at or near the junction. The fraction of particles represented by the subset may be dependent upon the relative flow directions of the outlet channels relative to the inlet channels. These flow directions may be generally orthogonal to an inlet flow stream, being directed in opposite directions, to form a "T-junction." Alternatively, outlet flow directions may form angles of less than and/or greater than 90 degrees.

[0226] The dividing-flow focusing feature, with two or more outlet channels, may be used as a portioned-flow mechanism. Specifically, fluid, particles, and/or reagents carried to the channel junction may be portioned according to fluid flow through the two or more outlet channels. Accordingly, the fractional number or volume of particles or reagent that enters the two or more channels may be regulated by the relative sizes of the channels and/or the flow rate of fluid through the channels, which in turn may be regulated by valves, or other suitable flow regulatory-mechanisms. In a first set of embodiments, outlet channels may be of very unequal sizes, so that only a small fraction of particle and/or reagents are directed to the smaller channel. In a second set of embodiments, valves may be used to forms desired dilutions of reagents. In a third set of embodiments, valves may be used to selectively direct particles to one of two or more fluid paths.

[0227] Locally widened channels may promote stochastic positioning by producing regions of decreased flow rate lateral to a main flow stream. The decreased flow rate may deposit a subset of inputted particles at a region of decreased flow rate. Such widened channels may include nonlinear channels that curve or bend at an angle. Alternatively, or in addition, widened regions may be formed by recesses formed in a channel wall(s), chambers

that intersect channels, and/or the like, particularly at the outer edge of a curved or bent channel.

[0228] Transverse positioning of particles and/or reagents also may be mediated at least in part by a centrifugal focusing feature. In centrifugal focusing features, particles may experience a centrifugal force determined by a change in velocity, for example, by moving through a bend in a fluid path. Size and/or density of particles may determine the rate of velocity change, distributing distinct sizes and/or densities of particle to distinct transverse positions.

Drain Features

10 [0229] In certain embodiments, the capture site also includes a drain feature. Where mechanical capture is employed, for example, the drain feature can include one or more interruptions in a capture feature that is/are sized to permit fluid flow, but not particle flow, through and/or around the capture feature. Thus, for example, the capture feature can include two physical barriers, separated by a space (the drain feature), wherein the space is sufficiently large to permit particle-free fluid to flow between the barriers with sufficiently low impedance to direct cells toward the barriers, thereby enhancing the probability of particle capture. The space between the physical barriers should generally be sufficiently small and/or suitably configured such that the particles to be captured at the capture site will not pass between the barriers. In a specific, illustrative embodiment, the capture feature includes two concave physical barriers, with first and second ends, wherein the barriers are arranged with a small space between first ends of the barriers, forming a drain feature, and a larger space between the second ends of the barriers. See Figure 22B (where d_3 is greater than d_1 , which forms a drain). In this configuration, the barriers form a “cup” suitably sized to capture a particle, with a drain at the base of the cup. By virtue of the drain, particles flow toward the cup, as long as it is unoccupied. Once a particle flows into the cup, the drain is “plugged,” which tends to enhance particle flow around the cup and on to the next capture feature in the microfluidic device.

Non-Optimized Single-Particle Capture

[0230] In particular embodiments, a capture technique, such as limiting dilution is used to capture particles in separate reaction volumes. In this type of capture, there is no

use of any capture feature, such as binding affinity or a mechanical feature(s), e.g., in a microfluidic device, that preferentially retains only a single cell at a capture site. For example, limiting dilution can be carried out by preparing a series of dilutions of a particle suspension, and distributing aliquots from each dilution into separate reaction volumes. The number of particles in each reaction volume is determined, and the dilution that produces the highest fraction of reaction volumes having only a single particle is then selected and used to capture particles for the parameter measurements described herein.

Optimized Single-Particle Capture

[0231] In some embodiments, the methods entail the use of an optimized capture technique to increase the expected fraction of separate reaction volumes having only one particle above that achieved using a method such as limiting dilution (i.e., above about 33 percent). In variations of these embodiments, capturing is optimized such that the expected fraction of separate reaction volumes with only one particle each is at least about 35 percent, at least about 40 percent, at least about 45 percent, at least about 50 percent, at least about 55 percent, at least about 60 percent, at least about 65 percent, at least about 70 percent, at least about 75 percent, at least about 80 percent, at least about 85 percent, at least about 90 percent, or at least about 95 percent of the total number of separate reaction volumes. In specific embodiments, the expected fraction of separate reaction volumes with only one particle each falls within a range bounded by any two percentages listed above. The expected fraction of separate reaction volume with only one particle each can be determined by empirical or statistical means, depending on the particular capture technique (e.g., limiting dilution produces reaction volumes having only one particle in a manner consistent with the Poisson distribution). As used herein, the term “optimizing” does not imply that an optimal result is achieved, but merely that some measure is taken to increase the expected fraction of separate reaction volumes with only one particle above about 33 percent. In particular embodiments, optimized single-particle capture can be achieved, for example, using a size-based mechanism that excludes retention of more than one particle at in each reaction volume (capture site).

[0232] In certain embodiments, mechanical capture is used alone or in combination with one or more other capture features to preferentially capture a single particle in each separate reaction volume (i.e., each capture site within a microfluidic device). For example,

each capture site can include one or more physical barrier(s) sized to contain only one particle. The shape of the physical barrier can be designed to enhance the retention of the particle. For example, where the particles are cells, the physical barrier(s) can be sized and configured to form a concave surface suitable for retaining just one cell. In such

5 embodiments, the physical barrier(s) can be designed so as to permit the flow of fluid through the capture site, when it is not occupied by a cell, and/or the capture site may include a drain feature that facilitates this flow. In particular embodiments, a microfluidic device contains a plurality of suitably sized/configured physical barriers, whereby a plurality of individual particles is retained within the device, one particle being retained by

10 each physical barrier. In illustrative embodiments, the physical barriers can be located within separate compartments within a microfluidic device, one region per compartment. The compartments can be arranged to form an array, such as, for example, the microfluidic arrays available from Fluidigm Corp. (South San Francisco, CA) and described herein. See also Figure 24A-G.

15 **[0233]** In certain embodiments, affinity-based capture is used alone or in combination with one or more other capture features, e.g., mechanical capture, to preferentially capture a single cell in each separate reaction volume (i.e., each capture site within a microfluidic device). For example, a discrete region of a microfluidic device surface that contains a binding partner for a particle or particle component may be sized so

20 that only one particle can bind to the region, with the binding of subsequent particles blocked by steric hindrance. In particular embodiments, a microfluidic device contains a plurality of suitably sized regions, whereby a plurality of individual particles, one at each region, is retained within the device. In illustrative embodiments, these regions can be located within separate compartments within a microfluidic device, one region per

25 compartment. The compartments can be arranged to form an array, such as, for example, the microfluidic arrays available from Fluidigm Corp. (South San Francisco, CA) and described herein.

[0234] One approach to affinity-based, optimized single-particle capture is based on capturing a support including a binding partner that binds the particle to be assayed. In

30 illustrative embodiments, the support can be a bead that has the binding partner distributed over its surface. See Figure 23A. The bead can be captured by mechanical capture using a cup-shaped capture feature to produce a single immobilized support (e.g., bead) at each

capture site. In addition to immobilizing the support, the capture feature can, in certain embodiments, reduce the surface area of the support (e.g., bead) that displays the binding partner. This surface can be sufficiently reduced that only one particle can bind to the area of the immobilized support (e.g., bead) that displays the binding partner. To facilitate particle-support binding, in some embodiments, the area of the immobilized support that displays the binding partners faces the flow path of the particles. In specific, illustrative embodiments, a flow channel of a microfluidic device contains a series of capture features. A suspension of beads bearing binding partners (e.g., cell-specific antibodies) is inputted into the channel to produce a series of immobilized beads at the capture sites. The channel is then washed to remove any free (i.e., non-immobilized) beads. Figure 23A. A cell suspension is then input into the channel. An individual cell can bind to the portion of each bead that displays binding partners. Each bound cell prevents any other cells from binding to the bead through steric occlusion. Washing of the channel removes unbound cells. See Figure 23B. Valves in between the capture sites can then be closed to create separate reaction volumes, each containing one capture site with one bound cell. One or more focusing features can be employed to direct bead, as well as, particle flow toward each capture site. Alternatively or in addition, the capture features can each include a drain feature that permits the flow of fluid through the capture site when the capture feature is not occupied by a bead.

Determination of Number and/or Characteristics of Particles Captured

[0235] In certain embodiments, it is advantageous to determine the number of particles in each separate reaction volume. This determination can be made when using limiting dilution to identify the dilution that produces the highest fraction of compartments having only a single particle. This determination can also be made after any capture technique to identify those reaction volumes that contain only one particle. For example, in some embodiments, the assay results can be sorted into multiple “bins,” based on whether they come from reaction volumes containing 0, 1, 2, or more cells, permitting separate analysis of one or more of these bins. In certain embodiments, any of the methods described herein can include determining whether any compartment includes more than a single particle; and not further analyzing, or disregarding, results from, any compartment that includes more than a single particle.

[0236] In some embodiments, the number of particles in each separate reaction volume is determined by microscopy. For example, where the separate reaction volumes are in compartments of a microfluidic device that is sufficiently transparent or translucent, simple brightfield microscopy can be used to visualize and count particles, e.g., cells, per compartment. See Example 5. The microfluidic devices described below and available from Fluidigm Corp. (South San Francisco, CA) are suitable for use in this brightfield microscopy approach.

[0237] In certain embodiments a stain, dye, or label can be employed to detect the number of particles in each separate reaction volume. Any stain, dye, or label that can be detected in the separate reaction volumes can be used. In illustrative embodiments, a fluorescent stain, dye, or label can be used. The stain, dye, or label employed can be tailored to the particular application. Where the particles are cells, and the parameter to be measured is a feature of the cell surface, the stain, dye, or label can be a cell-surface stain, dye, or label that need not penetrate the cells. For example, a labeled antibody specific for a cell-surface marker can be employed to detect the number of cells in each separate reaction volume. Where the particles are cells, and the parameter to be measured is an internal feature of the cell (e.g., nucleic acid), the stain, dye, or label can be a membrane-permeant stain, dye, or label (e.g., a double-stranded DNA binding dye).

[0238] In particular embodiments, a characteristic of a cell can be detected in each separate reaction volume, with or without a determination of the number of cells in each reaction volume. For example, a stain, dye, or label can be employed to determine whether any reaction volume (e.g., any compartment in a microfluidic device) includes a particle having the characteristic. This step can increase assay efficiency by permitting subsequent analysis of the reaction results of only those compartments that include a particle having the particular characteristic. Illustrative characteristics that can be detected in this context include, for example, a specific genomic rearrangement, copy number variation, or polymorphism; expression of a specific gene; and expression of a specific protein.

Analysis of Nucleic Acids in Single Particles

[0239] In particular embodiments, the methods described herein are used in the analysis of one or more nucleic acids. For example, the presence and/or level of a particular target nucleic acid can be determined, as can a characteristic of the target nucleic acid, e.g.,

the nucleotide sequence. In illustrative embodiments, a population of particles with one or more sample nucleic acids in or associated with the particle is captured in separate reaction volumes, each preferably containing only a single particle. Reactions, such ligation and/or amplification for DNA, or reverse transcription and/or amplification for RNA are carried out, which produce reaction products for any reaction volume containing one or more target nucleic acids. These reaction products can be analyzed within the reaction volumes, or the reaction volumes can be recovered, separately or in pools, for subsequent analysis, such as DNA sequencing.

[0240] In certain embodiments, the reactions incorporate one or more nucleotide sequences into the reaction products. These sequences can be incorporated by any suitable method, including ligation, transposase-mediated incorporation, or amplification using one or more primers bearing one or more nucleotide tags that include the sequence to be incorporated. These incorporated nucleotide sequence(s) can serve any function that facilitates any assay described herein. For example, one or more nucleotide sequences can be incorporated into a reaction product to encode an item of information about that reaction product, such as the identity of the reaction volume that was the source of the reaction product. In this case, the reactions are referred to herein as “encoding reactions.” Multi-primer methods for adding “barcode” nucleotide sequences to target nucleic acids can be employed for this purpose and are described above. In specific embodiments, nucleic acid amplification is carried out using at least two amplification primers, wherein each amplification primer includes a barcode nucleotide sequence, and the combination of barcode nucleotide sequences encodes the identity of the reaction volume that was the source of the reaction product (termed “combinatorial barcoding”). These embodiments are conveniently employed when the separate reaction volumes are in separate compartments of a matrix-type microfluidic device, e.g., like those available from Fluidigm Corp. (South San Francisco, CA) and described below (see “Microfluidic Devices”). Each separate compartment can contain a combination of barcode nucleotide sequences that identifies the row and column of the compartment in which the encoding reaction was carried out. If the reaction volumes are recovered and subjected to further analysis that includes detection of the barcode combination, the results can be associated with a particular compartment and, thereby, with a particle in the compartment. This association can be carried out for all

compartments that contain a single particle to permit single-particle (e.g., single-cell) analysis for a population of particles.

[0241] The following sections discuss suitable nucleic acid samples, and within these, target nucleic acids suitable for analysis in the methods described herein.

5 Amplification primer design and illustrative amplification methods are then described. The remaining sections discuss various labeling strategies and removal of undesired reaction components. These sections are described with respect to methods that employ amplification for incorporating nucleic acid sequences into target nucleic acids and/or analyzing them. However, those of skill in the art will recognize, based on the guidance
10 herein, that amplification is not critical to carrying out many of the methods described herein. For example, nucleic acid sequences can be incorporated by other means, such as ligation or using a transposase.

Sample Nucleic Acids

[0242] Preparations of nucleic acids (“samples”) can be obtained from biological
15 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
20 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
25 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, formalin-fixed and/or paraffin-embedded tissue samples, and needle biopsies. Nucleic acids useful in the methods described herein can also be derived
30 from one or more nucleic acid libraries, including cDNA, cosmid, YAC, BAC, P1, PAC libraries, and the like.

[0243] 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 reactions of interest 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.

Target Nucleic Acids

[0244] Target nucleic acids useful in the methods described herein can be derived from any of the sample nucleic acids described above. In typical embodiments, at least some nucleotide sequence information will be known for the target nucleic acids. For example, if PCR is employed as the encoding reaction, 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, target-specific sequences in primers could be replaced by random or degenerate nucleotide sequences.

[0245] 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, rearranged, 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). When multiple target nucleic acids are employed, these can be on the same or different chromosome(s).

[0246] In various embodiments, a target nucleic acid to be amplified can be, e.g., 25 bases, 50 bases, 100 bases, 200 bases, 500 bases, or 750 bases. In certain embodiments of the methods described herein, 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 nucleic acids ranging from one or a few kilobases (kb) to over 50 kb. In various embodiments, the target nucleic acids 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 nucleic acids can also fall within any range having any of these values as endpoints (e.g., 25 bases to 100 bases or 5-15 kb).

Primer Design

[0247] 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.

[0248] 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.

[0249] 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.

[0250] 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 described herein.

Amplification Methods

5 [0251] Nucleic acids can be amplified in accordance with the methods described herein for any useful purpose, e.g., to increase the concentration of target nucleic acids for subsequent analysis, and/or to incorporate one or more nucleotide sequences, and/or to detect and/or quantify and/or sequence one or more target nucleic acids. Amplification can be carried out in droplets, in emulsions, in vessels, in wells of a microtiter plate, in
10 compartments of a matrix-type microfluidic device, etc.

Amplification to Increase the Concentration of Target Nucleic Acids

[0252] Amplification to increase the concentration of target nucleic acids can be aimed at amplifying all nucleic acids in a reaction mixture, all nucleic acids of a particular type (e.g., DNA or RNA), or specific target nucleic acids. In specific, illustrative
15 embodiments, whole genome amplification can be carried out to increase the concentration of genomic DNA; RNA can be amplified, optionally preceded by a reverse transcription step; and/or general or target-specific preamplification.

Whole Genome Amplification

[0253] To analyze genomic DNA, the sample nucleic acids can be amplified using a
20 whole genome amplification (WGA) procedure. Suitable WGA procedures include primer extension PCR (PEP) and improved PEP (I-PEP), degenerated oligonucleotide primed PCR (DOP-PCR), ligation-mediated PCR (LMP), T7-based linear amplification of DNA (TLAD), and multiple displacement amplification (MDA). These techniques are described in U.S. Patent Publication No. 20100178655, published July 15, 2010 (Hamilton et al.),
25 which is incorporated herein by reference in its entirety and specifically for its description of methods useful in single-cell nucleic acid analysis.

[0254] Kits for WGA are available commercially from, e.g., Qiagen, Inc. (Valencia, CA USA), Sigma-Aldrich (Rubicon Genomics; e.g., Sigma GenomePlex® Single Cell Whole Genome Amplification Kit, PN WGA4-50RXN). The WGA step of the methods

described herein can be carried out using any of the available kits according to the manufacturer's instructions.

[0255] In particular embodiments, the WGA step is limited WGA, i.e., WGA is stopped before a reaction plateau is reached. Typically, WGA is performed for more than
5 two amplification cycles. In certain embodiments, WGA is performed for fewer than about 10 amplification cycles, e.g., between four and eight cycles, inclusive. However, WGA can be performed for 3, 4, 5, 6, 7, 8, or 9 cycles or for a number of cycles falling within a range defined by any of these values.

RNA Amplification

10 [0256] In certain embodiments, RNA from single cell or a small population of cells can be analyzed for one or more RNA targets. Suitable RNA targets include mRNA, as well as non-coding RNA, such as small nucleolar RNA (snoRNA), microRNA (miRNA), small interfering RNA (siRNA), and Piwi-interacting RNAs (piRNA). In particular
15 embodiments, the RNA of interest is converted to DNA, e.g., by reverse transcription or amplification.

[0257] For example, to analyze mRNA of a single cell or a small population of cells, the mRNA is generally converted to a DNA representation of the mRNA population. In certain embodiments, the method(s) employed preferably yield(s) a population of cDNAs, wherein the relative amounts of each cDNA is approximately the same as the relative
20 amounts of the corresponding mRNAs in the sample population.

[0258] In particular embodiments, reverse transcription can be employed to produce cDNA from the mRNA template, utilizing reverse transcriptase according to standard techniques. Reverse transcription of a cell's mRNA population can be primed, e.g., with the use of specific primers, oligo-dT, or random primers. To synthesize a cDNA library
25 representative of cellular mRNA, a first strand of cDNA complementary to the sample cellular RNA can be synthesized using reverse transcriptase. This can be done using the commercially available BRL Superscript II kit (BRL, Gaithersburg, Md.) or any other commercially available kit. Reverse transcriptase preferentially utilizes RNA as a template, but can also utilize single-stranded DNA templates. Accordingly, second strand cDNA
30 synthesis can be carried out using reverse transcriptase and suitable primers (e.g., poly-A,

random primers, etc.). Second strand synthesis can also be carried out using E. coli DNA polymerase I. The RNA can be removed at the same time the second cDNA strand is synthesized or afterwards. This is done by, for example, treating the mixture to an RNase such as E. coli RNase H, that degrades the RNA.

5 **[0259]** In other embodiments, an amplification method is employed to produce cDNA from the mRNA template. In such embodiments, an amplification method that produces a population of cDNA that is representative of the mRNA population is typically employed.

10 **[0260]** The analysis of non-coding RNA from a single cell or a small population of cells also typically begins with the conversion of the RNA of interest to DNA. This conversion can be carried out by reverse transcription or amplification. In certain embodiments, the method(s) employed preferably yield(s) a population of DNAs, wherein the relative amounts of each DNA is approximately the same as the relative amounts of the corresponding mRNAs in the sample population. The target RNAs can be selectively
15 reverse-transcribed or amplified using primers that anneal preferentially to the RNAs of interest. Suitable primers are commercially available or can be designed by those of skill in the art. For example, Life Technologies sells MegaPlex™ Pools of primers for microRNA (miRNA) targets. These primers can be used for both reverse transcription (RT) and specific target amplification (STA). See, e.g., Example 6B.

20 **Preamplification**

25 **[0261]** Preamplification can be carried to increase the concentration of nucleic acid sequences in a reaction mixture, generally, e.g, using a set of random primers, primers that are specific for one or more sequences common to a plurality of, or all, nucleic acids present (e.g., poly-dT to prime poly-A tails), or a combination of a set of random primers and a
30 specific primer. Alternatively, preamplification can be carried out using one or more primer pairs specific for the one or more target nucleic acids of interest. In specific, illustrative embodiments, an amplified genome produced by WGA or the DNA produced from RNA (e.g., cDNA) can be preamplified to produce a preamplification reaction mixture that includes one or more amplicons specific for one or more target nucleic acids of interest.

30 Preamplification is typically carried out using preamplification primers, a suitable buffer

system, nucleotides, and DNA polymerase enzyme (e.g., a polymerase enzyme modified for “hot start” conditions).

[0262] In particular embodiments, the preamplification primers are the same sequence as those to be used in an amplification assay for which the sample is being prepared although generally in reduced concentration. The primer concentration can, e.g., be about 10 to about 250 times less than the primer concentrations used in the amplification assay. Embodiments include the use of primers that are about 10, 20, 35, 50, 65, 75, 100, 125, 150, 175, and 200 times less than that of the primer concentration in the amplification assay.

[0263] In specific embodiments, preamplification is carried out for at least two cycles. In certain embodiments, preamplification is carried out for fewer than about 20 cycles, e.g., between 8 and 18 cycles, inclusive. However, preamplification can be performed for 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 cycles or for a number of cycles falling within a range defined by any of these values. In an exemplary embodiment, preamplification is carried out for about 14 cycles in order to increase the amplicons to be detected by about 16,000 fold.

Amplification for Detection and/or Quantification of Target Nucleic Acids

[0264] Any method of detection and/or quantification of nucleic acids can be used in the methods described herein 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). 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.

[0265] 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
5 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 methods described herein are not limited to use of these probes; any suitable probe can be used.

[0266] Other detection/quantification methods that can be employed in the present
10 invention include FRET and template extension reactions, molecular beacon detection, Scorpion detection, Invader detection, and padlock probe detection.

[0267] 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
15 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.
20 Patent No. 5,945,283 and PCT Publication WO 97/22719.

[0268] 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
25 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
30 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).

5 **[0269]** 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
10 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
15 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
20 DNA polymerase.

[0270] 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
25 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
30 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.

[0271] 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.

[0272] 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.

[0273] 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 Life Technologies, Foster City, Calif.).

[0274] In 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. 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 various embodiments, 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.

[0275] By acquiring fluorescence over different temperatures, it is possible to follow the extent of hybridization. Moreover, the temperature-dependence of PCR product hybridization can be used for the identification and/or quantification of PCR products. Accordingly, the methods described herein encompass the use of melting curve analysis in detecting and/or quantifying amplicons. Melting curve analysis is well known and is described, for example, in U.S. Patent Nos. 6,174,670; 6,472,156; and 6,569,627, each of which is hereby incorporated by reference in its entirety, and specifically for its description of the use of melting curve analysis to detect and/or quantify amplification products. In illustrative embodiments, melting curve analysis is carried out using a double-stranded DNA dye, 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).

[0276] In certain embodiments, multiplex detection is carried out in individual amplification mixture, e.g., in individual reaction compartments 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 compartment.

[0277] 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.

[0278] Devices have been developed that can perform a thermal cycling reaction with compositions containing a fluorescent dye, 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.

[0279] 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.

[0280] 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.

Amplification for DNA Sequencing

[0281] In certain embodiments, amplification methods are employed to produce amplicons suitable for automated DNA sequencing. 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 incorporate adaptor sequences, as described in greater detail below.

[0282] In addition, the ability of the above-described methods to provide substantially uniform amplification 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.

[0283] In certain embodiments, at least three primers can be employed to produce amplicons suitable for DNA sequencing: forward, reverse, and barcode primers. However, one or more of the forward primer, reverse primer, and barcode primer can include 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, as discussed above, can indicate sample origin.

[0284] In other embodiments, at least four primers are employed to produce amplicons suitable for DNA. For example, inner primers can be used with outer primers that additionally include first and second primer binding sites that are capable of being bound by DNA sequencing primers. Amplification yields a molecule having the following elements: 5'-first primer binding site-second barcode nucleotide sequence-first nucleotide tag sequence-first barcode nucleotide sequence-target nucleotide sequence-first barcode nucleotide sequence-second nucleotide tag sequence-second barcode nucleotide sequence-second primer binding site-3'. Because this molecule contains the barcode combination at either end, sequence can be obtained from either end of the molecule to identify the barcode combination.

[0285] In a similar manner, six primers can be employed to prepare DNA for sequencing. More specifically, inner and stuffer primers, as discussed above, can be used with outer primers that additionally include first and second primer binding sites that are capable of being bound by DNA sequencing primers. Amplification yields a molecule having the following elements: 5'-first primer binding site-second barcode nucleotide sequence-third nucleotide tag sequence-first barcode nucleotide sequence-first nucleotide tag sequence-target nucleotide sequence-second nucleotide tag sequence-first barcode nucleotide sequence-fourth nucleotide tag sequence-second barcode nucleotide sequence-second primer binding site-3'. Because this molecule contains the barcode combination at either end, sequence can be obtained from either end of the molecule to identify the barcode combination.

[0286] The methods described herein 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. As discussed above, 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. In vitro clonal amplification can also be

carried out by “bridge PCR,” where fragments are amplified upon primers attached to a solid surface. DNA molecules that are physically bound to a surface can be sequenced in parallel, for example, by a pyrosequencing or sequencing-by-synthesis method, as discussed above.

5 **Labeling Strategies**

[0287] Any suitable labeling strategy can be employed in the methods described herein. 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
10 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
15 probes can be conveniently incorporated into the tagged target nucleotide sequences during amplification.

[0288] 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
20 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*
25 Research Chemicals, Molecular Probes, Eugene (1992).
30

Removal of Undesired Reaction Components

[0289] 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.

[0290] 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.

[0291] 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.

[0292] 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.

5 **Microfluidic Devices**

[0293] In certain embodiments, methods described herein can be carried out using a microfluidic device. In illustrative embodiments, the device is a matrix-type microfluidic device that allows the simultaneous combination of a plurality of substrate solutions with reagent solutions in separate isolated reaction compartments. It will be recognized, that a
10 substrate solution can include one or a plurality of substrates (e.g., target nucleic acids) and a reagent solution can include 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
15 compartments. In various embodiments, the number of separate reaction compartments 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.

[0294] In particular embodiments, the matrix-type microfluidic device is a DYNAMIC ARRAY™ IFC (“DA”) microfluidic device. A DA microfluidic device is a
20 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 No. WO05107938A2
25 (Thermal Reaction Device and Method For Using The Same) and U.S. Patent Publication No. 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
30 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. In certain embodiments, DA microfluidic devices may be used for reacting M number of different samples with N number of different reagents.

5 **[0295]** Although the DA microfluidic devices described in WO05107938 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 in its entirety) includes a
10 diagram illustrating the 48.48 DYNAMIC ARRAY™ IFC, 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.

15 **[0296]** In specific embodiments, the microfluidic device can be a DIGITAL ARRAY™ IFC 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 compartments. In some embodiments, the DIGITAL ARRAY™ IFC microfluidic device is fabricated, at least in part, from an elastomer. Illustrative DIGITAL ARRAY™ IFC microfluidic devices are described in
20 copending U.S. Applications owned by Fluidigm Corp. (South San Francisco, CA), 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 compartments with a total volume
25 of 4.59 µL per panel. Microfluidic channels can connect the various reaction compartments on the panels to fluid sources. Pressure can be applied to an accumulator in order to open and close valves connecting the reaction compartments 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 chip when pressure is
30 applied to accumulator. Additionally, two or more inlets can be provided to provide hydration to the chip.

[0297] While the DIGITAL ARRAY™ IFC microfluidic devices are well suited for carrying out certain amplification methods described herein, one of ordinary skill in the art would recognize many variations and alternatives to these devices. The geometry of a given DIGITAL ARRAY™ IFC microfluidic device will depend on the particular application.

5 Additional description related to devices suitable for use in the methods described herein is provided in U.S. Patent Publication No. 20050252773, incorporated herein by reference for its disclosure of DIGITAL ARRAY™ IFC microfluidic devices.

[0298] In certain embodiments, the methods described herein can be performed using a microfluidic device that provides for recovery of reaction products. Such devices
10 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) and sold by Fluidigm Corp. as ACCESS ARRAY™ IFC (Integrated Fluidic Circuit).

[0299] In an illustrative device of this type, independent sample inputs are combined
15 with primer inputs in an MxN array configuration. Thus, each reaction is a unique combination of a particular sample and a particular reagent mixture. Samples are loaded into sample compartments in the microfluidic device through sample input lines arranged as columns in one implementation. Assay reagents (e.g., primers) are loaded into assay compartments in the microfluidic device through assay input lines arranged as rows
20 crossing the columns. The sample compartments and the assay compartments 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 compartments 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
25 pairwise combinations, producing one or more reaction product(s) in each compartment. The reaction products are harvested and can then be used for subsequent 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,
30 “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.

[0300] According to particular embodiments, the reaction products from the MxN pairwise combinations can be recovered from the microfluidic device in discrete pools, e.g., one for each of 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 $\pm 25\%$ within a sample and no more than $\pm 25\%$ 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. In certain embodiments, 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.

[0301] In some embodiments, reaction products are recovered by dilation pumping. 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 compartments 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 $\mu\text{l}/\text{hour}$. (i.e., $48 \times 1.5 / 0.5$ hour). In other embodiments, the removal rate of the reaction products is performed at a rate of less than 90 $\mu\text{l}/\text{hr}$, 80 $\mu\text{l}/\text{hr}$, 70 $\mu\text{l}/\text{hr}$, 60 $\mu\text{l}/\text{hr}$, 50 $\mu\text{l}/\text{hr}$, 40 $\mu\text{l}/\text{hr}$, 30 $\mu\text{l}/\text{hr}$, 20 $\mu\text{l}/\text{hr}$, 10 $\mu\text{l}/\text{hr}$, 9 $\mu\text{l}/\text{hr}$, less than 8 $\mu\text{l}/\text{hr}$, less than 7 $\mu\text{l}/\text{hr}$, less than 6 $\mu\text{l}/\text{hr}$, less than 5 $\mu\text{l}/\text{hr}$, less than 4 $\mu\text{l}/\text{hr}$, less than 3 $\mu\text{l}/\text{hr}$, less than 2 $\mu\text{l}/\text{hr}$, less than 1 $\mu\text{l}/\text{hr}$, or less than 0.5 $\mu\text{l}/\text{hr}$.

[0302] 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 compartments (e.g., sample compartments) 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 compartments.

[0303] The methods described herein may use microfluidic devices with a plurality of “unit cells” that generally include a sample compartment and an assay compartment.

5 Such unit cells can have 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 compartments and the assay compartments are selected to provide amounts of materials sufficient for desired processes while reducing sample and assay
10 usage. As examples, sample compartments 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
15 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 compartments can have similar dimensional ranges, typically providing similar steps sizes over smaller ranges than the smaller compartment volumes. In some
20 embodiments, the ratio of the sample compartment volume to the assay compartment volume is about 5:1, 10:1, 15:1, 20:1, 25:1, or 30:1. Smaller compartment volumes than the listed ranges are included within the scope of the invention and are readily fabricated using microfluidic device fabrication techniques.

[0304] Higher density microfluidic devices will typically utilize smaller
25 compartment volumes in order to reduce the footprint of the unit cells. In applications for which very small sample sizes are available, reduced compartment volumes will facilitate testing of such small samples.

[0305] For single-particle analysis, microfluidic devices can be designed to facilitate
loading and capture of the particular particles to be analyzed. Figure 9 shows the unit cell
30 architecture for an illustrative microfluidic device for analyzing mammalian cells. Each unit cell has a “cell channel” (i.e., sample compartment) and an “assay channel” (i.e., assay

compartment). The cell channel is rounded for loading mammalian cells, with dimensions on the order of tens microns in diameter to a hundred of several hundred microns in length. Diameters can be about 15 μm , about 20 μm , about 25 μm , about 30 μm , about 35 μm , about 40 μm , or about 45 μm or more, or can fall within a range having any of these values as endpoints, depending on the size of the cells being analyzed. Lengths can be about 60 μm , about 90 μm , about 120 μm , about 150 μm , about 170 μm , about 200 μm , about 230 μm , about 260 μm , about 290 μm or more, or can fall within a range having any of these values as endpoints, depending on the size of the cells being analyzed. In an illustrative microfluidic device based on the ACCESS ARRAY™ IFC platform (the “MA006”), a unit cell for loading mammalian cells can be about 30 $\mu\text{m} \times 170 \mu\text{m}$. Such a device can be equipped to provide, or to facilitate providing, heat to cell channels after loading to lyse the cells. As shown in Figure 9, the device can include assay channels separate from cell channels for conducting reactions such as nucleic acid amplification. 170 $\mu\text{m} \times 170$ containment valves can be used to close cell channels.

[0306] Co-pending U.S. App. No. 61/605,016, filed February 29, 2012, and entitled “Methods, Systems, And Devices For Multiple Single-Particle or Single-Cell Processing Using Microfluidics,” describes methods, systems, and devices for multiple single-particle or single-cell processing utilizing microfluidics. Various embodiments provide for capturing, partitioning, and/or manipulating individual particles or cells from a larger population of particles of cells along with generating genetic information and/or reaction(s) related to each individual particle or cell. Some embodiments may be configured for imaging the individual particles or cells or associated reaction products as part of the processing. This application is incorporated by reference herein in its entirety and, in particular, for its description of microfluidic devices configured for multiple single-particle or single-cell processing and related systems.

[0307] In specific embodiments, a microfluidic device is employed that facilitates assays having 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.

[0308] 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 devices 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.

Data Output and Analysis

[0309] In certain embodiments, when the methods described herein 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 compartment 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 product, 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

[0310] In particular embodiments, the methods described herein are used in the analysis of one or more nucleic acids, e.g. (in some embodiments), in or associated with a particle. 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, rearrangements, 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, cancer, pharmacogenomics

5 (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 described herein can be applied in the identification of sequences indicative of particular conditions or organisms in biological or environmental
10 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.

[0311] In certain embodiments, these methods can also be employed in
15 determinations of 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
20 disease states) and/or at different developmental stages.

[0312] In addition, the methods can be employed to prepare nucleic acid samples for further analysis, such as, e.g., DNA sequencing.

[0313] Furthermore, nucleic acid samples can be tagged as a first step, prior
subsequent analysis, to reduce the risk that mislabeling or cross-contamination of samples
25 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
30 another could be used to establish chain of custody of the sample.

[0314] As discussed above, the methods described herein can be used in the analysis of other parameters of particles besides nucleic acids, such as, for example, the expression level(s) of one or more proteins in or associated with each particle. In some embodiments, one or more nucleic acids are analyzed, together with one or more other parameters, for each particle.

[0315] The ability to associate assay results for multiple parameters with each particle in a population of particles can be exploited in a variety of different types of investigations. In various embodiments, the methods described herein can be employed to identify two or more of a variation such as a copy number variation, a mutation, an expression level variation, or a splice variant, wherein the variations are, together, correlated with a phenotype. The phenotype can, for example, be risk, presence, severity, prognosis, and/or responsiveness to a specific therapy of a disease or resistance to a drug. The methods described here can also be used to detect the co-occurrence of particular nucleic acid sequences, which can indicate genomic recombination, co-expression of particular splice variants, co-expression of particular light and heavy chains in B cells. The methods are also applicable to detecting presence of a particular pathogen in a particular host cell, e.g., where both pathogen-specific and host cell-specific nucleic acids (or other parameter) co-occur in the same cell. The methods can also be employed for targeted re-sequencing from circulating tumor cells, e.g., at mutation hot spots in different cancers.

Kits

[0316] Kits according to the invention can include one or more reagents useful for practicing one or more assay methods described herein. 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. In specific embodiments, the kit includes one or more matrix-type microfluidic devices discussed above.

[0317] In certain embodiments, the invention includes kits for performing the above-described method of adding adaptor molecules to each end of a plurality of target

nucleic acids that include sticky ends. These embodiments are useful, for example, in fragment generation for high-throughput DNA sequencing. Such kits can include a plurality of adaptor molecules that are designed to be used in this method (see above) and one or more components selected from the group consisting of a DNase enzyme, an exonuclease, an endonuclease, a polymerase, and a ligase.

[0318] In particular embodiments, the invention includes kits for combinatorial barcoding. A kit for performing a four-primer method, for example, can include a polymerase and:

(i) inner primers including:

10 a forward, inner primer including a first nucleotide tag, a first barcode nucleotide sequence, and a target-specific portion; and

a reverse, inner primer including a target-specific portion, a first barcode nucleotide sequence, and a second nucleotide tag; and

(ii) outer primers including:

15 a forward, outer primer including a second barcode nucleotide sequence and a first nucleotide tag-specific portion; and

a reverse, outer primer including a second nucleotide tag-specific portion and a second barcode nucleotide sequence, wherein the outer primers are in excess of the inner primers. A kit for performing a six-primer, combinatorial barcoding method can include a polymerase and:

(i) inner primers including:

a forward, inner primer including a first nucleotide tag and a target-specific portion; and

25 a reverse, inner primer including a target-specific portion and a second nucleotide tag;

(ii) stuffer primers including:

a forward, stuffer primer including a third nucleotide tag, a first barcode nucleotide sequence, and a first nucleotide tag-specific portion; and

a reverse, stuffer primer including a second nucleotide tag-specific portion, a first barcode nucleotide sequence, a fourth nucleotide tag; and

(iii) outer primers including:

5 a forward, outer primer including a second barcode nucleotide sequence and a third nucleotide tag-specific portion; and

a reverse, outer primer including a fourth nucleotide tag-specific portion and a second barcode nucleotide sequence, wherein the outer primers are in excess of the stuffer primers, which are in excess of the inner primers.

[0319] In other embodiments, the invention includes kits for combinatorial ligation-based tagging. These kits include a plurality of adaptors including:

a plurality of first adaptors, each comprising the same endonuclease site, N different barcode nucleotide sequences, wherein N is an integer greater than 1, a first primer binding site and a sticky end;

15 a second adaptor comprising a second primer binding site and a sticky end; and

a plurality of third adaptors including a second barcode nucleotide sequence and sticky ends complementary to those produced upon cutting the first adaptors at the endonuclease site, wherein the plurality of third adaptors include M different second barcode nucleotide sequences, wherein M is an integer greater than 1. Such kits can optionally include an endonuclease specific for the endonuclease site in the first adaptors and/or a ligase.

[0320] The invention also provides kits for tagging by insertional mutagenesis, which can also be employed for combinatorial tagging, as described above. In certain embodiments, such kits include:

25 one or more nucleotide tags(s); and

a plurality of barcode primers, wherein each barcode primer includes:

a first portion that is specific for a first portion of the nucleotide tag(s) linked to;

a barcode nucleotide sequence that does not anneal to the nucleotide tag(s) linked to;

5 a second portion that is specific for a second portion of the nucleotide tag(s), wherein the barcode primers in the plurality each include the same first and second tag-specific portions, but M different second barcode nucleotide sequences, wherein M is an integer greater than one. In specific embodiments, the nucleotide tag(s) include transposon ends, and the kit additionally includes a transposase, which can add transposon ends to target nucleic acids. Such kits can also, optionally, include a polymerase.

[0321] The invention includes kits useful in bidirectional nucleic acid sequencing.

10 In particular embodiments, such a kit can include:

a first set of outer primers, wherein the set includes:

a first outer, forward primer including a portion specific for a first primer binding site; and

15 a first outer, reverse primer including a barcode nucleotide sequence and a portion specific for a second primer binding site, wherein the first and second primer binding sites are different;

a second set of outer primers, wherein the set includes:

a second outer, forward primer including a barcode nucleotide sequence and a portion specific for the first primer binding site; and

20 a second outer, reverse primer including a portion specific for the second primer binding site. The first and second primer binding sites can, in certain embodiments, be binding sites for DNA sequencing primers. In some embodiments, the outer primers can each additionally include an additional nucleotide sequence, wherein:

25 the first outer, forward primer includes a first additional nucleotide sequence, and the first outer, reverse primer includes a second additional nucleotide sequence; and

the second outer, forward primer includes the second additional nucleotide sequence, and the second outer, reverse primer includes the first additional nucleotide sequence; and the first and second additional nucleotide sequences are different.

30 In a specific, illustrative embodiment, the first set of outer primers includes PE1-CS1 and

PE2-BC-CS2, and the second set of outer primers includes PE1-CS2 and PE2-BC-CS1 (Table 1, Example 9).

[0322] Bidirectional nucleic acid sequencing kits including the two sets of outer primers can also, optionally include a set of inner primers, wherein the set includes:

5 an inner, forward primer including a target-specific portion and the first primer binding site; and

an inner, reverse primer including a target-specific portion and the second primer binding site. In certain embodiments, the kit can include a plurality of sets of inner primers, each specific for a different target nucleic acid.

10 **[0323]** Any of these bidirectional nucleic acid sequencing kits can also, optionally, include DNA sequencing primers that:

bind to the first and second primer binding sites and prime sequencing of the target nucleotide sequence(s); and/or

15 bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequence(s). In particular embodiments, both types of DNA sequencing primer are included in the kit, and the primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequences(s) are reverse complements of the primers that prime sequencing of the target nucleotide sequences. In a specific, illustrative embodiment, the kit includes DNA sequencing primers
20 CS1, CS2, CS1rc, and CS2rc (Table 2, Example 9).

[0324] Kits generally include instructions for carrying out one or more of the methods described herein. Instructions included in kits 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
25 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.

30 **[0325]** 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.

[0326] In addition, all other publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

5

EXAMPLES

Example 1

A General Library Preparation Method for DNA Sequencing

[0327] Current methods of preparing libraries for nucleic acid sequencing are cumbersome and require multiple steps. The essence of the methods involves random
10 fragmentation of the DNA (for example), followed by end repair, polishing of fragment ends and ligation of end adaptors. These steps each require specific reaction conditions and purification of products between each step.

[0328] This Example and Figures 1 and 2 describe an alternative approach to library preparation. This approach uses a degenerate sequencing adaptor, which can be a double
15 stranded DNA molecule that comprises the end adaptors (or portions thereof) for a given sequencer, a restriction enzyme digestion site (or other specific cleavage site), and flanking degenerate sequences at the 3' end of both strands. Alternatively, the adaptors can be hairpin sequences, or double stranded oligonucleotides. It would also be possible for the end adaptors to be single-stranded oligonucleotides with degenerate sequences at the 3' end.

20 [0329] DNA would be fragmented using standard methods (enzymatic digest, nebulization, sonication, for example). Enzymatic digests would be preferred, as they cause less damage to the DNA molecules for downstream steps. For example, DNase I would be added to the DNA to be sequenced. This reaction could be stopped by heat treatment.

[0330] Double stranded DNA would then be digested back to single-stranded DNA
25 at the ends using T4 polymerase in the absence of NTPs, or a strand-specific exonuclease without polymerase activity. An exonuclease would be preferred, as it could work in concert with a ligase (e.g., a thermostable ligase) and polymerase (e.g. PHUSION®) within a single reaction. However, the prep method would still work in multiple steps if T4 polymerase were used.

[0331] The nuclease digestion would expose one strand at the ends of the DNA. Adaptor sequences would be added in the presence of a polymerase and a ligase. Adaptor sequences will anneal to the digested DNA, and gaps will be filled and repaired with the polymerase/ligase mixture. In one version of this protocol, the adaptor sequences would be made from hairpin structures, so that during the digestion/ligation/polymerisation, the end product would be circularized DNA. This would be protected from further degradation by the exonuclease, resulting in the accumulation of end product.

Example 2

Combinatorial Ligation-Based Barcoding for Illumina Sequencing

10 [0332] Prepare DNA sequencing libraries, with standard PE2-BC-tag sequence replaced by RE-1-BC-tag.

[0333] PE2 tag sequence downstream of barcode sequence replaced with recognition site (RE-1) for restriction enzyme, (e.g. BsrD1) which leaves short overhang:

5'-TGCATAGCAATGNN|CTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-Target-3' (SEQ ID NO:2)

15 3'-ACGTATCGTTAC|NNGATCCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-Target-5' (SEQ ID NO:3)

[0334] Cut library with enzyme.

[0335] Ligate adapter molecules containing the appropriate overhang and a second barcode sequence:

5'-CAAGCAGAAGACGGCATACGAGATAGCTNN+CTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-Target-3'

20 (SEQ ID NO:4)

3'-GTTCTGCTTCTGCGTATGCTCTATCGA NNGATCCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-Target-5'

(SEQ ID NO:5)

[0336] Ligation will result in the following construct:

5'-CAAGCAGAAGACGGCATACGAGATAGCTNNCTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-Target-3'

25 (SEQ ID NO:6)

3'-GTTCTGCTTCTGCGTATGCTCTATCGANNNGATCCACTGACCTCAAGTCTGCACACGAGAAGGCTAGA-Target-5'

(SEQ ID NO:7)

[0337] Remove left-over adaptor molecules before sequencing using standard cleanup methods.

[0338] During the index read on the sequencing run, the index sequence reported back will be: CTAGNNAGCT (SEQ ID NO:8).

5

Example 3

Single-Cell Analysis of Gene Expression

[0339] **Problem:** To obtain single cell gene expression data for a panel of genes using a DYNAMIC ARRAY™ IFC, the cell is first be isolated in a tube off-chip. The methods to isolate this cell are difficult to perform and/or require a large number of cells.

10 Where cells are limited, such as primary cells from tissue and/or cells from drug screening experiments in mini-well plates, this last obstacle becomes more of a barrier to obtaining gene expression data from single cells using the BioMark.

[0340] **Solution:** An ACCESS ARRAY™ IFC (“chip”), or similar chip that allows recovery of reaction mixtures, can be used to load single cells via limiting dilution (MA006 chip, for example.) By using the chip as an apparatus to sort and prepare the cells for downstream gene expression analysis, a limited number of cells can be prepped for the DYNAMIC ARRAY™ IFC with ease, thus providing a solution to the problems outlined above. The steps of the invention are as follows:

[0341] 1) Load cells in limiting dilution in an ACCESS ARRAY™ IFC. Load primer sets as shown in Figure 7A. Any given cell will be exposed to all gene-specific primers and a single unique barcode primer.

[0342] 2) Do reverse transcription and preamplification in the chip. An example of an amplicon generated is shown in Figure 7B. This is a 3 primer approach. The advantage of using this approach is that only one set of 96 primer pairs (or more, for as many genes that are desired) needs to be designed and ordered for a particular experiment. The BC reverse primers are universal and used in all experiments. Any given cell will have amplified all genes and all amplicons will have have been tagged with a single barcode. (See possible variations below).

[0343] 3) Export the reaction products by pool (90 degrees to different primer sets, i.e. by sample). Pool N now contains a preamp of 96 genes (or more or less), with a mixture of barcodes, where one barcode is matched with one cell. The pools are kept separate, such that even

30

though multiple cells are tagged with the same barcode, they are distinguishable because they belong to different pools.

[0344] 4) Load a DYNAMIC ARRAY™ IFC as shown in Figure 7C. Note: Single cells can be tracked on the ACCESS ARRAY™ chip via a variety of methods. This provides the information regarding which pool and which barcode pre-amp reaction had a single cell, i.e., which should be loaded on a DYNAMIC ARRAY™ IFC. This selection allows us to read only ACCESS ARRAY™ IFC chambers which contained a cell, resulting in efficient use of the DYNAMIC ARRAY™ IFC. Moreover, if cells of interest are delineated by using cell-specific stains, i.e., antibody for a cell-surface marker, then only this subset of cells could be selected for loading in the DYNAMIC ARRAY™ IFC. This could become important where cells are rare in a heterogeneous population of cells, i.e., stem cells, cancer stem cells, cancer cells.

[0345] 5) Run qPCR, with EvaGreen for detection. By amplifying a combination of one BC primer and one gene specific primer, gene expression for a single cell (whose amplicons were tagged with a BC primer during preamplification in the ACCESS ARRAY™ IFC) for a given gene (whose amplification will be detected by the gene specific primer in the DYNAMIC ARRAY™ IFC) can be obtained.

[0346] **Possible variations:** There are different detection methods, that have the common end result of preamplifying a set of genes and tagging individual cells with a unique barcode. Examples are as follows:

[0347] Doing the same as above but use a 2-primer approach.

[0348] Use Fen-Ligase Chain Reaction.

[0349] Use Melting Temperature strategy.

Example 4

Alternative Methods of Detecting Reaction Products from Example 3

[0350] Instead of detecting BC-tagged amplicons from preamplification in the ACCESS ARRAY™ IFC using qPCR with EvaGreen, ligase chain reaction is carried out in a DYNAMIC ARRAY™ IFC (e.g., M96) with real time detection.

[0351] An illustrative amplicon has the structure: 5'-forward primer sequence-target nucleotide sequence-reverse primer sequence-barcode nucleotide sequence-3'. In this

case, one primer can anneal to the reverse primer sequence, and the other primer can anneal to the adjacent barcode nucleotide sequence, which is followed by ligation and repeated cycles of annealing and ligation. See Figure 8A. Amplicons in the pool that have either a different reverse primer (“R”) are derived from a different target nucleic acids (here, messenger RNA), and amplicons in the pool that have a different barcode primer (“BC”) will not be amplified. Therefore, amplification of pool_N with BC_M amplifies barcoded target nucleic acids from the chamber in ACCESS ARRAY™ IFC row N, column M. The use of R₁ as the other primer in this amplification amplifies the amplicon derived from the target nucleic acid corresponding to R₁.

[0352] One method of real time detection is flap endonuclease-ligase chain reaction, which uses a 5' flap endonuclease and labeled BC_n primers, as shown in Figure 8B. This reaction employs a labeled probe and an unlabeled probe, wherein the simultaneous hybridization of the probes to a reaction product results in the formation of a flap at the 5' end of the labeled probe, and cleavage of the flap can cleave a fluorophore from a quencher, producing a signal. Since the BCs are not amplicon-specific, these primers need only be made once. One set of, e.g., 96 BCs would suffice for any number of different sets of F_nR_n amplicons.

[0353] Advantages of this strategy:

[0354] Selection of a pool and BC allows analysis of only those ACCESS ARRAY™ IFC chambers that contained a single cell (where single-cell analysis is the goal). Unlabeled cells can be detecting using brightfield or fluorescence imaging of the ACCESS ARRAY™ IFC. In addition, cells can be stained with a dye and/or a labeled antibody, prior to or upon loading into the ACCESS ARRAY™ IFC to identify cells of interest (e.g., stem cells, cancer cells, cancer stem cells, etc.). Selection of a pool and BC allows analysis of only those ACCESS ARRAY™ IFC chambers that contained a cell of interest, improving efficiency.

[0355] This strategy requires far fewer cells than FACS, which makes it possible for use in analyses that cannot be carried out using FACS, such as analyses of population of primary cells or cells from screening experiments.

Example 5**Method to Prepare Nucleic Acids for Sequencing from Single Cells using ACCESS
ARRAY™ IFC Adapted for Cell Handling (“MA006”)****Summary of General Approach**

5 [0356] A “chip,” herein referred to as MA006, has been developed using the
ACCESS ARRAY™ IFC platform as have methods using MA006 that integrate cell
handling and sample preparation for nucleic acid sequencing. See Figure 9 for a schematic
diagram of the MA006 unit cell architecture, showing on-chip processes. This integration
simplifies the steps required to execute the experiment. Moreover, only hundreds of cells are
10 required to load the chip.

[0357] The MA006 chip has the following features:

Unit cell with 170 x 30 pm rounded channel to load mammalian cells

48.48 matrix format;

Use heat to lyse cells in cell channels;

15 Separate reaction chamber for amplification reaction;

170 x 170 pm containment valves to close cell channels;

Extra resist layer: PourOB - 30 gm rounded resist;

Chip fabrication: Use current AA48.48 processes;

65 pm alignment tolerance;

20 130 pm punch diameter;

65 x 85 pm valve size; and

3-layer design process.

There are no cell capture features on the MA006 chip. The result is that a limiting dilution
strategy is used to obtain the desired number of cells per chamber. However, cell capture
25 features can be designed into the chip. They can be physical (for example, cups, or chalice
structures), biological (for example, spotted peptides), or chemical (for example, charged
ions).

[0358] Cell Handling off of the chip: Cells to be analyzed are prepared to a density such that a desired number of cells per sample chamber (“cell channel” in Figure 9) is obtained. Since the MA006 chip uses a limiting dilution strategy, the number of cells per chamber follows a Poisson distribution, both theoretical and real. Since, in the first instance, a maximum number of chambers containing a single cell was desired, the optimal cell density was 300-600 cells per microliter. Minimal volumes of one to two microlitres can be applied to the inlets. Therefore, experiments can be carried out with only hundreds of cells. Any cell type (i.e., mammalian, bacterial, etc.) from any source can be used (i.e., living organisms, tissue culture, etc.). Any form or extent of preparation, washing, and/or staining can be used, as long as this is compatible with downstream applications.

[0359] Cell tracking in the chip: In the absence of any polymerase/amplification dependent chemistry, the cells in the chip can be monitored for position, identity, and/or content using brightfield or fluorescence microscopy. The cells can be stained with any stain (i.e., nucleic acid-specific staining, such as SYTO10; immunodetection, such as Cy5 conjugated anti-CD19; etc.) as long as this is compatible with downstream applications. This can be used, for example, to identify rare cells, i.e. cancer stem cells, in a heterogeneous cell population.

[0360] Chemistry: After the cells are loaded into the MA006, the assays are loaded in the assay chamber (“assay channel” in Figure 9), and the interface valves are released to mix the contents of the sample and assay chambers. The chip is subjected to thermal cycling according to the selected chemistry and imaged in real-time or at the end point if this is required and/or supported by the chemistry. This procedure is not limited to gene-specific amplification, i.e. non-specific degenerate primers can be used, or RNA-specific amplification can be carried out. In the case of gene-specific amplification, more than one gene can be targeted simultaneously using a “multiplex” strategy. The chemistry is flexible, provided that the output is a substrate for sequencing, and should not be restricted to polymerase chain reaction or even amplification.

Cell Handling

Cell Counting: Brightfield Imaging

[0361] RAMOS cells were handled as follows:

- (1) Harvest cells.
- (2) Wash 2-3X in ice-cold Tris Saline BSA buffer.
- (3) Count and make appropriate dilution. The theoretical distribution (Poisson distribution) for various cell densities is shown in Figure 10.
- 5 (4) Push cells into MA006 chip.
- (5) Image by brightfield.

Figure 11A-B shows the results of cell counting in the chip using brightfield (A) to image, as compared to the theoretical distribution (B). Cell density in the chip, based on brightfield imaging, is close to, but lower than, the Poisson distribution, with this tendency exacerbated at higher cell densities. This may be due, in part, to “shadowing” created by chip features, which can reduce the measurable area within which cells can be detected using brightfield imaging.

Cell Counting: Post-PCR Fluorescence

[0362] Cells were loaded into the MA006 chip at 0.15E6/ml and subjected to RT-PCR using Cells-Direct™ RT PCR components, Rox, and EVA green. Figures 12A-B show that fluorescent cell “ghost” images (A) permit detection of more cells than pre-PCR brightfield, so that the cell density more closely approximates the Poisson distribution (B). Based on these results, if 4000 cells are applied per inlet (e.g., 4 µl of 1000 cell/µl) of the MA006 chip and distributed throughout, approximately 1/3 of 2304 (48 × 48) or 800 chambers have a single cell.

More Specific Approaches

[0363] More specific methods for detecting cells in the chip that can be used include, e.g., the use of a cell membrane-permeant nucleic acid stain and/or cell-specific surface marker detection with an antibody. Thus, for example, RAMOS cells could be handled as follows:

- (1) Harvest cells.
- (2) Wash 2-3X in ice-cold Tris Saline BSA buffer.
- (3) Stain with Syto10 DNA stain and/or Cy5-labeled anti-CD19 antibodies.

- (4) Wash 2-3X in ice-cold Tris Saline BSA buffer.
- (5) Count and make appropriate dilution.
- (6) Push cells into MA006 chip.
- (7) Image.

5 The results of these more specific approaches are shown for a cell density of 1E6/ml in Figure 13. Figure 14A shows a comparison between pre-RT-PCR nucleic acid stain (Syto10 DNA stain) versus post RT-PCR ghost images (Cell ghost), and Figure 14B shows that Syto10 does not inhibit RT-PCR of GAPDH. A workflow for cell detection in the chip could include staining cells with a DNA stain and/or antibody, followed by counting pre-
10 RT-PCR and then counting cell ghosts as a back-up post-RT-PCR.

Chemistry: One-Step Gene-Specific RT-PCR

[0364] Different chemistries were investigated to find an efficient chemistry to convert gene-specific RNA in cells into amplicons in the MA006 chip. Cells are pushed into cell channels in Tris Saline BSA (0.5 µg/ml) buffer. Reagents loaded into assay
15 channels included:

Primers (500 nM final concentration)

CellsDirect™ One-Step qRT-PCR kit components (available from Life Technologies, Foster City, CA)

Reaction Mix

20 Enzyme Mix: Superscripte III + Platinum Taq Polymerase

Buffer

Rox

EVA Green

25 Loading Reagent – AA or GE (available from Fluidigm Corp., South San Francisco, CA) to prevent non-specific absorption by PDMS (“depletion effect”) and to lyse cells.

[0365] RT-PCR of GAPDH was carried out with or without AA or GE loading reagent. The results showed that both loading reagents inhibited RT-PCR. The loading reagents contain: Prionex (AA) or BSA (GE) and 0.5% Tween-20. RT-PCR of GAPDH was carried out in the presence of Prionex or BSA. Prionex, but not BSA, was found to inhibit RT-PCR. RT-PCR of GAPDH was carried out in the presence of 0.5% Tween 20 or 0.5% NP40 (the latter is a cell lysis reagent). The results of this study are shown in Figure 15. Neither 0.5% Tween 20 or 0.5% NP40 inhibited RT-PCR of GAPDH significantly.

[0366] To determine that the reaction conditions developed for RT-PCR of GAPDH from cells would permit RT-PCR of other genes, expressed at different levels, RT-PCR of 11 genes covering a range of expression levels was carried out with 10 ng/μl of RNA and the reagents described above, except that 0.5% NP40 was substituted for AA/GE Loading Reagent. The thermal protocol was 50°C for 30 minutes; 55°C for 30 minutes; 95°C for 2 minutes; and then 45 cycles of: 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 60 seconds. Standard curve amplification of these 11 genes, carried out in the MA006 chip, is shown in Figure 16. These results demonstrate that the CellsDirect™ One-Step qRT-PCR kit can be used with 0.5% NP40 (for cell lysis and to prevent the depletion effect in the chip) to convert gene-specific RNA in cells into amplicons in the MA006 chip.

Sequencing

[0367] To facilitate sequencing of gene-specific amplicons generated in the MA006 chip, a barcoding method was employed to distinguish amplicons from different chambers (e.g., cells). More specifically, a four-primer, combinatorial barcoding method was employed to put a combination of two barcodes on either end of each amplicon. This method is shown schematically in Figure 17. Inner primers include target-specific portions (“TS-F” in the forward primer and “TS-R” in the reverse primer), a barcode nucleotide sequence (“bc2”), and different nucleotide tags. Outer primers include tag-specific portions (“CS1” and “CS2”), a different barcode nucleotide sequence (“bc1”), and primer binding sites for sequencing primers (“A” and “B”). Figure 18A-B illustrates how 4-primer barcoding can be carried out on a chip, such as the MA006. Amplification is carried out on-chip with inner primers, where each row of chambers has the same pair of inner primers with the same barcode. Reaction products from each column of chambers can be harvested as a pool and each pool subjected to amplification using a different pair of outer primers.

This amplification produces amplicons having barcode combinations at either end of the amplicon that uniquely identify the chamber (by row and column) in which the initial amplification was carried out. The reaction products were sequenced and the number of reads of each sequence for each reaction chamber was determined. This determination was carried out for RAMOS cells and for spleen RNA. Figure 19 shows a comparison of the results obtained, expressed as number of reads for each gene-specific amplicon (red), as compared to that for total RNA. As is apparent from this figure, the representation of these RNAs is different when measured in individual cells, as compared to that observed in the total RNA.

Example 6

Size-Based Microfluidic Single-Particle Capture

[0368] One approach to discretely capturing single cells from suspension as they flow through a microfluidic device is to define a microfluidic geometry that guides flow of a suspension of particles (such as cells or beads) over a capture site in a manner that the capture site catches a single particle, efficiently captures single particles (e.g., the probability of the capture of a particle passing near a capture site is high), and/or guides the remaining suspension around the capture site. The geometries can be size-based, i.e., the capture site is just large enough to contain one particle (and no more), but still permit the flow of particle-free suspension through the site at reasonably low fluidic impedance, such that an empty capture site would guide the flow of particles toward it rather than around it. This goal can be accomplished by the use of a drain. Additional geometries can also focus the flow of particles in a manner that increases the likelihood of particles coming in close enough proximity to the capture site for high probability of successful capture. Variations on these geometries have focused on controlling the flow resistance of the fluidics surrounding the capture site and drain, including the drain itself, as well as varying the aperture of focusing geometry in attempts to position the flow of particles close to the capture site. Figure 20A-B illustrates a capture site with a capture feature and drain. Panel A shows a site without baffles to focus flow, whereas panel B shows a site with baffles. Additional capture site designs are shown in Figure 21.

Example 7

Surface Marker-Based Capture of Particles

[0369] Single-cell studies within microfluidic architectures require the isolation of individual cells into individual reaction partitions (chambers, droplets, particles). Limiting dilution is one method for achieving this isolation. Cells are loaded at concentrations of less than one cell per partition on average, and distribute into those partitions in a pattern described by Poisson statistics. Another approach is to rely on mechanical traps to capture cells. These traps are designed to capture cells of a given size range (see Example 6). This results in a biased selection of cells from the population within that size range.

[0370] For some applications, an ideal capture method would use biological markers expressed on the surface of cells. Antibodies can be patterned in specific locations on a microfluidic array, although this approach may not be simple, depending on the structure of the microfluidic array.

[0371] This example describes a method for capture of single particles (e.g., cells) based on the initial capture of a single, affinity-reagent-coated bead in a specific location in a microfluidic device. The surface area presented by this bead at the opening of a capture site provides a defined surface of affinity reagent accessible for cell binding. The bead size and capture site can be chosen/designed such that once a single cell is bound to the bead, the rest of the accessible surface area of the bead is sterically blocked by the first-bound cell. Selection of an appropriate sized bead capture site also provides for capture of a broad range of cell sizes. As long as the cell is larger than the exposed capture area, and expresses the appropriate surface marker or binding partner for the affinity reagent, it should be possible to capture that cell.

[0372] Capture architectures can be designed to maximize the probability that cells will come into contact with the surface markers. For example, baffles on one or more channel walls can be used to direct beads towards capture feature. See Figure 22A for illustrative capture feature/baffle combinations. Performance of the capture feature can be adjusted by adjusting one or more variables, including angle of baffles, distance of baffles from capture site, length of baffles, size and shape of capture feature, size of drain in capture feature (if present). See Figures 22B and C illustrating the variables for, and

performance of, capture feature/baffle combinations. In Figure 22B, baffles on the channel wall are used to direct beads towards a capture feature. In Figure 22C, the capture feature is coupled to a baffle on a channel wall; individual capture feature/baffle combinations can be located on alternate walls to focus flow towards the adjacent capture feature/baffle combination. These combinations can be located at sites that, in use, are separable (e.g., using valves) to form separate reaction chambers.

[0373] Figures 23A and B illustrate (in simplified form, lacking baffles) a strategy for using capture features to catch single, affinity-reagent-coated beads, which then display the affinity reagent (e.g., antibody) so as to capture single particles (e.g., cells). In

Figure 23A-1, flow is initiated in a channel containing capture features. In panel A-2, antibody-bound beads flow toward the capture features until a bead lodges in the capture feature, as shown in panel A-3. The channel is then washed to remove non-captured beads. Subsequently, as shown in Figure 23B-1, cells bearing a cell-surface marker to which the antibody binds are flowed into the channel containing the captured beads. Panel B-2 illustrates how cells bearing the marker interact with and bind to antibodies displayed by the captured bead. The display area is sized so that a bound cell will inhibit other cells from interacting with the captured bead through steric occlusion, such that only one cell binds to each captured bead. The channel is then washed to remove non-bound cells, as shown in panel B-3, leaving one cell immobilized at each capture site.

Example 8

Microfluidic Device for Cell Capture ("CCap")

[0374] Figure 24A shows a schematic of a microfluidic device designed to capture single cells at discrete locations (niches). Flow is designed to be stronger over niches than through an overflow channel. Niches contain small gaps (~3 μm tall). See Figure 24B.

When a cell enters niche, it blocks the niche and prevents any more flow into the niche. Flow passes through next unoccupied niche, until it too is blocked by a cell. In theory, every niche should capture one cell before cells pass through the overflow channel and out to waste. Referring to Figure 24C-F for more detail, a buffer inlet converges with a cell inlet so as to force cells to a side of a feeder channel that is closest to a series of transverse cell capture channels. See Figure 24D. The resistance of the transverse cell capture channels is lower than that of a cell overflow channel to induce preferential flow of cells

into niches versus into the cell overflow channel. See Figure 24E. As shown in Figure 24F, each niche is large enough to capture just one cell. The niche gap is sufficiently small that cells are captured at the operational pressure/flow levels. If the latter are too high and/or the niche gaps are too large, cells may deform and be pushed through the niche gaps. The presence of a cell in a niche raises the resistance of that particular circuit, and flow is therefore directed to circuits without cell. Figure 24G shows an actual device with captured human umbilical vein endothelial cells (HUVEC) located in niches.

Example 9

Bidirectional DNA Sequencing Amplicon Tagging for Illumina Sequencers using the **48.48 ACCESS ARRAY™ IFC – Protocol 1**

Introduction

[0375] The following protocol outlines a bidirectional sequencing strategy on the Illumina Genome GAI, HiSeq, and MiSeq Sequencers for amplicon libraries that have been generated on the ACCESS ARRAY™ System. The goal of this protocol is to sequence both ends of PCR products with a single read sequencing run. In a standard 4-primer amplicon tagging approach (see Example 6), tagged target-specific (TS) primer pairs were combined with sample-specific primer pairs containing a barcode sequence (BC) and the adaptor sequences used by the Illumina sequencers (PE1 and PE2, Figure 25A). Here, in the bidirectional sequencing amplicon tagging strategy, by contrast, tagged target-specific primer pairs were combined with two sets of sample-specific primer pairs. The sample-specific primer pairs were comprised of common sequence tags CS1 or CS2, appended with the Illumina adaptor sequences in both permutations (PE1 and PE2, Figure 25B). This approach required only one set of target-specific primer pairs while the sample-specific barcode primers were universal and could be used in multiple experiments.

[0376] Bidirectional sequencing amplicon tagging generated two types of PCR products per target region: one PCR product that allowed for sequencing of the 5' end of the target region (product A) and one PCR product that allowed for sequencing of the 3' end of the target region (product B). Because both PCR products were present on the flow cell at the same time, one sequencing read yielded sequence information for both ends of the target region. The main difference between this strategy and paired-end sequencing (Example 6)

is that the 5' read and the 3' read were not derived from the same cluster, i.e., from the same template molecule. Instead, an average of the template population was derived.

[0377] Amplification of multiple target sequences can be done prior to adding the Bidirectional barcode. In short, the protocol adopts a two-step approach: the PCR on the ACCESS ARRAY IFC was run in the presence of multiplexed, tagged, target-specific primers only. The harvested PCR product pools were then used as template in a second PCR with the sample-specific barcode primers. The two sets of barcode primers were added in independent PCR reactions as described below.

[0378] Sample-specific barcode primer pairs were segregated out into two separate PCR reactions (Figure 26; see also Table 1).

Table 1. Barcode primers used in the segregated-primer PCR strategy.

Primer	Sequence
PE1-CS1	5'-AATGATACGGCGACCACCGAGATCTACACTGACGACATGGTTCTACA-3' (SEQ ID NO:9)
PE2-BC-CS2	5'-CAAGCAGAAGACGGCATACGAGAT-[BC]-TACGGTAGCAGAGACTTGGTCT-3' (SEQ ID NO:10)
PE1-CS2	5'-AATGATACGGCGACCACCGAGATCTTACGGTAGCAGAGACTTGGTCT-3' (SEQ ID NO:11)
PE2-BC-CS1	5'-CAAGCAGAAGACGGCATACGAGAT-[BC]-ACACTGACGACATGGTTCTACA-3' (SEQ ID NO:12)

[0379] After the barcoding PCR, the PCR products of both the 5' reaction and the 3' reaction were combined and used as template for cluster formation on the flow cell.

Because both PCR product types were present and formed clusters on the flow cell, an equimolar mixture of the CS1 and CS2 sequencing primers allowed for simultaneous sequencing of both PCR product types (Figure 27). Similarly, the index read with an equimolar mixture of the CS1rc and CS2rc sequencing primers allowed for simultaneous sequencing of the barcodes of both PCR product types.

[0380] The *Fluidigm® IFC Controller for ACCESS ARRAY™ System User Guide* (PN 68000157) may be consulted as a reference for this protocol. The Illumina website may be consulted for up to date protocols, reagent and catalog number information.

Preparing and sequencing amplicons

[0381] The following reagents were used for this protocol and were stored at -20°C: FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001); 20X ACCESS ARRAY™ Loading Reagent (Fluidigm, PN 100-0883); Target-specific primer

pairs with universal tags (CS1 forward tag, CS2 reverse tag), including 50 μ M CS1-Tagged TS Forward Primer and 50 μ M CS2-Tagged TS Reverse Primer; and Bidirectional 384 Barcode Kit for the Illumina GAII, HiSeq and MiSeq Sequencers (Fluidigm, PN 100-3771). Additional reagents were stored at 4°C, including: Agilent DNA 1000 Kit Reagents

5 (Agilent, PN 5067-1504); and 1 X ACCESS ARRAY™ Harvest Solution (Fluidigm, PN 100-1031). Other reagents were stored at room temperature, including PCR Certified Water (Teknova, PN W330); DNA Suspension Buffer (10mM Tris HCl, 0.1mM EDTA, pH8.0) (Teknova, PN T0221); and Agilent DNA 1000 Chips (included in the Agilent DNA 1000 DNA kit) (Agilent).

10 [0382] The following equipment and consumables were used for this protocol: 1.5 mL or 2 mL microcentrifuge tubes; Microcentrifuge with rotor for 2 mL tubes; Microcentrifuge with rotor for 0.2 mL PCR tube strips; Centrifuge with plate carriers; Agilent 2100 BioAnalyzer (Agilent); 96-Well Reaction Plate; MicroAmp Clear Adhesive Film (Applied Biosystems, PN 4306311); IFC Controller AX (2 quantity, pre- and post-
15 PCR) (Fluidigm); FC1 Cycler (Fluidigm); 48.48 ACCESS ARRAY™ IFC s (Fluidigm); and Control Line Fluid Syringes (Fluidigm, PN 89000020).

[0383] Multiplex PCR on the ACCESS ARRAY™ IFC was performed according to the instructions as detailed in Chapter 6 - Multiplex PCR on the 48.48 ACCESS ARRAY™ IFC of the *Fluidigm ACCESS ARRAY™ System for Illumina Platform User Guide*.

20 [0384] Barcoding PCR was performed according to the instructions as detailed in Chapter 6 - Attaching Sequence Tags and Sample Barcodes of the *Fluidigm ACCESS ARRAY System for Illumina Platform User Guide*. The 100X dilution of the harvested PCR product pool served as template in two rather than one barcoding PCR reactions: one reaction generated PCR product A that allowed for sequencing of the 5' end of the target
25 region, the other reaction generated PCR product B that allowed for sequencing of the 3' end of the target region. The set up of the reaction was identical to "Attaching Sequence Tags and Sample Barcodes" in the *Fluidigm ACCESS ARRAY System for Illumina Platform User Guide*. However, the quantities in the Sample Pre-Mix Master Mix were doubled to compensate for the increase in the number of wells. After the second PCR had finished,
30 PCR Product A and PCR Product B pools were combined prior to sequencing. Chapter 8 of

the *Fluidigm ACCESS ARRAY™ System for Illumina Platform User Guide* provides methods describing post-PCR product library purification and quantitation.

[0385] The remainder of this Example provides the sequencing workflow used in the protocol.

- 5 [0386] The following instructions for preparing reagents are intended for use with Illumina TruSeq sequencing reagents. The Fluidigm reagents FL1 and FL2 contain equimolar mixtures of the CS1 and CS2 sequencing and indexing primers respectively. FL1 is the sequencing primer and contains 50 μ M each of the CS1 and CS2 primers. FL2 is the indexing primer and contains 50 μ M each of the CS1rc and CS2rc primers. Sequences for
10 these primers are shown in Table 2.

Table 2 Primers and sequences

Primer	Sequence
CS1	5'-ACACTGACGACATGGTTCTACA-3' (SEQ ID NO:13)
CS2	5'-TACGGTAGCAGAGACTTGGTCT-3' (SEQ ID NO:14)
CS1rc	5'-TGTAGAACCATGTCGTCAGTGT-3' (SEQ ID NO:15)
CS2rc	5'-AGACCAAGTCTCTGCTACCGTA-3' (SEQ ID NO:16)

- [0387] The sequencing primer HP6/FL1 was prepared by diluting Fluidigm reagent FL1 (which contains the custom sequencing primers) to a final concentration of 0.25 μ M in
15 TruSeq reagent HP6 in a DNase, RNase free 0.5 mL microfuge tube, as shown in Table 3. The primer was vortexed after mixing to ensure complete mixing.

Table 3 Instructions for Preparing HP6/FL 1 (per mL)

Reagent	Volume
TruSeq reagent HP6	995 μ L
FL1	5 μ L
Total	1000 μL

- [0388] The indexing primer HP8/FL2 was prepared by diluting Fluidigm reagent
20 FL2 (which contains the custom indexing primers) to a final concentration of 0.25 μ M in Truseq reagent HP8 in a DNase, RNase free 0.5ml microfuge tube, as shown in Table 4. The primer was vortexed after mixing to ensure complete mixing.

Table 4 Instructions for Preparing HP8/FL2 (per mL)

Reagent	Volume
TruSeq reagent HP8	995 μ L
FL2	5 μ L
Total	1000 μL

[0389] Clusters were generated using detailed instructions in the *Illumina cBot™*

5 *User Guide, Illumina Cluster Station User Guide, or Illumina MiSeq User Guide.* To hybridize the sequencing primer, the sequencing primer reagent HP6/FL1 was used for the first read.

[0390] Sequencing reagents were prepared and loaded onto the sequencer according to the manufacturer's instructions. For Read 1, the instructions provided by the

10 manufacturer were followed for conducting a multiplexed single-read sequencing run.

[0391] For the index read, the index reagent HP7/FL2 was substituted rather than the HP7 reagent. The barcode sequences used in the Fluidigm Bidirectional Primer Library were designed so that they could be distinguished even when sequencing errors are present. As more samples are run in parallel, the length of the index read required to distinguish the

15 barcode sequences unambiguously increases. Recommendations for index reads are described in Table 5.

Table 5 Index Read Recommendations

Number of samples per lane	1-96	97-384	385-1920
Length of index read	6 bases	8 bases	10 bases

[0392] When preparing the sequencing run, the length of the index read was

20 adjusted according to the guidelines in Table 5. The volumes of the sequencing reagents loaded onto the sequencer were ensured to be sufficient for the index cycles. These changes were implemented according to the manufacturer's recommendations.

Example 10**Detailed Procedure for Tagging Target Nucleic Acids for Bidirectional Illumina Sequencing Using a Microfluidic Device that Permits Recovery of Amplification****Products**

- 5 [0393] 394 primer pairs were designed to PCR amplify exons from the genes BRCA1, BRCA2, PTEN, PI3KCA, APC, EGFR, TP53 (See Table 6 below). Forward primers were appended with the Tag8 sequence, and reverse primers were appended with the Tag5 sequence. The 394 primers were arranged in 48 groups containing, on average, approximately 8 primer pairs per group, at a concentration of 1 μ M per primer in 0.05% Tween-20. Sample mixes were prepared from 48 cell-line genomic DNA samples (see
- 10 Table 7 below) by adding 1 μ l of sample (50 ng/ μ l) to 3 μ l pre-sample mix, which contained 1U Roche Faststart HiFi polymerase, 1x buffer, 100 μ M dNTPs, 4.5 mM MgCl₂, 5% DMSO, and 1x ACCESS ARRAY™ sample loading solution.
- 15 [0394] The ACCESS ARRAY™ IFC was run according to instructions in the ACCESS ARRAY™ User Guide. Sample mixes were loaded into the sample ports of an ACCESS ARRAY 48.48™ IFC. Groups of primers were loaded into the inlets of the ACCESS ARRAY 48.48™ IFC. PCR was carried out on a Fluidigm stand-alone thermal
- 20 cyclizer using the standard PCR protocol supplied with the thermal cyclizer. After PCR, products were harvested from the ACCESS ARRAY™ IFC using a separate controller. One microliter of each product was then transferred to a PCR plate and diluted 100x with PCR-grade water. Three PCR plates were then prepared containing 4 μ l of PCR mastermix (1U Roche Faststart HiFi polymerase, 1x buffer, 100 μ M dNTPs, 4.5 mM MgCl₂, 5% DMSO and barcode primers as described below in Table 8). Plate 1 contained a pair of
- 25 primers bearing barcodes FL001-FL0048 of the form PE2-CS1/PE1-BC-CS2, with each primer having a concentration of 400 nM. Plate 2 contained a pair of primers bearing barcodes FL001-FL0048 of the form PE2-CS2/PE1-BC-CS1, with each primer at a concentration of 400 nM. Plate 3 contained two pairs of primers bearing barcodes FL0049-FL0096 of the form PE2-CS1/PE2-CS2/PE1-BC-CS1/PE1-BC-CS2. All three plates were subjected to 15 cycles of PCR using the following thermal protocol (95°C 10 min ;15x(
- 30 95°C 15 s, 60°C 30 s, 72°C 90 s); 72°C 3 min).

[0395] Each of the reaction products from each plate was analyzed on an Agilent 1000 Bioanalyzer chip, and concentrations of the PCR product pool were measured based on electropherograms from the analysis (Figure 28). PCR products from each plate were pooled to equal concentrations using volumes adjusted according to concentrations obtained from the Agilent Bioanalyzer.

[0396] The pooled sample was cleaned up using AMPure beads (Beckman Coulter) with a bead to sample ratio of 1:1.

[0397] The amplicon pool was sequenced on two separate lanes of a Genome Analyzer II (Illumina). The first lane used CS1 and CS2 primers for the first read, and the CS1rc and CS2rc primers for the index read. Because the annealing temperatures of CS1 and CS2 are predicted to be 10°C below those of the standard Illumina Read 1 and Index sequencing primers, LNA (locked-nucleic acid) versions of CS1, CS2, CS1rc and CS2rc were used in order to optimize hybridization to the cluster under the standard conditions described in the Illumina Cluster Station and Genome Analyzer manuals.

[0398] For sequencing, the second lane used a pool of the target-specific forward and reverse primers assembled from primers that were used during amplification on the ACCESS ARRAY™ IFC (Figure 29). The CS1/CS2rc indexing primer was used for the index read. Due to their increased length, the target-specific primers have annealing temperatures higher than those of CS1 or CS2. This approach circumvented reading through the uninformative target-specific primer portion of the PCR products. Instead, sequencing information with the lowest error rate was obtained from an informative region of the PCR product where there was the least amount of overlap between the 5' and 3' reads. The approach also allowed for greater overlap where the sequencing error rate is the greatest (i.e., the middle of the PCR product), and an increase in PCR product size of 30-40 bp.

[0399] Sequence data were demultiplexed using Illumina software and aligned to the human genome reference sequence build hg19 using the aligner ELAND (Illumina). The per-base coverage of the gene EGFR for an illustrative sample is shown in Figure 30.

Table 6 Primers for Amplifying Exons from the Genes BRCA1, BRCA2, PTEN, PI3KCA, APC, EGFR, TP53

Primer plate well	Gene/chr	Name	F-primer	R-primer
A1	APC	APC_SE00000972224_1	ACACTGACGACATGGTTCTACACCTTATAGGTCCAAGGGTAGC (SEQ ID NO:17)	TACGGTAGCAGAGACTTGGTCTAAAGTCACAGTCTTGATACCTTCA (SEQ ID NO:18)
B1	APC	APC_972224_1_1	ACACTGACGACATGGTTCTACAGTTGAGGCACTGAAGATGG (SEQ ID NO:19)	TACGGTAGCAGAGACTTGGTCTTTTAAGTAAAGTGTCTTACCTCAAGTTT (SEQ ID NO:20)
C1	APC	APC_SE00000917796_1	ACACTGACGACATGGTTCTACAGCA TACTTAAATGTCAAGAAATACAGAATCA (SEQ ID NO:21)	TACGGTAGCAGAGACTTGGTCTTAAATCTACCTTTAAGACGCTCTAATAAAT (SEQ ID NO:22)
D1	APC	APC_SE00000917796_2	ACACTGACGACATGGTTCTACAGTACTTAAACAACTACAAGGAAGTATTGA (SEQ ID NO:23)	TACGGTAGCAGAGACTTGGTCTACC AACACCCAAATCGAGAGA (SEQ ID NO:24)
E1	APC	APC_972225_2	ACACTGACGACATGGTTCTACATCATGTCATGTATATTTGTGGTTAAATGT (SEQ ID NO:25)	TACGGTAGCAGAGACTTGGTCTCTCTCCCGGCTTCCATAAGA (SEQ ID NO:26)
F1	APC	APC_SE00000972225_7	ACACTGACGACATGGTTCTACAGCGGTCAAAAATGTCCCTCC (SEQ ID NO:27)	TACGGTAGCAGAGACTTGGTCTACTGGAGTACACAAGGCAATGTT (SEQ ID NO:28)
G1	APC	APC_SE00000917798_1	ACACTGACGACATGGTTCTACATCTTCTGCAGTCTTTATTAGCATTGT (SEQ ID NO:29)	TACGGTAGCAGAGACTTGGTCTAGTTTCAAATAAGTTGTACTGCCAAG (SEQ ID NO:30)
H1	APC	APC_SE00000972226_1	ACACTGACGACATGGTTCTACATGCTTTTTGCTTTTACTGATTAACG (SEQ ID NO:31)	TACGGTAGCAGAGACTTGGTCTCCTGTGCTCGTTTTTCCATATCC (SEQ ID NO:32)
A2	APC	APC_SE00000972226_2	ACACTGACGACATGGTTCTACATTACAAACAGATATGACCAGAAGGC (SEQ ID NO:33)	TACGGTAGCAGAGACTTGGTCTTAACAGAGCTgTAATTCATTTTATTCTCT (SEQ ID NO:34)
B2	APC	APC_SE00000972227_1	ACACTGACGACATGGTTCTACACCC TGAGCTTTTAAGTGGTAGC (SEQ ID NO:35)	TACGGTAGCAGAGACTTGGTCTACC CACAAACAAGAAAGGCAA (SEQ ID NO:36)
C2	APC	APC_SE00000972228_1	ACACTGACGACATGGTTCTACATGGGCTAAGAAAGCCTACACC (SEQ ID NO:37)	TACGGTAGCAGAGACTTGGTCTaCC TGACCATTACCAGAAGTTGC (SEQ ID NO:38)
E2	APC	APC_SE00000917801_1	ACACTGACGACATGGTTCTACACTTCATTTGGAGTACCTTAACA (SEQ ID NO:39)	TACGGTAGCAGAGACTTGGTCTACCTTGGTTCCCAGATGACT (SEQ ID NO:40)
F2	APC	APC_SE00000917801_2	ACACTGACGACATGGTTCTACATCTATAATGTGCTTAATTTTAGGGTTCA (SEQ ID NO:41)	TACGGTAGCAGAGACTTGGTCTAGATGTCTTAGCAAAGTAGTCATGG (SEQ ID NO:42)
G2	APC	APC_SE00000917803_1	ACACTGACGACATGGTTCTACATCACTTAATTGGTTTTTGGCTTTTGA (SEQ ID NO:43)	TACGGTAGCAGAGACTTGGTCTCCAGACTGTCGCATGGAT (SEQ ID NO:44)
H2	APC	APC_SE00000917803_2	ACACTGACGACATGGTTCTACATGTGTCAATGCTTGGTACTCAT (SEQ ID NO:45)	TACGGTAGCAGAGACTTGGTCTGCCCGGAGCCTCTTTACTG (SEQ ID NO:46)
A3	APC	APC_SE00000917803_3	ACACTGACGACATGGTTCTACACCTCATCCAGCTTTTACATGGC (SEQ ID NO:47)	TACGGTAGCAGAGACTTGGTCTGCCACTCCCAACAGGTTTC (SEQ ID NO:48)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
B3	APC	APC_SE0000 0917803_4	ACACTGACGACATGGTTCTACATGC AGCACTCCACAACATCA (SEQ ID NO:49)	TACGGTAGCAGAGACTTGGTCTGCT TTGAAACATGCACTACGA (SEQ ID NO:50)
C3	APC	APC_SE0000 0917804_1	ACACTGACGACATGGTTCTACATCA TTGCTCTTCAAATAACAAAGCAT (SEQ ID NO:51)	TACGGTAGCAGAGACTTGGTCTAAA AATCCACCAGTAATTGTCTATGTC (SEQ ID NO:52)
D3	APC	APC_SE0000 0917807_1	ACACTGACGACATGGTTCTACAGGT ACCAGTTTGTATTTATTTAGATGATT GT (SEQ ID NO:53)	TACGGTAGCAGAGACTTGGTCTACA TACCTTGTGGCTACATCTCC (SEQ ID NO:54)
E3	APC	APC_917807_7	ACACTGACGACATGGTTCTACACGA TATGCTGGAATGGCTT (SEQ ID NO:55)	TACGGTAGCAGAGACTTGGTCTCAG TCATTGTTTAATGAGGAGAGTG (SEQ ID NO:56)
F3	APC	APC_SE0000 0760099_1	ACACTGACGACATGGTTCTACAGCT TGGCTTCAAGTTGTCT (SEQ ID NO:57)	TACGGTAGCAGAGACTTGGTCTAGG TGAAATTCTAAATAGTACCTGCT (SEQ ID NO:58)
G3	APC	APC_SE0000 0917809_1	ACACTGACGACATGGTTCTACAGCT AGCATTAACAAAAACAAAAAGCAACT (SEQ ID NO:59)	TACGGTAGCAGAGACTTGGTCTAAA GCACATTCCATCAATGC (SEQ ID NO:60)
A4	APC	APC_SE0000 0972229_6	ACACTGACGACATGGTTCTACAATT AGATGACCCATATTCTGTTTCTTAC (SEQ ID NO:61)	TACGGTAGCAGAGACTTGGTCTCAA TAATGGCTAAAGTGTGTCTGG (SEQ ID NO:62)
B4	APC	APC_E00000 972229_15	ACACTGACGACATGGTTCTACAGTG CTGTAGATGGTGCACCTTG (SEQ ID NO:63)	TACGGTAGCAGAGACTTGGTCTATT AGGTCtTTTGTAGAGTATGAATTCTG (SEQ ID NO:64)
C4	APC	APC_SE0000 0972230_1	ACACTGACGACATGGTTCTACACTG CATAACATTGTGACCTT (SEQ ID NO:65)	TACGGTAGCAGAGACTTGGTCTCCC CCATGTCCCATAATGCTT (SEQ ID NO:66)
D4	APC	APC_SE0000 0972230_2	ACACTGACGACATGGTTCTACATGC ATGTGGAACCTTGTGGA (SEQ ID NO:67)	TACGGTAGCAGAGACTTGGTCTTGG CATCCTTGTACTTCGC (SEQ ID NO:68)
E4	APC	APC_SE0000 0972230_5	ACACTGACGACATGGTTCTACAATG ATTGCTATGGGAAGTGCT (SEQ ID NO:69)	TACGGTAGCAGAGACTTGGTCTCGA TGAGATGCCTTGGGACT (SEQ ID NO:70)
G4	APC	APC_E00000 972230_22	ACACTGACGACATGGTTCTACACAA GCAAAGTCTCTATGGTGAT (SEQ ID NO:71)	TACGGTAGCAGAGACTTGGTCTACT TCTATCTTTTTCAGAACGAGAACTAT (SEQ ID NO:72)
H4	APC	APC_E00000 972230_24	ACACTGACGACATGGTTCTACAGCA ACATGACTGTCCTTTCACC (SEQ ID NO:73)	TACGGTAGCAGAGACTTGGTCTGCA AACCTCGCTTTGAAGA (SEQ ID NO:74)
A5	APC	APC_E00000 972230_25	ACACTGACGACATGGTTCTACAGGC AACTACCATCCAGCAA (SEQ ID NO:75)	TACGGTAGCAGAGACTTGGTCTGCA GAGCTTCTTCTAAGTGCAT (SEQ ID NO:76)
B5	APC	APC_E00000 972230_27	ACACTGACGACATGGTTCTACAGTC ATGGAAGAAGTGTCAGC (SEQ ID NO:77)	TACGGTAGCAGAGACTTGGTCTTGT ATTCTAATTTGGCATAAGGCAT (SEQ ID NO:78)
C5	APC	APC_E00000 972230_28	ACACTGACGACATGGTTCTACAGCC CATAACATTCAAACACTTAC (SEQ ID NO:79)	TACGGTAGCAGAGACTTGGTCTCAG AATAGGATTCAATCGAGGGT (SEQ ID NO:80)
D5	APC	APC_E00000 972230_29	ACACTGACGACATGGTTCTACATCA AATGATAGTTTAAATAGTGTCAGTA GTAG (SEQ ID NO:81)	TACGGTAGCAGAGACTTGGTCTATA ATTTATTGGTGTATCTAGTTCTCCAT C (SEQ ID NO:82)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
E5	APC	APC_E00000 972230_30	ACACTGACGACATGGTTCTACAGCc GACCTAGCCCATAAA (SEQ ID NO:83)	TACGGTAGCAGAGACTTGGTCTCCT TGATTGTCTTTGCTCACTT (SEQ ID NO:84)
F5	APC	APC_E00000 972230_31	ACACTGACGACATGGTTCTACAACCT CTGGAAGGCAAAGTCCT (SEQ ID NO:85)	TACGGTAGCAGAGACTTGGTCTCCC CGTGACCTGTATGGAGA (SEQ ID NO:86)
G5	APC	APC_E00000 972230_33	ACACTGACGACATGGTTCTACATGG ACAGCAGGAATGTGTTT (SEQ ID NO:87)	TACGGTAGCAGAGACTTGGTCTGGT CTCTCTTCTTCTTCATGCT (SEQ ID NO:88)
H5	APC	APC_E00000 972230_35	ACACTGACGACATGGTTCTACATGA TAAGCCTACCAATTATAGTGAACG (SEQ ID NO:89)	TACGGTAGCAGAGACTTGGTCTGCT TTGTCCAGATGAACTCTTT (SEQ ID NO:90)
A6	APC	APC_E00000 972230_36	ACACTGACGACATGGTTCTACAGTT TAAATATGCCACAGATATTCCTTCA (SEQ ID NO:91)	TACGGTAGCAGAGACTTGGTCTGCC TTTTGAGGCTGACCACT (SEQ ID NO:92)
B6	APC	APC_E00000 972230_37	ACACTGACGACATGGTTCTACAGCA GTGAGAATACGTCCACAC (SEQ ID NO:93)	TACGGTAGCAGAGACTTGGTCTCAG CTGATGACAAAGATGATAATGAA (SEQ ID NO:94)
C6	APC	APC_E00000 972230_38	ACACTGACGACATGGTTCTACAGCC ACTTGCAAAGTTTCTTCT (SEQ ID NO:95)	TACGGTAGCAGAGACTTGGTCTAGC TGACCTAGTTCCAATCTTTT (SEQ ID NO:96)
D6	APC	APC_E00000 972230_39	ACACTGACGACATGGTTCTACAGAC GACACAGGAAGCAGAT (SEQ ID NO:97)	TACGGTAGCAGAGACTTGGTCTGGA GATTTCGCTCCTGAAGAA (SEQ ID NO:98)
E6	APC	APC_E00000 972230_40	ACACTGACGACATGGTTCTACATCC TGTGAGCGAAGTTCCA (SEQ ID NO:99)	TACGGTAGCAGAGACTTGGTCTACA TCTGCTAAACATGAGTGGG (SEQ ID NO:100)
F6	APC	APC_E00000 972230_41	ACACTGACGACATGGTTCTACACCC aAAAGTCCACCTGA (SEQ ID NO:101)	TACGGTAGCAGAGACTTGGTCTGCT TGGTGGCATGGTTTG (SEQ ID NO:102)
G6	APC	APC_E00000 972230_43	ACACTGACGACATGGTTCTACAGCC AGCTCCGTTCCAGAGT (SEQ ID NO:103)	TACGGTAGCAGAGACTTGGTCTGCA GCTTGCTTAGGTCCAC (SEQ ID NO:104)
H6	APC	APC_E00000 972230_45	ACACTGACGACATGGTTCTACAGCA CCTACTGCTGAAAAGAGAG (SEQ ID NO:105)	TACGGTAGCAGAGACTTGGTCTCCA CATCTTTCTGTATAAATGGCTCA (SEQ ID NO:106)
A7	APC	APC_E00000 972230_47	ACACTGACGACATGGTTCTACACCA CgGAAAGTAcTCCAGATG (SEQ ID NO:107)	TACGGTAGCAGAGACTTGGTCTGCC TCTTTCTTTGGTTTCA (SEQ ID NO:108)
B7	APC	APC_E00000 972230_52	ACACTGACGACATGGTTCTACACCA GTTTCAGGAAAATGACAATGGG (SEQ ID NO:109)	TACGGTAGCAGAGACTTGGTCTGG GCTGGCTTTTTTGCTT (SEQ ID NO:110)
C7	APC	APC_E00000 972230_59	ACACTGACGACATGGTTCTACATCT GCCATGCCAACAAAGTC (SEQ ID NO:111)	TACGGTAGCAGAGACTTGGTCTGTC CCTTCAACACAATACACCC (SEQ ID NO:112)
D7	APC	APC_E00000 972230_60	ACACTGACGACATGGTTCTACATCT ACCATCACAAAACAGGTTGC (SEQ ID NO:113)	TACGGTAGCAGAGACTTGGTCTGAC TGTGcCCTCCTCTA (SEQ ID NO:114)
E7	APC	APC_E00000 972230_61	ACACTGACGACATGGTTCTACAACCT TTTCCACAGCTACATCTCT (SEQ ID NO:115)	TACGGTAGCAGAGACTTGGTCTTGT CATCCAATTCAGGTATGGT (SEQ ID NO:116)
F7	APC	APC_E00000 972230_62	ACACTGACGACATGGTTCTACACCA TTCCTACAGAAGGCAGAA	TACGGTAGCAGAGACTTGGTCTAAG AaGAcGCAGATGCTTGC

Primer plate well	Gen e/chr	Name	F-primer	R-primer
			(SEQ ID NO:117)	(SEQ ID NO:118)
G7	APC	APC_E00000 972230_63	ACACTGACGACATGGTTCTACAGCA TTAATTCTGCTATGCCCAA (SEQ ID NO:119)	TACGGTAGCAGAGACTTGGTCTTTT TGAGTCTGCATTTTTCTTACAC (SEQ ID NO:120)
H7	APC	APC_E00000 972230_64	ACACTGACGACATGGTTCTACATGC ACCCAACAAAAATCAGTTAG (SEQ ID NO:121)	TACGGTAGCAGAGACTTGGTCTAGC TTATCATTGAAGaCCTTGGAA (SEQ ID NO:122)
B8	APC	APC_E00000 972230_70	ACACTGACGACATGGTTCTACAGAG TCAGAGGAAGTTTTGCTTTTG (SEQ ID NO:123)	TACGGTAGCAGAGACTTGGTCTGgT TAGTTCTGTGTGGCTGGT (SEQ ID NO:124)
C8	APC	APC_E00000 972230_71	ACACTGACGACATGGTTCTACATTC CAGGGAAAAGGCTGAATTA (SEQ ID NO:125)	TACGGTAGCAGAGACTTGGTCTGGT ATGTCTTTGGATGACTGGG (SEQ ID NO:126)
D8	APC	APC_E00000 972230_85	ACACTGACGACATGGTTCTACACCC ATACTTCAGAAACAATCCACT (SEQ ID NO:127)	TACGGTAGCAGAGACTTGGTCTGG GCTCAGTCTCTTTGATAGGT (SEQ ID NO:128)
E8	APC	APC_E00000 972230_87	ACACTGACGACATGGTTCTACATCT CTCAgTGACATTGACCAAGAA (SEQ ID NO:129)	TACGGTAGCAGAGACTTGGTCTGCA ACAGGTCATCTTCAGAGT (SEQ ID NO:130)
F8	APC	APC_E00000 972230_93	ACACTGACGACATGGTTCTACAtcC AAGAAACAGTTCTCTCAGTT (SEQ ID NO:131)	TACGGTAGCAGAGACTTGGTCTGTT CTGAATCTGGTCTCTGTATATCT (SEQ ID NO:132)
G8	APC	APC_E00000 972230_95	ACACTGACGACATGGTTCTACAGG GTGATAATGAAAAACATAGTCCA (SEQ ID NO:133)	TACGGTAGCAGAGACTTGGTCTGCA GCAGCAGCTTGATGTAA (SEQ ID NO:134)
H8	APC	APC_972230_95_3	ACACTGACGACATGGTTCTACAGCT ATTCAGGAAGGTGCAAA (SEQ ID NO:135)	TACGGTAGCAGAGACTTGGTCTCCC TGGTTTTAGAATTCTGTTGGG (SEQ ID NO:136)
A9	APC	APC_0000097 2230_116	ACACTGACGACATGGTTCTACATGA TTCAGATTCCATCCTTTCCCT (SEQ ID NO:137)	TACGGTAGCAGAGACTTGGTCTACT TTCAGATTCTATCTTTTAGTTTCCA A (SEQ ID NO:138)
B9	APC	APC_0000097 2230_126	ACACTGACGACATGGTTCTACAGGC CCACGAATTCTAAAACCA (SEQ ID NO:139)	TACGGTAGCAGAGACTTGGTCTGTC CTGCCTCGAGAGATTG
C9	APC	APC_972230_126_2	ACACTGACGACATGGTTCTACATGA AAGTAAAGGAATCAAAGGAGGA (SEQ ID NO:140)	TACGGTAGCAGAGACTTGGTCTACA GGACTTGTAATTGAGGAGC (SEQ ID NO:141)
E9	APC	APC_0000097 2230_140	ACACTGACGACATGGTTCTACAGGA GTTGAAATAGCTCCTCAAG (SEQ ID NO:142)	TACGGTAGCAGAGACTTGGTCTAGA AGGTGCTTTACTTGACCCA (SEQ ID NO:143)
F9	APC	APC_0000097 2230_141	ACACTGACGACATGGTTCTACATCC TAGAGGAGcCAAGCCAT (SEQ ID NO:144)	TACGGTAGCAGAGACTTGGTCTAGG ACTTATTCCATTTCTACCAGGG (SEQ ID NO:145)
G9	APC	APC_0000097 2230_142	ACACTGACGACATGGTTCTACATCT AGAGATTcGACCCCTTCA (SEQ ID NO:146)	TACGGTAGCAGAGACTTGGTCTCAT CTGTCTACCTGGAGATGTATATG (SEQ ID NO:147)
H9	APC	APC_0000097 2230_143	ACACTGACGACATGGTTCTACATCA ACTTCCAAGGACATCATCCC (SEQ ID NO:148)	TACGGTAGCAGAGACTTGGTCTGG CTCCATTACCATTATTCATCTG (SEQ ID NO:149)
A10	APC	APC_0000097 2230_144	ACACTGACGACATGGTTCTACAGCC AACAGAACCTTACCAAACA (SEQ ID NO:150)	TACGGTAGCAGAGACTTGGTCTGAC TGGCGTACTAATACAGGT (SEQ ID NO:151)

Primer plate well	Gene/chr	Name	F-primer	R-primer
B10	APC	APC_0000097 2230_145	ACACTGACGACATGGTTCTACATCA ACTAAATCAAGTGAAGTGAATCTG (SEQ ID NO:152)	TACGGTAGCAGAGACTTGGTCTAAC TGGAGTTTGTGCCTGGG (SEQ ID NO:153)
C10	APC	APC_0000097 2230_147	ACACTGACGACATGGTTCTACAAGA AGAAAATTGGAGGAATCTGC (SEQ ID NO:154)	TACGGTAGCAGAGACTTGGTCTCCA TCATTATACTATAGTGGGACTG (SEQ ID NO:155)
D10	APC	APC_0000097 2230_148	ACACTGACGACATGGTTCTACAGTC CTTCCCTTCTGATATGTCTC (SEQ ID NO:156)	TACGGTAGCAGAGACTTGGTCTGTG CTCACGTTTCCAGTT (SEQ ID NO:157)
E10	APC	APC_0000097 2230_149	ACACTGACGACATGGTTCTACACCA gCAAAGCGCCATGATA (SEQ ID NO:158)	TACGGTAGCAGAGACTTGGTCTCAC TTTTTGCTTTTCACTGGATT (SEQ ID NO:159)
F10	APC	APC_0000097 2230_150	ACACTGACGACATGGTTCTACATTC ATCATCCCTTCTCTG (SEQ ID NO:160)	TACGGTAGCAGAGACTTGGTCTCCA TGTTCCCTTTGCGGAT (SEQ ID NO:161)
G10	APC	APC_0000097 2230_152	ACACTGACGACATGGTTCTACAGGA ACCAAACAAAGTAAAGAAAACCA (SEQ ID NO:162)	TACGGTAGCAGAGACTTGGTCTGGA CAGTCCTCAATTCTCACCC (SEQ ID NO:163)
H10	APC	APC_0000097 2230_153	ACACTGACGACATGGTTCTACACCG TTTCCTCAGGTGCTACAA (SEQ ID NO:164)	TACGGTAGCAGAGACTTGGTCTGAA TCTTTAATGTTTGGATTGCTT (SEQ ID NO:165)
A11	APC	APC_0000097 2230_155	ACACTGACGACATGGTTCTACACGG TGATTGACAGTGTTCAG (SEQ ID NO:166)	TACGGTAGCAGAGACTTGGTCTCTG ATACAGGGACAGGATTA (SEQ ID NO:167)
B11	APC	APC_0000097 2230_157	ACACTGACGACATGGTTCTACATGG ATGCCCTGACCAAAA (SEQ ID NO:168)	TACGGTAGCAGAGACTTGGTCTGCT TTTCCTAGGGCTTGGGT (SEQ ID NO:169)
C11	APC	APC_0000097 2230_159	ACACTGACGACATGGTTCTACATCT AGCAGCTCAAGCAAACAC (SEQ ID NO:170)	TACGGTAGCAGAGACTTGGTCTCCA CTGGATTCTGTGCTGTC (SEQ ID NO:171)
D11	APC	APC_0000097 2230_160	ACACTGACGACATGGTTCTACACGC AGATAGCACTTCAGCTC (SEQ ID NO:172)	TACGGTAGCAGAGACTTGGTCTAGA ATTTTCTTAGTTTCATTCTTCTCTC (SEQ ID NO:173)
A1	PTEN	PTEN_E0000 1456562_1	ACACTGACGACATGGTTCTACAGCA GCTTCTGCCATCTCTCT (SEQ ID NO:174)	TACGGTAGCAGAGACTTGGTCTCCG TCTACTCCACGTTCT (SEQ ID NO:175)
B1	PTEN	PTEN_E0000 1156351_1	ACACTGACGACATGGTTCTACATGC TGCATATTTAGATATTTCTTCTT A (SEQ ID NO:176)	TACGGTAGCAGAGACTTGGTCTATG AAAACACAACATGAATATAAACATCA AT (SEQ ID NO:177)
C1	PTEN	PTEN_E0000 1156344_1	ACACTGACGACATGGTTCTACAATC TGTCTTTTGGTTTTCTTGATAGT (SEQ ID NO:178)	TACGGTAGCAGAGACTTGGTCTAAT AGTTGTTTGAAGATATTTGCAAGC (SEQ ID NO:179)
D1	PTEN	PTEN_E0000 1156337_4	ACACTGACGACATGGTTCTACATAT ATCACTTTTAACTTTCTTTTAGTT GTGC (SEQ ID NO:180)	TACGGTAGCAGAGACTTGGTCTCGA TAATCTGGATGACTCATTATTGTT (SEQ ID NO:181)
E1	PTEN	PTEN_E0000 1156330_1	ACACTGACGACATGGTTCTACATtCT TATTCTGAGGTTATCTTTTACCAC (SEQ ID NO:182)	TACGGTAGCAGAGACTTGGTCTCAT TACACCAGTTCGTCCCT (SEQ ID NO:183)
F1	PTEN	PTEN_E0000 1156330_3	ACACTGACGACATGGTTCTACATGA CCAATGGCTAAGTGAAGATGA (SEQ ID NO:184)	TACGGTAGCAGAGACTTGGTCTCCA GGAAGAGGAAAGGAAAAACA (SEQ ID NO:185)

Primer plate well	Gene/chr	Name	F-primer	R-primer
G1	PTEN	PTEN_E00001156327_1	ACACTGACGACATGGTTCTACATCTTAAATGGCTACGACCCAG (SEQ ID NO:186)	TACGGTAGCAGAGACTTGGTCTCCAGATGATTCTTTAACAGGTAGC (SEQ ID NO:187)
H1	PTEN	PTEN_E00001156327_4	ACACTGACGACATGGTTCTACAGTCAGAGGCGCTATGTGT (SEQ ID NO:188)	TACGGTAGCAGAGACTTGGTCTAGATATGGTTAAGAAACTGTTCCA (SEQ ID NO:189)
A2	PTEN	PTEN_E00001156321_1	ACACTGACGACATGGTTCTACATGACAGTTTGACAGTTAAAGGCAT (SEQ ID NO:190)	TACGGTAGCAGAGACTTGGTCTCACACACAGGTAACGGCTGA (SEQ ID NO:191)
B2	PTEN	PTEN_E00001156321_2	ACACTGACGACATGGTTCTACATGTGGTCTGCCAGCTAAAGG (SEQ ID NO:192)	TACGGTAGCAGAGACTTGGTCTCCCAATGAAAGTAAAGTACAAACC (SEQ ID NO:193)
C2	PTEN	PTEN_E00001156321_4	ACACTGACGACATGGTTCTACATCCACAAACAGAACAAGATGCT (SEQ ID NO:194)	TACGGTAGCAGAGACTTGGTCTGGCCTTTTCTTCAAACAGGATT (SEQ ID NO:195)
D2	PTEN	PTEN_E00001156315_1	ACACTGACGACATGGTTCTACAGCAACAGATACTCAGATTGCCTT (SEQ ID NO:196)	TACGGTAGCAGAGACTTGGTCTGTTTCTCTGGTCTGGTATGA (SEQ ID NO:197)
E2	PTEN	PTEN_E00001156315_5	ACACTGACGACATGGTTCTACAGGACAAAATGTTTCACTTTTGGGTAA (SEQ ID NO:198)	TACGGTAGCAGAGACTTGGTCTACTAGATATTCCTTGTCTATTATCTGCAC (SEQ ID NO:199)
F2	PTEN	PTEN_E00001156315_7	ACACTGACGACATGGTTCTACACCTCAGAAAAgTAGAAAATGGAAGTC (SEQ ID NO:200)	TACGGTAGCAGAGACTTGGTCTACAGTCAaCAACCCCCACA (SEQ ID NO:201)
G2	PTEN	PTEN_E00001456541_1	ACACTGACGACATGGTTCTACAGATGAGTcATATTTGTGGGTTTCA (SEQ ID NO:202)	TACGGTAGCAGAGACTTGGTCTGGATCAGAGTCAGTGGT (SEQ ID NO:203)
H2	PTEN	PTEN_E00001456541_2	ACACTGACGACATGGTTCTACAGTAGAGGAGCCGTCAAATCCA (SEQ ID NO:204)	TACGGTAGCAGAGACTTGGTCTTCATGGTGTATCCCTCTTGA (SEQ ID NO:205)
A3	PIK3CA	PIK3CA_0001825398_1	ACACTGACGACATGGTTCTACAGGTTCCTGCTTTGGGACAACC (SEQ ID NO:206)	TACGGTAGCAGAGACTTGGTCTCCTCACGGAGGCATTCTAAAG (SEQ ID NO:207)
B3	PIK3CA	PIK3CA_0001825398_3	ACACTGACGACATGGTTCTACAGTaGAATGTTTACTACCAAATGGAATG (SEQ ID NO:208)	TACGGTAGCAGAGACTTGGTCTCACAAAGTCGTCTTGTTCATCA (SEQ ID NO:209)
C3	PIK3CA	PIK3CA_0001825398_4	ACACTGACGACATGGTTCTACAGCAGAAAATACCCCTCCA (SEQ ID NO:210)	TACGGTAGCAGAGACTTGGTCTGATCTTTTCTTCACGGTTGCC (SEQ ID NO:211)
D3	PIK3CA	PIK3CA_1825398_3	ACACTGACGACATGGTTCTACAGACGACTTTGTGACCTTCGG (SEQ ID NO:212)	TACGGTAGCAGAGACTTGGTCTTTTAAAAAGGGACAACAGTTAAGC (SEQ ID NO:213)
E3	PIK3CA	PIK3CA_0000997375_1	ACACTGACGACATGGTTCTACATGTTCTATGCTGTGTATGTAATAGAA (SEQ ID NO:214)	TACGGTAGCAGAGACTTGGTCTACATACATTGCTCTACTATGAGGTGA (SEQ ID NO:215)
F3	PIK3CA	PIK3CA_0000997375_3	ACACTGACGACATGGTTCTACATTCgAAcGTTTGTAAAGAAGCTGT (SEQ ID NO:216)	TACGGTAGCAGAGACTTGGTCTGATAGAGTAGATTAGTCATTTTCTTACCTT (SEQ ID NO:217)
G3	PIK3CA	PIK3CA_997375_3	ACACTGACGACATGGTTCTACACCAGAATTGCCAAAGCACA (SEQ ID NO:218)	TACGGTAGCAGAGACTTGGTCTATTAGCACTCACTATATCTTGTGAC (SEQ ID NO:219)
H3	PIK3CA	PIK3CA_0001	ACACTGACGACATGGTTCTACACCC	TACGGTAGCAGAGACTTGGTCTCAG

Primer plate well	Gen e/chr	Name	F-primer	R-primer
	A	077693_1	TTGAAAAATGAAAGAGAGATGGT (SEQ ID NO:220)	AGGATAGCAACATACTTCGAG (SEQ ID NO:221)
A4	PIK3C A	PIK3CA_1077693_2	ACACTGACGACATGGTTCTACACCA TGACTGTGTACCAGAAC (SEQ ID NO:222)	TACGGTAGCAGAGACTTGGTCTAAC TTGTTACTCACCTTATACTGACT (SEQ ID NO:223)
B4	PIK3C A	PIK3CA_0001077692_1	ACACTGACGACATGGTTCTACATGA AAAACCTTACAGGAAATGGC (SEQ ID NO:224)	TACGGTAGCAGAGACTTGGTCTGTG GAAATGCGTCTGGAATAAGA (SEQ ID NO:225)
C4	PIK3C A	PIK3CA_0001077692_3	ACACTGACGACATGGTTCTACAGCC TTTATTCTCAACTGCCAA (SEQ ID NO:226)	TACGGTAGCAGAGACTTGGTCTAGC ATCAGCATTTGACTTTACCT (SEQ ID NO:227)
D4	PIK3C A	PIK3CA_0001077692_4	ACACTGACGACATGGTTCTACATCT ACAAAATCCCTTTGGGT (SEQ ID NO:228)	TACGGTAGCAGAGACTTGGTCTGAA AACATACTACAGGTCAACAGA (SEQ ID NO:229)
E4	PIK3C A	PIK3CA_0001077694_1	ACACTGACGACATGGTTCTACATCG AGTGTGTGCATATGTGT (SEQ ID NO:230)	TACGGTAGCAGAGACTTGGTCTACT GCTAAACATAATATAACCTTTGGA (SEQ ID NO:231)
F4	PIK3C A	PIK3CA_0001077691_5	ACACTGACGACATGGTTCTACAATT TTACATAGGTGGAATGAATGGCTGA (SEQ ID NO:232)	TACGGTAGCAGAGACTTGGTCTATC AGCGGTATAATCAGGAGTTTTTAAAGG (SEQ ID NO:233)
G4	PIK3C A	PIK3CA_0001128465_1	ACACTGACGACATGGTTCTACAGG GAAGAAAAGTGTGTTGAAATGTGT (SEQ ID NO:234)	TACGGTAGCAGAGACTTGGTCTACA CCAATAGGGTTCAGCAA (SEQ ID NO:235)
H4	PIK3C A	PIK3CA_0001128465_3	ACACTGACGACATGGTTCTACATCT TTGGCCAGTACCTCATGG (SEQ ID NO:236)	TACGGTAGCAGAGACTTGGTCTCAC ACTGCTGAACCACTCAA (SEQ ID NO:237)
A5	PIK3C A	PIK3CA_0001128465_5	ACACTGACGACATGGTTCTACATGG CAGTCAAACCTTCTCTCTT (SEQ ID NO:238)	TACGGTAGCAGAGACTTGGTCTACC AGTCCTGCGTGGGAA (SEQ ID NO:239)
B5	PIK3C A	PIK3CA_1128465_2	ACACTGACGACATGGTTCTACAGAC TGGTTCAGCAGTGTGGT (SEQ ID NO:240)	TACGGTAGCAGAGACTTGGTCTGCC AGTAAATATAAGGATCCTTTTCCA (SEQ ID NO:241)
C5	PIK3C A	PIK3CA_0001077674_1	ACACTGACGACATGGTTCTACATGC TTTTCTGTAAATCATCTGTGAA (SEQ ID NO:242)	TACGGTAGCAGAGACTTGGTCTGTA GAAATTGCTTTGAGCTGTTCTT (SEQ ID NO:243)
D5	PIK3C A	PIK3CA_0000826291_1	ACACTGACGACATGGTTCTACATCA ACCTTTTGAACAGCATGCAA (SEQ ID NO:244)	TACGGTAGCAGAGACTTGGTCTGAG AGAAAACAATTTAAGTGACATACCA (SEQ ID NO:245)
E5	PIK3C A	PIK3CA_0000826292_1	ACACTGACGACATGGTTCTACAGGC AGTGTGTTTAGATGGCTCA (SEQ ID NO:246)	TACGGTAGCAGAGACTTGGTCTCCA AGCACcGAACAGCAAAA (SEQ ID NO:247)
F5	PIK3C A	PIK3CA_0000826292_2	ACACTGACGACATGGTTCTACAiTCT CATACACAGATGATTGCTTGG (SEQ ID NO:248)	TACGGTAGCAGAGACTTGGTCTaCc TGTAAGCTGAATTAATACTGAGAAA (SEQ ID NO:249)
G5	PIK3C A	PIK3CA_0000826293_1	ACACTGACGACATGGTTCTACATCG GCCATGCAGAACTGA (SEQ ID NO:250)	TACGGTAGCAGAGACTTGGTCTACC TTAAGAATTTAATGGGAAAATAATTAGACTT (SEQ ID NO:251)
H5	PIK3C A	PIK3CA_0000826294_1	ACACTGACGACATGGTTCTACAATG AGTGTGTTAAATTGTTAGCAAAGATT A	TACGGTAGCAGAGACTTGGTCTGCC TcGACTTGCCATTCA (SEQ ID NO:253)

Primer plate well	Gene/chr	Name	F-primer	R-primer
			(SEQ ID NO:252)	
A6	PIK3CA	PIK3CA_0000826294_2	ACACTGACGACATGGTTCTACACCA GAGGTTTGGCCTGCTT (SEQ ID NO:254)	TACGGTAGCAGAGACTTGGTCTGAA AAGAGTCTCAAACACAaACTAGAGT CA (SEQ ID NO:255)
B6	PIK3CA	PIK3CA_0000826295_1	ACACTGACGACATGGTTCTACATGT GAGAAAGAGATTAGCAGTTAGTT (SEQ ID NO:256)	TACGGTAGCAGAGACTTGGTCTCAA TGAAACCCCAAGAAAGT (SEQ ID NO:257)
C6	PIK3CA	PIK3CA_0000826296_1	ACACTGACGACATGGTTCTACATcAG GGTAAATAATAATAAGCAAAGGT (SEQ ID NO:258)	TACGGTAGCAGAGACTTGGTCTACT CTTCCTTACCATCCCCAT (SEQ ID NO:259)
D6	PIK3CA	PIK3CA_0000826296_2	ACACTGACGACATGGTTCTACAGGC CACTGTGGTTGAATTGG (SEQ ID NO:260)	TACGGTAGCAGAGACTTGGTCTGG CTTTCAGTAGTTTTcATGGTTCA (SEQ ID NO:261)
E6	PIK3CA	PIK3CA_0000826297_1	ACACTGACGACATGGTTCTACATGA TGGCGTGATCCCCAAAT (SEQ ID NO:262)	TACGGTAGCAGAGACTTGGTCTACT TTCAACATACAGGTTGCCTT (SEQ ID NO:263)
F6	PIK3CA	PIK3CA_0000826298_1	ACACTGACGACATGGTTCTACACTA ATAAAATACTCATGTTTtagcctggt (SEQ ID NO:264)	TACGGTAGCAGAGACTTGGTCTTTC AAGCCGCCTTTGC (SEQ ID NO:265)
G6	PIK3CA	PIK3CA_0000826298_2	ACACTGACGACATGGTTCTACATGT GGGACTTATTGAGGTGGTG (SEQ ID NO:266)	TACGGTAGCAGAGACTTGGTCTACA CAAACACCgACAGACTCA (SEQ ID NO:267)
H6	PIK3CA	PIK3CA_0000826299_1	ACACTGACGACATGGTTCTACATCT CAAGTTGGCCTGAATCACT (SEQ ID NO:268)	TACGGTAGCAGAGACTTGGTCTCCA AAACATTTTAAACAGAGAAAACCA (SEQ ID NO:269)
C7	PIK3CA	PIK3CA_0001139987_1	ACACTGACGACATGGTTCTACATGC TCCAACTGACCAAACTG (SEQ ID NO:270)	TACGGTAGCAGAGACTTGGTCTGCT AGgGTCTTTCGAATGTATGC (SEQ ID NO:271)
A1	EGFR	EGFR_E00001633131_1	ACACTGACGACATGGTTCTACAGCC AACGCCACAACCAC (SEQ ID NO:272)	TACGGTAGCAGAGACTTGGTCTggcg AGACACGCCCTTAC (SEQ ID NO:273)
B1	EGFR	EGFR_E00001882478_1	ACACTGACGACATGGTTCTACACCT GGACCTTGAGGGATTG (SEQ ID NO:274)	TACGGTAGCAGAGACTTGGTCTTCC CAAGGACCACCTCACA (SEQ ID NO:275)
C1	EGFR	EGFR_E00001882478_2	ACACTGACGACATGGTTCTACACGC AGTTGGGCACTTTTG (SEQ ID NO:276)	TACGGTAGCAGAGACTTGGTCTCCC AGGCcTTCTCCACTTA (SEQ ID NO:277)
D1	EGFR	EGFR_E00001704157_1	ACACTGACGACATGGTTCTACAGGC TCCCTGGACCCATTTTA (SEQ ID NO:278)	TACGGTAGCAGAGACTTGGTCTACT GCTAAGGCATAGGAATTTTCG (SEQ ID NO:279)
E1	EGFR	EGFR_E00001704157_2	ACACTGACGACATGGTTCTACACAG TGGAGCgAATTCCTTT (SEQ ID NO:280)	TACGGTAGCAGAGACTTGGTCTGG GAGCCATCGGAAGT (SEQ ID NO:281)
F1	EGFR	EGFR_E00001688856_1	ACACTGACGACATGGTTCTACATGC TCACCGCAGTTCCATTC (SEQ ID NO:282)	TACGGTAGCAGAGACTTGGTCTCCC CCATAGGAGCTGGAG (SEQ ID NO:283)
G1	EGFR	EGFR_E00001683983_1	ACACTGACGACATGGTTCTACAGG GAAAGGGCGTCATCAGTT (SEQ ID NO:284)	TACGGTAGCAGAGACTTGGTCTAGC AAGTGAAGGAAGAGAGGG (SEQ ID NO:285)
H1	EGFR	EGFR_E00001652975_1	ACACTGACGACATGGTTCTACATGA TCCTACCCTCACTCTTCA (SEQ ID NO:286)	TACGGTAGCAGAGACTTGGTCTCCA GGGAGGCTGCT (SEQ ID NO:287)

Primer plate well	Gene/chr	Name	F-primer	R-primer
A2	EGFR	EGFR_E0000 1623732_1	ACACTGACGACATGGTTCTACAGTG TGGCgCTGAGTGTACTT (SEQ ID NO:288)	TACGGTAGCAGAGACTTGGTCTAGG TGGCACCAAAGCTGTAT (SEQ ID NO:289)
B2	EGFR	EGFR_E0000 1623732_2	ACACTGACGACATGGTTCTACACCA TAGGTCTGCCGCAAATTC (SEQ ID NO:290)	TACGGTAGCAGAGACTTGGTCTGAC AGAGCGGGACAAGGATG (SEQ ID NO:291)
C2	EGFR	EGFR_E0000 1694314_1	ACACTGACGACATGGTTCTACACCT GGTGCCACCGTCATC (SEQ ID NO:292)	TACGGTAGCAGAGACTTGGTCTCAG CAGCcGAGAACAAG (SEQ ID NO:293)
D2	EGFR	EGFR_E0000 1639282_1	ACACTGACGACATGGTTCTACAGTG GATCCCTAGCTATTCTTAATCCA (SEQ ID NO:294)	TACGGTAGCAGAGACTTGGTCTAAC CTGTGACTCACCCCTA (SEQ ID NO:295)
E2	EGFR	EGFR_E0000 1639282_2	ACACTGACGACATGGTTCTACACTC ACTCTCCATAAATGCTACGA (SEQ ID NO:296)	TACGGTAGCAGAGACTTGGTCTGTG TGAAGGAGTCACTGAAACA (SEQ ID NO:297)
F2	EGFR	EGFR_E0000 1639282_3	ACACTGACGACATGGTTCTACATGC TTGTATAAAGAAAAACAAATCTGC (SEQ ID NO:298)	TACGGTAGCAGAGACTTGGTCTCTC TCTAAACACTGATTTCCCA (SEQ ID NO:299)
G2	EGFR	EGFR_E0000 1084926_1	ACACTGACGACATGGTTCTACAGTC CCTGAGAGTCTAGAGTAATGT (SEQ ID NO:300)	TACGGTAGCAGAGACTTGGTCTACC AGGCTTTGGCTGTG (SEQ ID NO:301)
H2	EGFR	EGFR_E0000 1084941_1	ACACTGACGACATGGTTCTACAAAG TTTTCAGGGATACATTGTTTTAT (SEQ ID NO:302)	TACGGTAGCAGAGACTTGGTCTACA GTTTTTCCAGTTTATTGTATTGTC (SEQ ID NO:303)
A3	EGFR	EGFR_E0000 1084941_2	ACACTGACGACATGGTTCTACATCC TTGGGATTACGCTCCCT (SEQ ID NO:304)	TACGGTAGCAGAGACTTGGTCTGGA CCCATTAGAACCAACTCCA (SEQ ID NO:305)
B3	EGFR	EGFR_E0000 1084939_1	ACACTGACGACATGGTTCTACATCT CCTCCGGCCCCCTC (SEQ ID NO:306)	TACGGTAGCAGAGACTTGGTCTAAC CTCCTACCCCTCCAGAA (SEQ ID NO:307)
C3	EGFR	EGFR_E0000 1084939_2	ACACTGACGACATGGTTCTACATCT CTTGCCGGAATGTCAGC (SEQ ID NO:308)	TACGGTAGCAGAGACTTGGTCTCCC CACAGAAAACCCA (SEQ ID NO:309)
D3	EGFR	EGFR_E0000 1084927_1	ACACTGACGACATGGTTCTACATTG AAGAGGTGATTTGTGTTCTGT (SEQ ID NO:310)	TACGGTAGCAGAGACTTGGTCTGAc GTGGATAGCAGCAAGGG (SEQ ID NO:311)
E3	EGFR	EGFR_E0000 1627115_1	ACACTGACGACATGGTTCTACACTT CCATTTTGAAAGAGAAAAGAAAGAG (SEQ ID NO:312)	TACGGTAGCAGAGACTTGGTCTgGC GTCTGCGTACTTCCA (SEQ ID NO:313)
F3	EGFR	EGFR_E0000 1627115_2	ACACTGACGACATGGTTCTACATTG ACGGCCCCCAC (SEQ ID NO:314)	TACGGTAGCAGAGACTTGGTCTTGC CgGAAACTTGGGAGA (SEQ ID NO:315)
G3	EGFR	EGFR_E0000 1802824_3	ACACTGACGACATGGTTCTACAAAA ATGTTAGTGGTCATTTTCTAATGTC T (SEQ ID NO:316)	TACGGTAGCAGAGACTTGGTCTAGT GTCAGGACTTTATTTGAAGCA (SEQ ID NO:317)
H3	EGFR	EGFR_E0000 1371541_1	ACACTGACGACATGGTTCTACAtGT ACTTGTCCATCTTTCTCCAG (SEQ ID NO:318)	TACGGTAGCAGAGACTTGGTCTCCC AACCCAGCTGAAACTCT (SEQ ID NO:319)
A4	EGFR	EGFR_E0000 1371541_3	ACACTGACGACATGGTTCTACATAA TGATGGCAGCGTGTCCC (SEQ ID NO:320)	TACGGTAGCAGAGACTTGGTCTGG GAACAGACAcGTGAAGGC (SEQ ID NO:321)
B4	EGFR	EGFR_E0000	ACACTGACGACATGGTTCTACATGC	TACGGTAGCAGAGACTTGGTCTAGG

Primer plate well	Gen e/chr	Name	F-primer	R-primer
		1768076_1	CAAAGAAGTAGAATGAGAAAAATG (SEQ ID NO:322)	ACAGTCAGAAATGCAGGA (SEQ ID NO:323)
C4	EGFR	EGFR_E0000 1699330_1	ACACTGACGACATGGTTCTACAGGA AAAgTGTGCCTGGTAGGG (SEQ ID NO:324)	TACGGTAGCAGAGACTTGGTCTGGA GGAACAAGGAAGGGTG (SEQ ID NO:325)
D4	EGFR	EGFR_E0000 1699330_2	ACACTGACGACATGGTTCTACAGCT ACATAGTGTCTCACTTTCCAA (SEQ ID NO:326)	TACGGTAGCAGAGACTTGGTCTGAT CCCCAGGGCCACCA (SEQ ID NO:327)
E4	EGFR	EGFR_E0000 1699330_4	ACACTGACGACATGGTTCTACAGCC CTCCTCTTGCTGCT (SEQ ID NO:328)	TACGGTAGCAGAGACTTGGTCTGTA TcTAACATACAACTGCTAATGG (SEQ ID NO:329)
F4	EGFR	EGFR_E0000 1778519_1	ACACTGACGACATGGTTCTACATGG TGAGGGCTGAGGTG (SEQ ID NO:330)	TACGGTAGCAGAGACTTGGTCTcCT GTGCCAGGGACCTTAC (SEQ ID NO:331)
G4	EGFR	EGFR_Exon1 9	ACACTGACGACATGGTTCTACATCA CAATTGCCAGTTAAGTCT (SEQ ID NO:332)	TACGGTAGCAGAGACTTGGTCTCCA CACAGCAAAGCAGAAAC (SEQ ID NO:333)
H4	EGFR	EGFR_E0000 1601336_1	ACACTGACGACATGGTTCTACAGCG TCTTCACCTGGAAGGG (SEQ ID NO:334)	TACGGTAGCAGAGACTTGGTCTCCG GACATAGTCCAGGAGG (SEQ ID NO:335)
A5	EGFR	EGFR_E0000 1601336_2	ACACTGACGACATGGTTCTACAGCG TGGACAACCCCCAC (SEQ ID NO:336)	TACGGTAGCAGAGACTTGGTCTGG CTCCTTATCTCCCCTCC (SEQ ID NO:337)
B5	EGFR	EGFR_E0000 1681524_1	ACACTGACGACATGGTTCTACAGGA TGCAGAGCTTCTCCCA (SEQ ID NO:338)	TACGGTAGCAGAGACTTGGTCTTTC TCTCCGCACCCAG (SEQ ID NO:339)
C5	EGFR	EGFR_E0000 1681524_2	ACACTGACGACATGGTTCTACAGGT CTTCTCTGTTTCAGGGCAT (SEQ ID NO:340)	TACGGTAGCAGAGACTTGGTCTGCT GACCTAAAGCCACCTCC (SEQ ID NO:341)
D5	EGFR	EGFR_E0000 1631695_1	ACACTGACGACATGGTTCTACAGTG TCACTCGTAATTAGGTCCA (SEQ ID NO:342)	TACGGTAGCAGAGACTTGGTCTGG CCTCAGTACAAACTCATTAGC (SEQ ID NO:343)
E5	EGFR	EGFR_E0000 1779947_1	ACACTGACGACATGGTTCTACATGT TCATTCATGATCCCACTGC (SEQ ID NO:344)	TACGGTAGCAGAGACTTGGTCTCCA CCAGTCACTCACTTG (SEQ ID NO:345)
F5	EGFR	EGFR_E0000 1779947_2	ACACTGACGACATGGTTCTACATCC CTGCCAGCGAGAT (SEQ ID NO:346)	TACGGTAGCAGAGACTTGGTCTAGG GATGCAAAGGCCTCA (SEQ ID NO:347)
G5	EGFR	EGFR_E0000 1790701_1	ACACTGACGACATGGTTCTACAGCC TTCTTTAAGCAATGCCATCTTTAT (SEQ ID NO:348)	TACGGTAGCAGAGACTTGGTCTCAA TGGAAGCaCAGACTGCAA (SEQ ID NO:349)
H5	EGFR	EGFR_E0000 1801208_1	ACACTGACGACATGGTTCTACACCC CTGCTCCTATAGCCAA (SEQ ID NO:350)	TACGGTAGCAGAGACTTGGTCTATG AGGTACTCGTCGGCATC (SEQ ID NO:351)
A6	EGFR	EGFR_E0000 1801208_2	ACACTGACGACATGGTTCTACAACT TCTACCGTGCCCTGA (SEQ ID NO:352)	TACGGTAGCAGAGACTTGGTCTGTT CAAATGAGTAGACACAGCTT (SEQ ID NO:353)
B6	EGFR	EGFR_E0000 1773562_1	ACACTGACGACATGGTTCTACATAC CCTCCATGAGGCACAC (SEQ ID NO:354)	TACGGTAGCAGAGACTTGGTCTGGA GAgCTGTAAATTCTGGCTT (SEQ ID NO:355)
C6	EGFR	EGFR_E0000 1610532_1	ACACTGACGACATGGTTCTACaccct gACCGGAGTAACCTTC (SEQ ID NO:356)	TACGGTAGCAGAGACTTGGTCTAGG AGCAGGACTGTTTCCAG (SEQ ID NO:357)

Primer plate well	Gene/chr	Name	F-primer	R-primer
D6	EGFR	EGFR_E00001610532_4	ACACTGACGACATGGTTCTACAGACGACACCTTCCTCCAGT (SEQ ID NO:358)	TACGGTAGCAGAGACTTGGTCTGCA GGCTCGGTCATGTGTTA (SEQ ID NO:359)
E6	EGFR	EGFR_E00001245887_1	ACACTGACGACATGGTTCTACATGTGAGGACATTCACAGGGT (SEQ ID NO:360)	TACGGTAGCAGAGACTTGGTCTGGT CCTGGTAGTGTGGGTCT (SEQ ID NO:361)
F6	EGFR	EGFR_E00001245887_2	ACACTGACGACATGGTTCTACAGCTCTGTGCAGAATCCTGTCT (SEQ ID NO:362)	TACGGTAGCAGAGACTTGGTCTTTG GTGGcTGCCTTTCTGG (SEQ ID NO:363)
G6	EGFR	EGFR_E00001245887_3	ACACTGACGACATGGTTCTACAGCAACCCGAGTATCTCAACA (SEQ ID NO:364)	TACGGTAGCAGAGACTTGGTCTGCGACCCCTAGGTATTCT (SEQ ID NO:365)
H6	EGFR	EGFR_Exon2 8-2	ACACTGACGACATGGTTCTACATGTCAACAGCACATTCGACAG (SEQ ID NO:366)	TACGGTAGCAGAGACTTGGTCTGGT CCTGGGTATCGAAAGAGT (SEQ ID NO:367)
A1	TP53	TP53_E00001757276_1	ACACTGACGACATGGTTCTACAGACCCAAAACCCAAATGGC (SEQ ID NO:368)	TACGGTAGCAGAGACTTGGTCTCCCTGCTTCTGCTCCTAC (SEQ ID NO:369)
B1	TP53	TP53_E00001728015_1	ACACTGACGACATGGTTCTACAGGAATCCTATGGCTTCCAACC (SEQ ID NO:370)	TACGGTAGCAGAGACTTGGTCTCCCTCCTCTGTTGCTG (SEQ ID NO:371)
C1	TP53	TP53_00001404886_13	ACACTGACGACATGGTTCTACAtctgTATCAGGCAAAGTCATAGAA (SEQ ID NO:372)	TACGGTAGCAGAGACTTGGTCTGCC TCAAAGACATGGCTCC (SEQ ID NO:373)
D1	TP53	TP53_E00001789298_1	ACACTGACGACATGGTTCTACAGAAACGGCATTTCGAGTGT (SEQ ID NO:374)	TACGGTAGCAGAGACTTGGTCTAAG GGTGCAGTTATGCCTCA (SEQ ID NO:375)
E1	TP53	TP53_E00001789298_2	ACACTGACGACATGGTTCTACACTGTGtGTGGGAGT (SEQ ID NO:376)	TACGGTAGCAGAGACTTGGTCTATC TCCgCAAGAAAGGGGAG (SEQ ID NO:377)
F1	TP53	TP53_E00001789298_3	ACACTGACGACATGGTTCTACATGTCCTGCTTGCTTACCTCG (SEQ ID NO:378)	TACGGTAGCAGAGACTTGGTCTGCC TCTTGCTTCTCTTTTCT (SEQ ID NO:379)
G1	TP53	TP53_E00001665758_1	ACACTGACGACATGGTTCTACAGGGGTCAGaGGCAAGCAG (SEQ ID NO:380)	TACGGTAGCAGAGACTTGGTCTTGG GCCTGTGTTATCTCC (SEQ ID NO:381)
H1	TP53	TP53_E00001255919_1	ACACTGACGACATGGTTCTACAGAGAAAGCCCCCTACTGC (SEQ ID NO:382)	TACGGTAGCAGAGACTTGGTCTAGC ATCTTATCCGAGTGGAAGG (SEQ ID NO:383)
A2	TP53	TP53_E00001255919_3	ACACTGACGACATGGTTCTACATCCAAATACTCCACGCAAA (SEQ ID NO:384)	TACGGTAGCAGAGACTTGGTCTgCT GCCCCACCATGAG (SEQ ID NO:385)
B2	TP53	TP53_E00001255919_5	ACACTGACGACATGGTTCTACAGCTGCTCACCATCGCTA (SEQ ID NO:386)	TACGGTAGCAGAGACTTGGTCTCCA ACTGgCCAAGACCT (SEQ ID NO:387)
C2	TP53	TP53_E00001255919_6	ACACTGACGACATGGTTCTACATGTGCTGTGACTGCTTGTAG (SEQ ID NO:388)	TACGGTAGCAGAGACTTGGTCTGCC CTGACTTTCAACTCTGT (SEQ ID NO:389)
D2	TP53	TP53_E00001612188_1	ACACTGACGACATGGTTCTACATACGGCCAGGCATTGAAGT (SEQ ID NO:390)	TACGGTAGCAGAGACTTGGTCTCCT CCTGGCCCCGTGTC (SEQ ID NO:391)
E2	TP53	TP53_E00001612188_2	ACACTGACGACATGGTTCTACAGGAACCGTAGCTGCCCTG	TACGGTAGCAGAGACTTGGTCTAAG ACCCAGGTCCAGATGAA

Primer plate well	Gen e/chr	Name	F-primer	R-primer
			(SEQ ID NO:392)	(SEQ ID NO:393)
G2	TP53	TP53_E00001 596491_1	ACACTGACGACATGGTTCTACATTT CGCTTCCCACAGGTCTC (SEQ ID NO:394)	TACGGTAGCAGAGACTTGGTCTCAG CCAGACTGCCTTCCG (SEQ ID NO:395)
A1	BRCA 1	BRCA1_210_ 28486_164	ACACTGACGACATGGTTCTACAATA TTTAGTAGCCAGGACAGTAGAAG (SEQ ID NO:396)	TACGGTAGCAGAGACTTGGTCTCtT AccAgTGCCAGGAGC (SEQ ID NO:397)
B1	BRCA 1	BRCA1_210_ 28486_165	ACACTGACGACATGGTTCTACAATC TGGGGTATCAGGTAGGTGTC (SEQ ID NO:398)	TACGGTAGCAGAGACTTGGTCTCCT GGAGTCGATTGATTAGAGCC (SEQ ID NO:399)
C1	BRCA 1	BRCA1_210_ 41199761	ACACTGACGACATGGTTCTACAaag GACCCCATATAGCACAGGTA (SEQ ID NO:400)	TACGGTAGCAGAGACTTGGTCTACA CTTTGAATGCTCTTTCCTTCC (SEQ ID NO:401)
D1	BRCA 1	BRCA1_210_ 44080_1	ACACTGACGACATGGTTCTACAGGT GCCAGTCTTGCTCACAG (SEQ ID NO:402)	TACGGTAGCAGAGACTTGGTCTGTA GAGGGCCTGGGTAAAGTATG (SEQ ID NO:403)
E1	BRCA 1	BRCA1_210_ 30488_1	ACACTGACGACATGGTTCTACATCA ACTTGAGGGAGGGAGCTTTA (SEQ ID NO:404)	TACGGTAGCAGAGACTTGGTCTATA TGACGTGTCTGCTCCACTTC (SEQ ID NO:405)
F1	BRCA 1	BRCA1_210_ 95889_1	ACACTGACGACATGGTTCTACAGAA AGTGGTGCATTGATGGAAGG (SEQ ID NO:406)	TACGGTAGCAGAGACTTGGTCTAAG AGCACGTTCTTCTGCTGTAT (SEQ ID NO:407)
G1	BRCA 1	BRCA1_210_ 14034_1	ACACTGACGACATGGTTCTACATGC AATTCTGAGGTGTTAAAGGGA (SEQ ID NO:408)	TACGGTAGCAGAGACTTGGTCTGGA CAGCAcTTCCTGATTTTGT (SEQ ID NO:409)
H1	BRCA 1	BRCA1_210_ 78044_1	ACACTGACGACATGGTTCTACAgcc TCATGTGGTTTATGCAG (SEQ ID NO:410)	TACGGTAGCAGAGACTTGGTCTACT AGTATTCTGAGCTGTGTGC (SEQ ID NO:411)
A2	BRCA 1	BRCA1_210_ 97215_1	ACACTGACGACATGGTTCTACATAC CtACATAAACTCTTCCAGAATGT (SEQ ID NO:412)	TACGGTAGCAGAGACTTGGTCTGCA atgGAAGAAgtGTGAGCAG (SEQ ID NO:413)
B2	BRCA 1	BRCA1_210_ 97215_2	ACACTGACGACATGGTTCTACATGT TAAGTCTTAGTCATTAGGGAGATAC A (SEQ ID NO:414)	TACGGTAGCAGAGACTTGGTCTAGa GTcCAgctGCTGCTCATA (SEQ ID NO:415)
C2	BRCA 1	BRCA1_210_ 23203_1	ACACTGACGACATGGTTCTACAAAG cGTCAATTCTgGCTTCTCC (SEQ ID NO:416)	TACGGTAGCAGAGACTTGGTCTcAa CCTCTGCATTGaAAGTTCCC (SEQ ID NO:417)
D2	BRCA 1	BRCA1_210_ 23203_2	ACACTGACGACATGGTTCTACATGC ATTATAcCCAgCAGTATCAG (SEQ ID NO:418)	TACGGTAGCAGAGACTTGGTCTAAc cCcTTAcCtGGAATCTGGA (SEQ ID NO:419)
E2	BRCA 1	BRCA1_210_ 97215_16	ACACTGACGACATGGTTCTACAtCA ATGCAGAGGtTgAAGATGGT (SEQ ID NO:420)	TACGGTAGCAGAGACTTGGTCTACT TTGTAATTCAACATTCATCGTTGTG (SEQ ID NO:421)
F2	BRCA 1	BRCA1_210_ 21142_1	ACACTGACGACATGGTTCTACATGT AGGATTcAGAGTAAATCAAAGTGT (SEQ ID NO:422)	TACGGTAGCAGAGACTTGGTCTTGC TCTGGGAGTCTcAGAATAGA (SEQ ID NO:423)
G2	BRCA 1	BRCA1_210_ 26566_1	ACACTGACGACATGGTTCTACAGTG TTTGTCCAATACAGCAGATGA (SEQ ID NO:424)	TACGGTAGCAGAGACTTGGTCTTAC ATGCAcagTTGCTCTGGG (SEQ ID NO:425)
H2	BRCA 1	BRCA1_210_ 26566_2	ACACTGACGACATGGTTCTACATcC TcttGAGATGGGtAGTTTCTAT (SEQ ID NO:426)	TACGGTAGCAGAGACTTGGTCTCTG CCCAGcAAGTATGATTTGTC (SEQ ID NO:427)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
A3	BRCA 1	BRCA1_210_21142_12	ACACTGACGACATGGTTCTACAtgTG CATGTAcCACcTaTCATCTAA (SEQ ID NO:428)	TACGGTAGCAGAGACTTGGTCTGG GCTCTTTTTT GCCAGTCATTT (SEQ ID NO:429)
B3	BRCA 1	BRCA1_210_16775_1	ACACTGACGACATGGTTCTACAGAT GTCAGATACCACAGCATCTT (SEQ ID NO:430)	TACGGTAGCAGAGACTTGGTCTTGT TTTCTCATTcCATTTAAAGCAGTA (SEQ ID NO:431)
C3	BRCA 1	BRCA1_210_1516237_1m	ACACTGACGACATGGTTCTACAGCC AGAACCACCATCTTTCAGTA (SEQ ID NO:432)	TACGGTAGCAGAGACTTGGTCTTTT TTGAACAGTACCCGTTCCCT (SEQ ID NO:433)
D3	BRCA 1	BRCA1_210_18525_1	ACACTGACGACATGGTTCTACAGG GAAGGAAAGAATTTTGCTTAAGAT (SEQ ID NO:434)	TACGGTAGCAGAGACTTGGTCTGaT AAAgCtCcAGCAGGAAATGG (SEQ ID NO:435)
E3	BRCA 1	BRCA1_210_18525_10	ACACTGACGACATGGTTCTACAGTT AGAAGGCTGGCTCCC (SEQ ID NO:436)	TACGGTAGCAGAGACTTGGTCTTGG AAAGCTTCTCAAAGTATTTTCATTTT (SEQ ID NO:437)
F3	BRCA 1	BRCA1_210_58415_1	ACACTGACGACATGGTTCTACATAC TGAATGCAAAGGACACCACA (SEQ ID NO:438)	TACGGTAGCAGAGACTTGGTCTCAG CAAGTTGCAGCGTTTATAGT (SEQ ID NO:439)
G3	BRCA 1	BRCA1_210_36588_2	ACACTGACGACATGGTTCTACAGTA AAATGTGCTCCCCAAAAGCA (SEQ ID NO:440)	TACGGTAGCAGAGACTTGGTCTGTC TGAAAGCCAgGGAGTTGG (SEQ ID NO:441)
H3	BRCA 1	BRCA1_210_36588_3	ACACTGACGACATGGTTCTACAAGT TTGAATCCATGCTTTGCTCT (SEQ ID NO:442)	TACGGTAGCAGAGACTTGGTCTCAC AGTGCAGTGAATTGGAAGAC (SEQ ID NO:443)
A4	BRCA 1	BRCA1_210_36588_4	ACACTGACGACATGGTTCTACAGGA TCCTGGGTGTTTGATTTGC (SEQ ID NO:444)	TACGGTAGCAGAGACTTGGTCTTGT CTAAGAACACAGAGGAGAATTTA (SEQ ID NO:445)
B4	BRCA 1	BRCA1_210_36588_5	ACACTGACGACATGGTTCTACAGCA GAACATTTTGTTCCTCACT (SEQ ID NO:446)	TACGGTAGCAGAGACTTGGTCTTCC CTGCTTCCAACACTTGTTAT (SEQ ID NO:447)
C4	BRCA 1	BRCA1_210_1_1	ACACTGACGACATGGTTCTACATCA ATGATAATAAATTCTCCTCTGTGTTCT (SEQ ID NO:448)	TACGGTAGCAGAGACTTGGTCTCTA GTGAGGATGAAGAGCTTCCC (SEQ ID NO:449)
D4	BRCA 1	BRCA1_210_1_2	ACACTGACGACATGGTTCTACAACC AAATAACAAGTGTGGAAGCA (SEQ ID NO:450)	TACGGTAGCAGAGACTTGGTCTAGT CCTAGCCCTTTCACCCATA (SEQ ID NO:451)
E4	BRCA 1	BRCA1_210_36588_6	ACACTGACGACATGGTTCTACAACA AGTGTGGAAGCAGGG (SEQ ID NO:452)	TACGGTAGCAGAGACTTGGTCTCAG GAGTCCTAGCCCTTCA (SEQ ID NO:453)
F4	BRCA 1	BRCA1_210_36588_7	ACACTGACGACATGGTTCTACAAGG GAAGCTCTTCATCCTCACTA (SEQ ID NO:454)	TACGGTAGCAGAGACTTGGTCTTGG TGAAATAAAGGAAGATACTAGTTTT G (SEQ ID NO:455)
G4	BRCA 1	BRCA1_210_36588_8	ACACTGACGACATGGTTCTACACTC CTTTCTGGACGCTTTTGCTA (SEQ ID NO:456)	TACGGTAGCAGAGACTTGGTCTTCA GACTGTTAATACAGATTTCTCTCCA (SEQ ID NO:457)
H4	BRCA 1	BRCA1_210_36588_9	ACACTGACGACATGGTTCTACAGTC TCAGAACAACCTGAGATGC (SEQ ID NO:458)	TACGGTAGCAGAGACTTGGTCTAGA TTAGGGGTTTTGCAACCTGA (SEQ ID NO:459)
A5	BRCA 1	BRCA1_210_36588_10	ACACTGACGACATGGTTCTACATCT TGCTTTTTTATTTT CAGGATGCTT (SEQ ID NO:460)	TACGGTAGCAGAGACTTGGTCTCCA GTACTAATGAAGTGGGCTCC (SEQ ID NO:461)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
B5	BRCA 1	BRCA1_210_36588_126	ACACTGACGACATGGTTCTACAtctA AGCATAGCATTCaATTTTGGC (SEQ ID NO:462)	TACGGTAGCAGAGACTTGGTCTGTG AGCACAATTAgCcgTAATAACA (SEQ ID NO:463)
C5	BRCA 1	BRCA1_210_36588_127	ACACTGACGACATGGTTCTACAGCA TTCAATTTTGGCCCTCTGTT (SEQ ID NO:464)	TACGGTAGCAGAGACTTGGTCTTGG GAAATGAGAACATTCCAAGTACA (SEQ ID NO:465)
D5	BRCA 1	BRCA1_210_36588_128	ACACTGACGACATGGTTCTACAGTT TTCATCACTGGAACCTATTTCA (SEQ ID NO:466)	TACGGTAGCAGAGACTTGGTCTTGC TAGAGGAAAACCTTGGAGGAACA (SEQ ID NO:467)
E5	BRCA 1	BRCA1_210_36588_129	ACACTGACGACATGGTTCTACAATA TTGCTTGAGCTGGCTTCTTT (SEQ ID NO:468)	TACGGTAGCAGAGACTTGGTCTTCG TATACCACCACTTTTCCCA (SEQ ID NO:469)
F5	BRCA 1	BRCA1_210_36588_130	ACACTGACGACATGGTTCTACATGT GCTCACTGTACTTGAATGT (SEQ ID NO:470)	TACGGTAGCAGAGACTTGGTCTTCT CAGTTCAGAGGCAACGAAAC (SEQ ID NO:471)
G5	BRCA 1	BRCA1_210_36588_131	ACACTGACGACATGGTTCTACAAGT GGTGGTATACGATATGGGT (SEQ ID NO:472)	TACGGTAGCAGAGACTTGGTCTAGA CAGTTAATCACTGCAGGCTT (SEQ ID NO:473)
H5	BRCA 1	BRCA1_210_36588_132	ACACTGACGACATGGTTCTACAACA AAACCTAGAGCCTCCTTTGA (SEQ ID NO:474)	TACGGTAGCAGAGACTTGGTCTCTG CCCCTCTGGGTCCTTA (SEQ ID NO:475)
A6	BRCA 1	BRCA1_210_36588_133	ACACTGACGACATGGTTCTACAACCT CATTCTTTCCTTGATTTTCTTCT (SEQ ID NO:476)	TACGGTAGCAGAGACTTGGTCTAGT GAACCTGATGCTCAGTATTTGC (SEQ ID NO:477)
B6	BRCA 1	BRCA1_210_36588_216	ACACTGACGACATGGTTCTACATCC TCTTcTGcATTTCCTGGATT (SEQ ID NO:478)	TACGGTAGCAGAGACTTGGTCTaAG TATCCATTGGGACATGAAGTTA (SEQ ID NO:479)
C6	BRCA 1	BRCA1_210_36588_217	ACACTGACGACATGGTTCTACACCT GGATTTGAAAACGGAGCAAA (SEQ ID NO:480)	TACGGTAGCAGAGACTTGGTCTAAC CCCAAGGGACTAATTCATGG (SEQ ID NO:481)
D6	BRCA 1	BRCA1_210_36588_218	ACACTGACGACATGGTTCTACATCT ATGCTTGTTCCTCCGACTGTG (SEQ ID NO:482)	TACGGTAGCAGAGACTTGGTCTGG CACTCAGGAAAGTATCTCGTT (SEQ ID NO:483)
E6	BRCA 1	BRCA1_210_36588_219	ACACTGACGACATGGTTCTACACTG CACACTGACTCACACATTTA (SEQ ID NO:484)	TACGGTAGCAGAGACTTGGTCTGCT GAAGACCCCAAAGATCTCAT (SEQ ID NO:485)
F6	BRCA 1	BRCA1_210_36588_267	ACACTGACGACATGGTTCTACACctg aGTGCCataATCAGTACCA (SEQ ID NO:486)	TACGGTAGCAGAGACTTGGTCTcAA ATACCAGTGAACCTTaaagAATTTGTC (SEQ ID NO:487)
G6	BRCA 1	BRCA1_210_36588_274	ACACTGACGACATGGTTCTACAttcTT CTcTTgGAagGCTAGGAT (SEQ ID NO:488)	TACGGTAGCAGAGACTTGGTCTtGg AAGGTAAAgacCTGCAACT (SEQ ID NO:489)
H6	BRCA 1	BRCA1_210_36588_282	ACACTGACGACATGGTTCTACAAGG TGcatttGttaACTTCAGCTC (SEQ ID NO:490)	TACGGTAGCAGAGACTTGGTCTCAA ATTgATaGTTGTTcTAGCAGTGA (SEQ ID NO:491)
A7	BRCA 1	BRCA1_210_46602_1	ACACTGACGACATGGTTCTACATAC TcttTtGGCTCCAGTTGC (SEQ ID NO:492)	TACGGTAGCAGAGACTTGGTCTGga gGaAGTCTtctaCCAGGCAT (SEQ ID NO:493)
B7	BRCA 1	BRCA1_210_46602_104	ACACTGACGACATGGTTCTACAcCa TGagtTgTaggTTTCTGCTG (SEQ ID NO:494)	TACGGTAGCAGAGACTTGGTCTGga gGaAGTCTtctaCCAGGCAT (SEQ ID NO:495)
C7	BRCA 1	BRCA1_210_46602_2	ACACTGACGACATGGTTCTACAACA ACTATcAATTTGCaATtcaGTACAATTA	TACGGTAGCAGAGACTTGGTCTAGC AGCaGTAtaAGCAATATGGA

Primer plate well	Gen e/chr	Name	F-primer	R-primer
			(SEQ ID NO:496)	(SEQ ID NO:497)
D7	BRCA 1	BRCA1_210_46602_3	ACACTGACGACATGGTTCTACATGC AATTCAGTACAATTAGGTGGG (SEQ ID NO:498)	TACGGTAGCAGAGACTTGGTCTGCT TTCAAAACGAAAGCTGAACC (SEQ ID NO:499)
E7	BRCA 1	BRCA1_210_46602_4	ACACTGACGACATGGTTCTACATTT TAGGTGCTTTTGAATTGTGGA (SEQ ID NO:500)	TACGGTAGCAGAGACTTGGTCTCG GAGCAGAATGGTCAAGTGAT (SEQ ID NO:501)
F7	BRCA 1	BRCA1_210_46602_105	ACACTGACGACATGGTTCTACATCC ATATTGCTTATACtGCTGCTTA (SEQ ID NO:502)	TACGGTAGCAGAGACTTGGTCTAGc AGAATGGTcAAGTGATGAAT (SEQ ID NO:503)
G7	BRCA 1	BRCA1_210_46602_106	ACACTGACGACATGGTTCTACACGA GTGATTCTATTGGGTTAGGATT (SEQ ID NO:504)	TACGGTAGCAGAGACTTGGTCTGAG ACCTACATCAGGCCCTTCATC (SEQ ID NO:505)
H7	BRCA 1	BRCA1_210_46602_48	ACACTGACGACATGGTTCTACACCA AATcTGcTTTctTGAtaAAATCCTC (SEQ ID NO:506)	TACGGTAGCAGAGACTTGGTCTCCC CAaCTtAAGCCaTGTAAGTGA (SEQ ID NO:507)
A8	BRCA 1	BRCA1_210_46602_49	ACACTGACGACATGGTTCTACAGG GGACGCTCTTGTATTATCTGT (SEQ ID NO:508)	TACGGTAGCAGAGACTTGGTCTGTG AAAGAGTTCACTCCAAATCAGT (SEQ ID NO:509)
B8	BRCA 1	BRCA1_210_46602_50	ACACTGACGACATGGTTCTACACCT TCTTCCGATAGGTTTTCCCA (SEQ ID NO:510)	TACGGTAGCAGAGACTTGGTCTTGA TGGGGAGTCTGAATCAAATG (SEQ ID NO:511)
C8	BRCA 1	BRCA1_210_46602_51	ACACTGACGACATGGTTCTACATGG CCAGTAAGTCTATTTCTCTGAAG (SEQ ID NO:512)	TACGGTAGCAGAGACTTGGTCTAGA GATACTGAAGATGTTCTTGGAT (SEQ ID NO:513)
D8	BRCA 1	BRCA1_210_46602_107	ACACTGACGACATGGTTCTACACCA TCATGTgAGTCATCAGAACCT (SEQ ID NO:514)	TACGGTAGCAGAGACTTGGTCTCTg ATcCCCTGTGTgAGAGAAAA (SEQ ID NO:515)
E8	BRCA 1	BRCA1_210_46602_103	ACACTGACGACATGGTTCTACATgA GTCATCAGAACCTAACAGTTCAT (SEQ ID NO:516)	TACGGTAGCAGAGACTTGGTCTCTg ATcCCCTGTGTgAGAGAAAA (SEQ ID NO:517)
F8	BRCA 1	BRCA1_210_46602_108	ACACTGACGACATGGTTCTACAcCA AGGAacatcTtcaGTATCTCTAGG (SEQ ID NO:518)	TACGGTAGCAGAGACTTGGTCTCAG ATgGgCTGGaAGTAAGGAAA (SEQ ID NO:519)
G8	BRCA 1	BRCA1_210_36588_437	ACACTGACGACATGGTTCTACAccAT TcTTTTCTCTcACACAGGG (SEQ ID NO:520)	TACGGTAGCAGAGACTTGGTCTcagc ATGAGAACAGCAGTTTATT (SEQ ID NO:521)
H8	BRCA 1	BRCA1_210_36588_454	ACACTGACGACATGGTTCTACAAGC CAGgctGTtTgctTTTATTAC (SEQ ID NO:522)	TACGGTAGCAGAGACTTGGTCTtGaT tTGAACAcCACTGAGAAGC (SEQ ID NO:523)
A9	BRCA 1	BRCA1_210_36588_467	ACACTGACGACATGGTTCTACAcA ATGGCTCCAcAtGCAAGTTT (SEQ ID NO:524)	TACGGTAGCAGAGACTTGGTCTTGA CAATTCAGTTTTTGTAGTACCTTGT (SEQ ID NO:525)
B9	BRCA 1	BRCA1_210_83677_1	ACACTGACGACATGGTTCTACATCT ACCCACTCTCTTTTCAGTGC (SEQ ID NO:526)	TACGGTAGCAGAGACTTGGTCTTCT TGGTCATTGACAGTTCTGC (SEQ ID NO:527)
C9	BRCA 1	BRCA1_210_83677_2	ACACTGACGACATGGTTCTACAAAT ATTAATAAATAGGAAAATACCAGC TTCA (SEQ ID NO:528)	TACGGTAGCAGAGACTTGGTCTTTT ATTTTTTGGGGGGAAATTTTTAGGA TCT (SEQ ID NO:529)
D9	BRCA 1	BRCA1_210_83677_3	ACACTGACGACATGGTTCTACAAGT TGCCTTATTAACGGTATCTTCA (SEQ ID NO:530)	TACGGTAGCAGAGACTTGGTCTACC ACTTCTCTGTATTACATACTAGCTTA (SEQ ID NO:531)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
E9	BRCA 1	BRCA1_210_86573_1	ACACTGACGACATGGTTCTACACAA AACTATAAGATAAGGAATCCAGCAA (SEQ ID NO:532)	TACGGTAGCAGAGACTTGGTCTTCT TTACCATACTGTTtaGCAGGAAA (SEQ ID NO:533)
F9	BRCA 1	BRCA1_210_86573_2	ACACTGACGACATGGTTCTACAGTtg TATCcgCTgCTTTGTCC (SEQ ID NO:534)	TACGGTAGCAGAGACTTGGTCTACA TGTTAGCTGACTGATGATGGT (SEQ ID NO:535)
G9	BRCA 1	BRCA1_210_17948_3	ACACTGACGACATGGTTCTACAACA AATGGTTTtACCAAGGAAGGAT (SEQ ID NO:536)	TACGGTAGCAGAGACTTGGTCTTCA TACATTTTtCTCTAAcTGCAAACA (SEQ ID NO:537)
H9	BRCA 1	BRCA1_210_17948_10	ACACTGACGACATGGTTCTACAGca cggTTTTctgAGCCCATA (SEQ ID NO:538)	TACGGTAGCAGAGACTTGGTCTACA CAACAAAGAGCATACATAGGGT (SEQ ID NO:539)
A10	BRCA 1	BRCA1_210_13831_1	ACACTGACGACATGGTTCTACATTC CTGAGTTTTcATGGACAGCA (SEQ ID NO:540)	TACGGTAGCAGAGACTTGGTCTTCA CTTGCTGAGTGTGTTTCTCA (SEQ ID NO:541)
B10	BRCA 1	BRCA1_210_45886_1	ACACTGACGACATGGTTCTACATTT CCTACTGTGGTTGCTTCCAA (SEQ ID NO:542)	TACGGTAGCAGAGACTTGGTCTTTC ATGGCTATTTGCCTTTTGAG (SEQ ID NO:543)
C10	BRCA 1	BRCA1_210_1751963_1	ACACTGACGACATGGTTCTACATGG AGCCACATAACACATTCAAA (SEQ ID NO:544)	TACGGTAGCAGAGACTTGGTCTACT CAGTCATAACAGCTCAAAGT (SEQ ID NO:545)
D10	BRCA 1	BRCA1_210_1751963_2	ACACTGACGACATGGTTCTACAACA TGTCTTTTCTTCCCTAGTATGT (SEQ ID NO:546)	TACGGTAGCAGAGACTTGGTCTATG TGTTAAAGTTCATTGGAACAGAA (SEQ ID NO:547)
A1	BRCA 2	BRCA2_210_1484009_1	ACACTGACGACATGGTTCTACATTT CCAGCGCTTCTGAGTTTAC (SEQ ID NO:548)	TACGGTAGCAGAGACTTGGTCTTGT CAATACCTGChTGTGTCAG (SEQ ID NO:549)
B1	BRCA 2	BRCA2_210_1484009_2	ACACTGACGACATGGTTCTACAAgC ATTGGAGGAATcGTAGGTAA (SEQ ID NO:550)	TACGGTAGCAGAGACTTGGTCTACA CTGTGACGTACTGGGTTTTT (SEQ ID NO:551)
C1	BRCA 2	BRCA2_210_890597_2	ACACTGACGACATGGTTCTACAgCA TTGGAGGAATATCGTAGGT (SEQ ID NO:552)	TACGGTAGCAGAGACTTGGTCTTTT TTAGAAAACACTTCTCGGTGT (SEQ ID NO:553)
D1	BRCA 2	BRCA2_210_0939160_1	ACACTGACGACATGGTTCTACAGTC ACTGGTAAAACTAAGGTGGG (SEQ ID NO:554)	TACGGTAGCAGAGACTTGGTCTtTG GAGtTgAAGcCAGCTGATTA (SEQ ID NO:555)
E1	BRCA 2	BRCA2_210_1_1	ACACTGACGACATGGTTCTACACTT TCTTCAGAAGCTCCACCCTA (SEQ ID NO:556)	TACGGTAGCAGAGACTTGGTCTGAG ATTGGTACAGCGGCAGAG (SEQ ID NO:557)
F1	BRCA 2	BRCA2_210_893213_24	ACACTGACGACATGGTTCTACAAAC CTATTTAAAACTCCACAaAGGAAAC C (SEQ ID NO:558)	TACGGTAGCAGAGACTTGGTCTATC TAATTCTTTTACAGGAGATTGGTACA (SEQ ID NO:559)
G1	BRCA 2	BRCA2_210_1_2	ACACTGACGACATGGTTCTACATGT TTCAGGAAGGAATGTTCCCA (SEQ ID NO:560)	TACGGTAGCAGAGACTTGGTCTaGC TTCATCATACCTTCACTAAGA (SEQ ID NO:561)
H1	BRCA 2	BRCA2_210_0939163_1	ACACTGACGACATGGTTCTACAAAA ATAACCTAAGGGATTGCTTTGT (SEQ ID NO:562)	TACGGTAGCAGAGACTTGGTCTTgA AACaAACTCCACATACCACT (SEQ ID NO:563)
B2	BRCA 2	BRCA2_210_900378_1	ACACTGACGACATGGTTCTACATGT TAATAAAAAATAAACTTAACAATTTT CCCCTT (SEQ ID NO:564)	TACGGTAGCAGAGACTTGGTCTATT ACTAAGTCATAAAAAaAAACCAGGTA GAATA (SEQ ID NO:565)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
C2	BRCA 2	BRCA2_210_0939164_1	ACACTGACGACATGGTTCTACATCC TTAATGATCAGGGCATTCT (SEQ ID NO:566)	TACGGTAGCAGAGACTTGGTCTTGC TCTTTCTTGAAATACACATTTGCT (SEQ ID NO:567)
D2	BRCA 2	BRCA2_210_900635_1	ACACTGACGACATGGTTCTACATCC TTAATGATCAGGGCATTCT (SEQ ID NO:568)	TACGGTAGCAGAGACTTGGTCTACC AAGACATATCAGGATCCACC (SEQ ID NO:569)
E2	BRCA 2	BRCA2_210_900635_3	ACACTGACGACATGGTTCTACAaCA TATTTCTGAAAGTCTAGGAGCTGA (SEQ ID NO:570)	TACGGTAGCAGAGACTTGGTCTTGC TCTTTCTTGAAATACACATTTGCT (SEQ ID NO:571)
F2	BRCA 2	BRCA2_210_0939165_1	ACACTGACGACATGGTTCTACATGT GTCaTGTAATCAAATAGTAGATGTG (SEQ ID NO:572)	TACGGTAGCAGAGACTTGGTCTAGC AATTTCAACAGTCTAATCAATGTC (SEQ ID NO:573)
G2	BRCA 2	BRCA2_210_0939166_1	ACACTGACGACATGGTTCTACActact ACTATATGTGCATTGAGAGTTTTT (SEQ ID NO:574)	TACGGTAGCAGAGACTTGGTCTACA GAGGACTTACCATGACTTGC (SEQ ID NO:575)
H2	BRCA 2	BRCA2_210_905055_1	ACACTGACGACATGGTTCTACATGT GCATTGAGAGTTTTTATaCTAGTGAT TT (SEQ ID NO:576)	TACGGTAGCAGAGACTTGGTCTTGA TTTGTGTTTTCACTGTCTGTC (SEQ ID NO:577)
A3	BRCA 2	BRCA2_210_905055_5	ACACTGACGACATGGTTCTACATCA TGATgAAAGTCTGAAGAAAAATGA (SEQ ID NO:578)	TACGGTAGCAGAGACTTGGTCTCAG AGGACTTACCATGACTTGC (SEQ ID NO:579)
B3	BRCA 2	BRCA2_210_0939167_1	ACACTGACGACATGGTTCTACATGG CTTATAAAATATTAATGTGCTTCTGT (SEQ ID NO:580)	TACGGTAGCAGAGACTTGGTCTacaa CTGTTTCAATACTTCATCTTCTAGG (SEQ ID NO:581)
C3	BRCA 2	BRCA2_210_2_1	ACACTGACGACATGGTTCTACATGG CTTATAAAATATTAATGTGCTTCTGT (SEQ ID NO:582)	TACGGTAGCAGAGACTTGGTCTTCT TCAGAGGTATCTACAACGTGTTTC (SEQ ID NO:583)
D3	BRCA 2	BRCA2_210_2_3	ACACTGACGACATGGTTCTACAACA GGATTTGGAAAAACATCAGGG (SEQ ID NO:584)	TACGGTAGCAGAGACTTGGTCTTTC CTAGTCTTGCTAGTTCTTACTTT (SEQ ID NO:585)
E3	BRCA 2	BRCA2_210_2_4	ACACTGACGACATGGTTCTACATGG AAAGTCAATGCCAAATGTCC (SEQ ID NO:586)	TACGGTAGCAGAGACTTGGTCTTCA CATTATCAGCGTTTGCTTC (SEQ ID NO:587)
F3	BRCA 2	BRCA2_210_3_3	ACACTGACGACATGGTTCTACAtgtag ATAcCTCTGAAGaAGATAGTTTTT (SEQ ID NO:588)	TACGGTAGCAGAGACTTGGTCTTGG ATCAGTATCATTTGGTTCCACT (SEQ ID NO:589)
H3	BRCA 2	BRCA2_210_39167_43	ACACTGACGACATGGTTCTACAaAa TcTcCaAGGAAGTTGTACCG (SEQ ID NO:590)	TACGGTAGCAGAGACTTGGTCTgGC TAGAAaTAcgTGGCAAAGAA (SEQ ID NO:591)
A4	BRCA 2	BRCA2_210_4_1	ACACTGACGACATGGTTCTACAACC CCTATTGCATATTTCTTCATGTG (SEQ ID NO:592)	TACGGTAGCAGAGACTTGGTCTTCT GTATGAGATTCAAGATGCTGCT (SEQ ID NO:593)
B4	BRCA 2	BRCA2_210_4_2	ACACTGACGACATGGTTCTACAGCC TACCAAAATCAGAGAAGCCAT (SEQ ID NO:594)	TACGGTAGCAGAGACTTGGTCTTGC ATTGAAAGTCTCTTTAGGTGAT (SEQ ID NO:595)
C4	BRCA 2	BRCA2_210_4_3	ACACTGACGACATGGTTCTACAGCA GTAAAGCAGGCAATATCTGG (SEQ ID NO:596)	TACGGTAGCAGAGACTTGGTCTGAG TCCTCCTTCTGTGAGCAAAC (SEQ ID NO:597)
D4	BRCA 2	BRCA2_210_4_5	ACACTGACGACATGGTTCTACACCT CTGAAAGTGGACTGGAAT (SEQ ID NO:598)	TACGGTAGCAGAGACTTGGTCTAAC TTATTTGTTTTCTTTTCAAAGTGGA T (SEQ ID NO:599)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
E4	BRCA 2	BRCA2_210_39167_124	ACACTGACGACATGGTTCTACATCTGTAGCTTTGAAGAATGCAGGT (SEQ ID NO:600)	TACGGTAGCAGAGACTTGGTCTTTGCAAATgtaAGTGGTGCTTC (SEQ ID NO:601)
F4	BRCA 2	BRCA2_210_5_2	ACACTGACGACATGGTTCTACAGTTCAGCCAGTTTGAAGCA (SEQ ID NO:602)	TACGGTAGCAGAGACTTGGTCTAAACACAGAAGGAATCGTCATCT (SEQ ID NO:603)
G4	BRCA 2	BRCA2_210_0939168_1	ACACTGACGACATGGTTCTACAAATATTAGTgAATGTGATTGATGGTACTTTA (SEQ ID NO:604)	TACGGTAGCAGAGACTTGGTCTAGAACATTTCCtCAGAATTGTCCCAAA (SEQ ID NO:605)
H4	BRCA 2	BRCA2_210_0939168_3	ACACTGACGACATGGTTCTACAACcAACTTTGTCTTAAGTCTCT (SEQ ID NO:606)	TACGGTAGCAGAGACTTGGTCTGGATCATTTTCACACTGTCCTTCC (SEQ ID NO:607)
A5	BRCA 2	BRCA2_210_39168_22	ACACTGACGACATGGTTCTACAactaCAgTTATttattACCCAGAAGC (SEQ ID NO:608)	TACGGTAGCAGAGACTTGGTCTagtcagGTATCACTGTATTCCACTTT (SEQ ID NO:609)
B5	BRCA 2	BRCA2_210_39168_32	ACACTGACGACATGGTTCTACATCAGATATAAAAGAAGAGGTCTTGGC (SEQ ID NO:610)	TACGGTAGCAGAGACTTGGTCTTGCCTCTAgAAATCAgACTAGGTTTG (SEQ ID NO:611)
C5	BRCA 2	BRCA2_210_39168_49	ACACTGACGACATGGTTCTACATGCGAGCAcTCTATTTTAACTCCT (SEQ ID NO:612)	TACGGTAGCAGAGACTTGGTCTACAGCTCAACgTTTTatAATTTTCATTT (SEQ ID NO:613)
D5	BRCA 2	BRCA2_210_6_1	ACACTGACGACATGGTTCTACACTGATGTTGAATTAACCAAAAATATTCCC (SEQ ID NO:614)	TACGGTAGCAGAGACTTGGTCTTGGATTACTCTTAGATTTGTGTTTTGG (SEQ ID NO:615)
E5	BRCA 2	BRCA2_210_6_2	ACACTGACGACATGGTTCTACAGTTGAGCTGTTGCCACCTGAAAA (SEQ ID NO:616)	TACGGTAGCAGAGACTTGGTCTTCTCAGAGTCTGGATTGACAGTTAT (SEQ ID NO:617)
F5	BRCA 2	BRCA2_210_39168_85m	ACACTGACGACATGGTTCTACATTCaCCaAAaCacaAATCTAAGAGTAA (SEQ ID NO:618)	TACGGTAGCAGAGACTTGGTCTTCGTTTACACAAGTCAAGTCTGTT (SEQ ID NO:619)
G5	BRCA 2	BRCA2_210_7_1m	ACACTGACGACATGGTTCTACAAATAATTTTGTCTTCCAAGTAGCTAATGA (SEQ ID NO:620)	TACGGTAGCAGAGACTTGGTCTATTGACACTTGGGTTGCTTGTTT (SEQ ID NO:621)
H5	BRCA 2	BRCA2_210_7_2	ACACTGACGACATGGTTCTACATCTTGCTTTAGGAAATACTAAGGAAC (SEQ ID NO:622)	TACGGTAGCAGAGACTTGGTCTACCTAGAGTCATTTTATATGCTGCTTT (SEQ ID NO:623)
A6	BRCA 2	BRCA2_210_7_3m	ACACTGACGACATGGTTCTACATAACAAGCAACCCAAGTGCAA (SEQ ID NO:624)	TACGGTAGCAGAGACTTGGTCTCCTGCCATTGTTCATGTAATC (SEQ ID NO:625)
B6	BRCA 2	BRCA2_210_39168_132	ACACTGACGACATGGTTCTACAAAAaGTGTAAAgcAgCATATAAAATGAC (SEQ ID NO:626)	TACGGTAGCAGAGACTTGGTCTTGTTCAGAGAgCTTgaTTTCCTTA (SEQ ID NO:627)
C6	BRCA 2	BRCA2_210_8_1	ACACTGACGACATGGTTCTACAAACAAATGGGCAGGACTCTTAGG (SEQ ID NO:628)	TACGGTAGCAGAGACTTGGTCTTTCACACAAGCTAACTAGTAGGA (SEQ ID NO:629)
D6	BRCA 2	BRCA2_210_8_3m	ACACTGACGACATGGTTCTACAAAGGAAATCAAGCTCTCTGAACA (SEQ ID NO:630)	TACGGTAGCAGAGACTTGGTCTACATCAGAAACAACTACACTACTCT (SEQ ID NO:631)
E6	BRCA 2	BRCA2_210_39168_171	ACACTGACGACATGGTTCTACACTTGtGTTGaAATTgTAaaTACCTTGG (SEQ ID NO:632)	TACGGTAGCAGAGACTTGGTCTTCTgCCTTTTgGCTAGGTG (SEQ ID NO:633)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
F6	BRCA 2	BRCA2_210_39168_188	ACACTGACGACATGGTTCTACAccctcAGatgTtatttTCCAAGCA (SEQ ID NO:634)	TACGGTAGCAGAGACTTGGTCTGCaCTTCAAATGTAcTCTTctGCAATATG (SEQ ID NO:635)
A7	BRCA 2	BRCA2_210_9_3	ACACTGACGACATGGTTCTACAACA GTTGAAATTAACGGAAGTTTGCT (SEQ ID NO:636)	TACGGTAGCAGAGACTTGGTCTGCA GAAGTTTCCTCACTAATATTCTCA (SEQ ID NO:637)
B7	BRCA 2	BRCA2_210_9_4m	ACACTGACGACATGGTTCTACATAC TGAAGCTCTGCAAAAAGCTG (SEQ ID NO:638)	TACGGTAGCAGAGACTTGGTCTTGA AACAACAGAATCATGACATTACTT (SEQ ID NO:639)
C7	BRCA 2	BRCA2_210_9_5	ACACTGACGACATGGTTCTACATGT CATGATTCTGTTGTTTCAATGT (SEQ ID NO:640)	TACGGTAGCAGAGACTTGGTCTTCT ACTGGCAGCAGTATATTTGTT (SEQ ID NO:641)
D7	BRCA 2	BRCA2_210_9_6	ACACTGACGACATGGTTCTACAGAA AATTACAAGAGAAATACTGAAAATG AAGAT (SEQ ID NO:642)	TACGGTAGCAGAGACTTGGTCTTCA GTAAATAGCAAGTCCGTTTCA (SEQ ID NO:643)
F7	BRCA 2	BRCA2_210_10_1m	ACACTGACGACATGGTTCTACAGCA GCAAGCAATTTGAAGGTACA (SEQ ID NO:644)	TACGGTAGCAGAGACTTGGTCTCAG CTTTTGCAGAGCTTCAGTA (SEQ ID NO:645)
H7	BRCA 2	BRCA2_210_10_4	ACACTGACGACATGGTTCTACACTG CAGAGGTACATCCAATAAGT (SEQ ID NO:646)	TACGGTAGCAGAGACTTGGTCTAGT ATTTCTCTGTAAATTTTCAGTAATTT CTTC (SEQ ID NO:647)
A8	BRCA 2	BRCA2_210_39168_291	ACACTGACGACATGGTTCTACAttgaT GGCAGTGATTCAAGTAAAAA (SEQ ID NO:648)	TACGGTAGCAGAGACTTGGTCTATG ACATGCTTcttGAGCTTTCG (SEQ ID NO:649)
B8	BRCA 2	BRCA2_210_39168_305m	ACACTGACGACATGGTTCTACATGG CcAGTTTATGAAGGAGGG (SEQ ID NO:650)	TACGGTAGCAGAGACTTGGTCTgcga CACTAATATTTTTCCCACTTG (SEQ ID NO:651)
E8	BRCA 2	BRCA2_210_39168_339	ACACTGACGACATGGTTCTACAtgTA AATTTCTTTGaTCaGAAACCAGAAG (SEQ ID NO:652)	TACGGTAGCAGAGACTTGGTCTCCC TGGAaggTCACTAGTTGATT (SEQ ID NO:653)
G8	BRCA 2	BRCA2_210_12_1	ACACTGACGACATGGTTCTACACCC AGTTGGTACTGGAAATCAAC (SEQ ID NO:654)	TACGGTAGCAGAGACTTGGTCTTCA CTAGTACCTTGCTCTTTTTCAT (SEQ ID NO:655)
A9	BRCA 2	BRCA2_210_39168_381	ACACTGACGACATGGTTCTACATGA AAAAgAGCAAGgtAcTAGTGAAAT (SEQ ID NO:656)	TACGGTAGCAGAGACTTGGTCTTtA GGTGGCACcacAGTCTCAAT (SEQ ID NO:657)
B9	BRCA 2	BRCA2_210_13_1	ACACTGACGACATGGTTCTACAAGC TGCCCCAAAGTGTAAGAA (SEQ ID NO:658)	TACGGTAGCAGAGACTTGGTCTTGA TGTTTTGAGATTTTCAGTTGTCT (SEQ ID NO:659)
C9	BRCA 2	BRCA2_210_13_3	ACACTGACGACATGGTTCTACACTG TGGTGCCACCTAAGCTCT (SEQ ID NO:660)	TACGGTAGCAGAGACTTGGTCTACA AGTTGCAGGACTTTTTGCTG (SEQ ID NO:661)
D9	BRCA 2	BRCA2_210_13_17	ACACTGACGACATGGTTCTACATGA AAGTTAAAGTACATGAAAATGTAGA AAAA (SEQ ID NO:662)	TACGGTAGCAGAGACTTGGTCTTGG TTGACCATCAAATATTCCTTCTC (SEQ ID NO:663)
E9	BRCA 2	BRCA2_210_13_25	ACACTGACGACATGGTTCTACAAAT CAGTCCCCTTATTCAGTCATT (SEQ ID NO:664)	TACGGTAGCAGAGACTTGGTCTTCA GCTATAGTACTGTTGAATTATTTTC AT (SEQ ID NO:665)
F9	BRCA	BRCA2_210_	ACACTGACGACATGGTTCTACATCA	TACGGTAGCAGAGACTTGGTCTTCA

Primer plate well	Gen e/chr	Name	F-primer	R-primer
	2	13_26	GCCTTAGCTTTTACACAAGT (SEQ ID NO:666)	GCTATAGTACTGTTGAATTATTTTCAT (SEQ ID NO:667)
G9	BRCA 2	BRCA2_210_13_27	ACACTGACGACATGGTTCTACAATT TGATGGTCAACCAGAAAGAATAAA (SEQ ID NO:668)	TACGGTAGCAGAGACTTGGTCTACCTCATCAGAATGGTAGGAATAGC (SEQ ID NO:669)
H9	BRCA 2	BRCA2_210_39168_458	ACACTGACGACATGGTTCTACAgAC AAAAATcATCTCTccGAAAAACAAG (SEQ ID NO:670)	TACGGTAGCAGAGACTTGGTCTTgCATCTTTTACAItGGATATTACTTTGGA A (SEQ ID NO:671)
A10	BRCA 2	BRCA2_210_39168_471m	ACACTGACGACATGGTTCTACAtgatT CTGgTATTGAGCCAGTATTGA (SEQ ID NO:672)	TACGGTAGCAGAGACTTGGTCTTtat TTTTGCAGGgtgAAGAGCTA (SEQ ID NO:673)
B10	BRCA 2	BRCA2_210_14_1	ACACTGACGACATGGTTCTACATCC AAAGTAATATCCAATGTAAAAGATG C (SEQ ID NO:674)	TACGGTAGCAGAGACTTGGTCTCAG GTGGCCCTACCTCAAAATTA (SEQ ID NO:675)
C10	BRCA 2	BRCA2_210_14_2m	ACACTGACGACATGGTTCTACAAC AGCTCTTCACCCGCAAAAA (SEQ ID NO:676)	TACGGTAGCAGAGACTTGGTCTTGA AACTGTCTGTAAATATGTCTTCACT (SEQ ID NO:677)
D10	BRCA 2	BRCA2_210_39168_501	ACACTGACGACATGGTTCTACAGGT AGGGCCACcGCATTTAG (SEQ ID NO:678)	TACGGTAGCAGAGACTTGGTCTtGA AgAATATcctcTGAATCATCCAAT (SEQ ID NO:679)
E10	BRCA 2	BRCA2_210_15_3	ACACTGACGACATGGTTCTACAGCC AAACGAAAATTATGGCAGGT (SEQ ID NO:680)	TACGGTAGCAGAGACTTGGTCTTGT TGTAATAATTTCTTCACTCTGAATGTC (SEQ ID NO:681)
F10	BRCA 2	BRCA2_210_3_1	ACACTGACGACATGGTTCTACATGA ATGTAGCACGCATTACATAA (SEQ ID NO:682)	TACGGTAGCAGAGACTTGGTCTTGC AGATGAGACTGACTTATGAAGC (SEQ ID NO:683)
G10	BRCA 2	BRCA2_210_15_12	ACACTGACGACATGGTTCTACAACC AAAATATGTCTGGATTGGAGAA (SEQ ID NO:684)	TACGGTAGCAGAGACTTGGTCTACA GATTTTCCACTTGCTGTGCT (SEQ ID NO:685)
H10	BRCA 2	BRCA2_210_0401_1237	ACACTGACGACATGGTTCTACATCA CCTTGTGATGTTAGTTTGGGA (SEQ ID NO:686)	TACGGTAGCAGAGACTTGGTCTGCA TCTGATAcCTGGACAGATTTT (SEQ ID NO:687)
A11	BRCA 2	BRCA2_210_0401_1280	ACACTGACGACATGGTTCTACAAGT GTTTTCTGAAATAGAAGATAGTACC AA (SEQ ID NO:688)	TACGGTAGCAGAGACTTGGTCTAGc CTTTTTGGGATATTAATGTTCTGG (SEQ ID NO:689)
B11	BRCA 2	BRCA2_210_15_26	ACACTGACGACATGGTTCTACATCA GTCTCATCTGCAAACTTGTG (SEQ ID NO:690)	TACGGTAGCAGAGACTTGGTCTTCT TGTGAGCTGGTCTGAATGTT (SEQ ID NO:691)
C11	BRCA 2	BRCA2_210_15_36	ACACTGACGACATGGTTCTACAAGG TATCAGATGCTTCATTACAAAACG (SEQ ID NO:692)	TACGGTAGCAGAGACTTGGTCTTGT TCTGGAGTACGTATAGCAGTATTT (SEQ ID NO:693)
D11	BRCA 2	BRCA2_210_0401_1299	ACACTGACGACATGGTTCTACAACC AGCTCACAAGAGAAGAAAA (SEQ ID NO:694)	TACGGTAGCAGAGACTTGGTCTACT TGCTTTCCACTTGCTGT (SEQ ID NO:695)
F11	BRCA 2	BRCA2_210_16_1	ACACTGACGACATGGTTCTACATGG TAAATTCATCTGCTTTCTCTGGA (SEQ ID NO:696)	TACGGTAGCAGAGACTTGGTCTCAC AGTGCTCTGGGTTTCTCTTA (SEQ ID NO:697)
H11	BRCA 2	BRCA2_210_39168_614m	ACACTGACGACATGGTTCTACACCC AGAGCaCTGtgtAAACTCAGAA (SEQ ID NO:698)	TACGGTAGCAGAGACTTGGTCTAGT GAcACTtgGTTCCCTAATACCA (SEQ ID NO:699)

Primer plate well	Gen e/chr	Name	F-primer	R-primer
			(SEQ ID NO:698)	(SEQ ID NO:699)
A12	BRCA 2	BRCA2_210_39168_631	ACACTGACGACATGGTTCTACATcTCCATatcTCTctcAATTTCACAA (SEQ ID NO:700)	TACGGTAGCAGAGACTTGGTCTagaa aAAGTTTcagTTTTACCAATTTCCA (SEQ ID NO:701)
B12	BRCA 2	BRCA2_210_17_2	ACACTGACGACATGGTTCTACAGG GAAAAGAACAGGCTTCACCTA (SEQ ID NO:702)	TACGGTAGCAGAGACTTGGTCTTGG CATGACTTGGCAGTTTAGAA (SEQ ID NO:703)
C12	BRCA 2	BRCA2_210_39168_659	ACACTGACGACATGGTTCTACAAC TTTGAAACagaaGCagtAGAAATTG (SEQ ID NO:704)	TACGGTAGCAGAGACTTGGTCTGG CAACACGAAAGGTAAAAATGAAC (SEQ ID NO:705)
D12	BRCA 2	BRCA2_210_39168_673	ACACTGACGACATGGTTCTACAttAC ATgtCcCGAAATGAGGAA (SEQ ID NO:706)	TACGGTAGCAGAGACTTGGTCTACT GACTACACAAAAATGGCTGA (SEQ ID NO:707)
E12	BRCA 2	BRCA2_210_0939169_1	ACACTGACGACATGGTTCTACAAGG TCACTATTGTGTAAGTATTTTTGT T (SEQ ID NO:708)	TACGGTAGCAGAGACTTGGTCTAGG ATTTTTcTTGATTTTcTATTATCCTGT C (SEQ ID NO:709)
F12	BRCA 2	BRCA2_210_0939169_2	ACACTGACGACATGGTTCTACACTT TTTAGgAGAAccCTCAaTCAAAAGAA (SEQ ID NO:710)	TACGGTAGCAGAGACTTGGTCTcaga aTATTATATACCATACCTATAGAGGG AGA (SEQ ID NO:711)
G12	BRCA 2	BRCA2_210_0939171_1	ACACTGACGACATGGTTCTACAAGC ATCtgTTACATTTCACTGAAAATTG (SEQ ID NO:712)	TACGGTAGCAGAGACTTGGTCTggtta aTCgGCTCTAAAGAAACATGA (SEQ ID NO:713)
H12	BRCA 2	BRCA2_210_18_1	ACACTGACGACATGGTTCTACAGTA TTTACAGTAACATGGATATTCTCTTA GATTT (SEQ ID NO:714)	TACGGTAGCAGAGACTTGGTCTACA TGTCTTACCGAAAGGGTACA (SEQ ID NO:715)
A1	BRCA 2	BRCA2_210_0939173_1	ACACTGACGACATGGTTCTACAATG TAGCAAATGAGGGTCTGCAA (SEQ ID NO:716)	TACGGTAGCAGAGACTTGGTCTCCA AAGTCaAgATGTTcAtACAAATGAGA (SEQ ID NO:717)
B1	BRCA 2	BRCA2_210_19_2	ACACTGACGACATGGTTCTACAAGG GTCTGCAACAAAGGCATA (SEQ ID NO:718)	TACGGTAGCAGAGACTTGGTCTTCC TGAAACTGCTAAATTGCTTG (SEQ ID NO:719)
C1	BRCA 2	BRCA2_210_19_1	ACACTGACGACATGGTTCTACACCA TTGCAGCACAACCTAAGGAAC (SEQ ID NO:720)	TACGGTAGCAGAGACTTGGTCTAAA ATGGATGTCCTGAAACTGCT (SEQ ID NO:721)
D1	BRCA 2	BRCA2_210_19_3	ACACTGACGACATGGTTCTACATTG TATGAACATCTGACTTTGGAAAAA (SEQ ID NO:722)	TACGGTAGCAGAGACTTGGTCTCTG TTCAACTCTGTGAAAATGTGA (SEQ ID NO:723)
E1	BRCA 2	BRCA2_210_0939173_	ACACTGACGACATGGTTCTACATcA AgCAATTTAGCAGTTTCAGG (SEQ ID NO:724)	TACGGTAGCAGAGACTTGGTCTTTG CtTTTGTCTGTTTCTCTCCA (SEQ ID NO:725)
F1	BRCA 2	BRCA2_210_39173_40	ACACTGACGACATGGTTCTACAACa gagTTGAACAGTGTGTTAGGA (SEQ ID NO:726)	TACGGTAGCAGAGACTTGGTCTAGT TACAGCTaCTgCTTGATTGGA (SEQ ID NO:727)
G1	BRCA 2	BRCA2_210_39173_47	ACACTGACGACATGGTTCTACATGA CAATGAgaTTCATCAGTTTAACAA (SEQ ID NO:728)	TACGGTAGCAGAGACTTGGTCTAGG GCTTTAAAATTACCACCACCA (SEQ ID NO:729)
H1	BRCA 2	BRCA2_210_20_2	ACACTGACGACATGGTTCTACAggcc AGGGGTTGTGCTTTTT (SEQ ID NO:730)	TACGGTAGCAGAGACTTGGTCTCGC GTTGCCTTTGTTTCTTCTTA (SEQ ID NO:731)
A2	BRCA	BRCA2_210_	ACACTGACGACATGGTTCTACAACA	TACGGTAGCAGAGACTTGGTCTTTA

Primer plate well	Gen e/chr	Name	F-primer	R-primer
	2	20_12	AGTCTTCAGAATGCCAGAGAT (SEQ ID NO:732)	TGAGAACACGCAGAGGGAAC (SEQ ID NO:733)
B2	BRCA 2	BRCA2_210_39174_21	ACACTGACGACATGGTTCTACAATc TTGCAAAaacATCCACTCTG (SEQ ID NO:734)	TACGGTAGCAGAGACTTGGTCTTTT CATTTCATCCATTCTGCACT (SEQ ID NO:735)
C2	BRCA 2	BRCA2_210_0939175_1	ACACTGACGACATGGTTCTACATTG TTTTTATTGTGTGATACATGTTTACT TT (SEQ ID NO:736)	TACGGTAGCAGAGACTTGGTCTAGC CaACTgTATTCTTTTCCAGT (SEQ ID NO:737)
D2	BRCA 2	BRCA2_210_0939175_2	ACACTGACGACATGGTTCTACAaTT GcaTAAAAaattaaCAGCAAAATGC (SEQ ID NO:738)	TACGGTAGCAGAGACTTGGTCTTGA GGGAATACATAAAAGTTAACACACA (SEQ ID NO:739)
E2	BRCA 2	BRCA2_210_1394102_1	ACACTGACGACATGGTTCTACAAGT TTTTGTACAGAGAATAGTTGTAGTT G (SEQ ID NO:740)	TACGGTAGCAGAGACTTGGTCTcaC ATTCCATAGCTGCCAGTTTC (SEQ ID NO:741)
F2	BRCA 2	BRCA2_210_21_1	ACACTGACGACATGGTTCTACATAT TTGTTCCAGGGCTCTGTGTGA (SEQ ID NO:742)	TACGGTAGCAGAGACTTGGTCTAGT TGAAGAAGCACCTTTCTGG (SEQ ID NO:743)
H2	BRCA 2	BRCA2_210_0939177_1	ACACTGACGACATGGTTCTACAGAT AAATTCAGTTTTTATTCTCAGTTATT CAGT (SEQ ID NO:744)	TACGGTAGCAGAGACTTGGTCTGAA caaGTGTTtTGCAGCTGTG (SEQ ID NO:745)
A3	BRCA 2	BRCA2_210_22_3	ACACTGACGACATGGTTCTACATCG GCTATAAAAAAGATAATGGAAAGG (SEQ ID NO:746)	TACGGTAGCAGAGACTTGGTCTACC CATCTGTAAGTTCAATAATGGC (SEQ ID NO:747)
B3	BRCA 2	BRCA2_210_22_4	ACACTGACGACATGGTTCTACAAGC AATAAACTAGTAGTGAGATACCC (SEQ ID NO:748)	TACGGTAGCAGAGACTTGGTCTCAA CTGTCAGTCTGCCATTCTTT (SEQ ID NO:749)
C3	BRCA 2	BRCA2_210_22_10	ACACTGACGACATGGTTCTACAATC CTCCCCTCTTAGCTGTCTTA (SEQ ID NO:750)	TACGGTAGCAGAGACTTGGTCTTTA ACATAAGAGATTCTGGGGCTT (SEQ ID NO:751)
D3	BRCA 2	BRCA2_210_39177_37	ACACTGACGACATGGTTCTACATTC TTCATGGAGCAGAACTGGTG (SEQ ID NO:752)	TACGGTAGCAGAGACTTGGTCTTCA GTAcATCTAAGAAATTGAGCATCC (SEQ ID NO:753)
E3	BRCA 2	BRCA2_210_39177_41	ACACTGACGACATGGTTCTACATGA TGCCTGTacACCTCTTGA (SEQ ID NO:754)	TACGGTAGCAGAGACTTGGTCTGCA CAAAAACTTTAACTGTCTGAAGAATA (SEQ ID NO:755)
F3	BRCA 2	BRCA2_210_0939178_1	ACACTGACGACATGGTTCTACACTT TTTAAAGTGAATATTTTAAAGGCAGT TCTA (SEQ ID NO:756)	TACGGTAGCAGAGACTTGGTCTAGG AAAaggTCtaGGGTCAGGAA (SEQ ID NO:757)
G3	BRCA 2	BRCA2_210_23_1	ACACTGACGACATGGTTCTACACTA ACAGTACTCGGCCTGCTC (SEQ ID NO:758)	TACGGTAGCAGAGACTTGGTCTACC TGTATAGGTATGCTCTTTGA (SEQ ID NO:759)
H3	BRCA 2	BRCA2_210_23_2	ACACTGACGACATGGTTCTACAGAC CTTTTCCTCTGCCCTTATCA (SEQ ID NO:760)	TACGGTAGCAGAGACTTGGTCTAGA AAGAAATATATGGTAAGTTTCAAGAA TACA (SEQ ID NO:761)
A4	BRCA 2	BRCA2_210_0939180_1	ACACTGACGACATGGTTCTACAATG TGACTTTTTTGGTGTGTGTAA (SEQ ID NO:762)	TACGGTAGCAGAGACTTGGTCTACC TTcATGTTCTTCAaATTCTCCT
B4	BRCA	BRCA2_210_	ACACTGACGACATGGTTCTACAAGA	TACGGTAGCAGAGACTTGGTCTTCA

Primer plate well	Gen e/chr	Name	F-primer	R-primer
	2	24_2	GGAAGAAAAGGAAGCAGCAA (SEQ ID NO:763)	TATTAGAAATAACAATGTGTACCATA TAACT (SEQ ID NO:764)
C4	BRCA 2	BRCA2_210_ 0939181_1	ACACTGACGACATGGTTCTACATTA GTTGCTTTTGAATTTAcAGTTTAGTG (SEQ ID NO:765)	TACGGTAGCAGAGACTTGGTCTAGC CTCATTATATGTCTCTTACTCTCT (SEQ ID NO:766)
D4	BRCA 2	BRCA2_210_ 0939183_1	ACACTGACGACATGGTTCTACATGG AACTTTTTTGTCTGATTGCT (SEQ ID NO:767)	TACGGTAGCAGAGACTTGGTCTCAc ggTTGTGACATcCCTTGATA (SEQ ID NO:768)
E4	BRCA 2	BRCA2_210_ 0939183_2	ACACTGACGACATGGTTCTACAATC ACAGGCAAATGTgAATGATAA (SEQ ID NO:769)	TACGGTAGCAGAGACTTGGTCTAAG TTAATAAAACTGATAAAAAACAAAGCA TTTAC (SEQ ID NO:770)
F4	BRCA 2	BRCA2_210_ 25_3	ACACTGACGACATGGTTCTACAAC TACTAATGCCACAAAGAGATAA (SEQ ID NO:771)	TACGGTAGCAGAGACTTGGTCTTTT TGAAGTTGCAAGATGATAAATTCTG (SEQ ID NO:772)
G4	BRCA 2	BRCA2_210_ 0939187_6	ACACTGACGACATGGTTCTACATgg CgTCCATCatcagATTATATTC (SEQ ID NO:773)	TACGGTAGCAGAGACTTGGTCTACT AACAAGCACTTATCAAAACTGAAA (SEQ ID NO:774)
H4	BRCA 2	BRCA2_210_ 26_1	ACACTGACGACATGGTTCTACAACC GGTACAAACCTTTCATTGT (SEQ ID NO:775)	TACGGTAGCAGAGACTTGGTCTGCT GAAAGTCTGGATCTAAAAATTTGCT (SEQ ID NO:776)
A5	BRCA 2	BRCA2_210_ 39187_47	ACACTGACGACATGGTTCTACAACgg gAGCcCCTTCACTT (SEQ ID NO:777)	TACGGTAGCAGAGACTTGGTCTGCC AACTGGTAGCTCCAACTAAT (SEQ ID NO:778)
B5	BRCA 2	BRCA2_210_ 27_2	ACACTGACGACATGGTTCTACAAGG CATATTAGAGTTTCCTTTCTTGC (SEQ ID NO:779)	TACGGTAGCAGAGACTTGGTCTTGC AGCAATTAACATATGAGGCTT (SEQ ID NO:780)
C5	BRCA 2	BRCA2_210_ 27_3	ACACTGACGACATGGTTCTACAAC TGCCCCCTTTCGTCTATTTGT (SEQ ID NO:781)	TACGGTAGCAGAGACTTGGTCTGCC CTCTTTTGGACTAGCAGAA (SEQ ID NO:782)
D5	BRCA 2	BRCA2_210_ 39189_30	ACACTGACGACATGGTTCTACACcA GTgGcgacCAGAATCC (SEQ ID NO:783)	TACGGTAGCAGAGACTTGGTCTTTC CTTGATACTGGACTGTCAAAA (SEQ ID NO:784)
E5	BRCA 2	BRCA2_210_ 0939192_1	ACACTGACGACATGGTTCTACAACA TTTAGGGTTTTTCATTCTTTTTTGGT (SEQ ID NO:785)	TACGGTAGCAGAGACTTGGTCTGGA CCAATTGGGATcATTTGCAT (SEQ ID NO:786)
F5	BRCA 2	BRCA2_210_ 0939192_3	ACACTGACGACATGGTTCTACAAAG CAGCTTTTCCAATTATTTCTT (SEQ ID NO:787)	TACGGTAGCAGAGACTTGGTCTATA ATATTCCTTGAGTTTACATTAACCTA CCA (SEQ ID NO:788)
G5	BRCA 2	BRCA2_210_ 28_1	ACACTGACGACATGGTTCTACATGT GAAGTAAATCACCTAACCTAT (SEQ ID NO:789)	TACGGTAGCAGAGACTTGGTCTTGA TAAAGGACTTTGATAATATATCTCAC AATTAG (SEQ ID NO:790)
H5	BRCA 2	BRCA2_210_ 28_2	ACACTGACGACATGGTTCTACAAC GTGTGTAATATTTGCGTGCTT (SEQ ID NO:791)	TACGGTAGCAGAGACTTGGTCTGTG GAAACAGACTTCCTTTTGGC (SEQ ID NO:792)
A6	BRCA 2	BRCA2_210_ 184789_7	ACACTGACGACATGGTTCTACACTc CTAATTgtGAGAtatAttaTCAAAGTCC (SEQ ID NO:793)	TACGGTAGCAGAGACTTGGTCTaGA AaTCCAAGGCTcTTCTCTTTT (SEQ ID NO:794)
B6	BRCA 2	BRCA2_210_ 184789_10	ACACTGACGACATGGTTCTACAAGA GATTGATGACCAAAGAAGTGC	TACGGTAGCAGAGACTTGGTCTTTA AATGGAGTCATCTGAGGAGAA

Primer plate well	Gene/chr	Name	F-primer	R-primer
			(SEQ ID NO:795)	(SEQ ID NO:796)
D6	BRCA2	BRCA2_210_29_2	ACACTGACGACATGGTTCTACAAGC TGACGAAGAAGCTTGCATTGA (SEQ ID NO:797)	TACGGTAGCAGAGACTTGGTCTCCT GGGAAGCTCCTGTTCTTTG (SEQ ID NO:798)
E6	BRCA2	BRCA2_210_184789_35	ACACTGACGACATGGTTCTACAGaA AAACAATTTaTatctGTCAGTGAATCC (SEQ ID NO:799)	TACGGTAGCAGAGACTTGGTCTTGT gTCCTGCTtAtTTTTCTCACA (SEQ ID NO:800)
F6	BRCA2	BRCA2_210_184789_48m	ACACTGACGACATGGTTCTACATCT GATCAaaGAACAGGAGAGTTC (SEQ ID NO:801)	TACGGTAGCAGAGACTTGGTCTTAA GTACTAATGTGTGGTTTGAAATTATA TTCCAGT (SEQ ID NO:802)

Table 7 Cell-line Genomic DNA Samples

Sample	BC
K562	FLD0001
MOLT-4	FLD0002
CCRF-CEM	FLD0003
RPMI-8226	FLD0004
HL-60(TB)	FLD0005
SR	FLD0006
SF-268	FLD0007
SF-295	FLD0008
SF-539	FLD0009
SNB-19	FLD0010
SNB-75	FLD0011
U251	FLD0012
BT-549	FLD0013
HS-578T	FLD0014
MCF7	FLD0015
NCI/ADR-RES	FLD0016
MBA-MB-231/ATCC	FLD0017
MDA-MB-435	FLD0018
T-47D	FLD0019
COLO 205	FLD0020
HCC-2998	FLD0021
HCT-116	FLD0022
HCT-15	FLD0023
HT-29	FLD0024
KM12	FLD0025
SW-620	FLD0026
A549/ATCC	FLD0027
EKVX	FLD0028
HOP-62	FLD0029
HOP-92	FLD0030
NCI-H322M	FLD0031
NCI-H226	FLD0032
NCI-H23	FLD0033
NCI-H460	FLD0034
NCI-H522	FLD0035
LOX IMVI	FLD0036
M14	FLD0037
MALME-3M	FLD0038
SK-MEL-2	FLD0039

Sample	BC
SK-MEL-28	FLD0040
SK-MEL-5	FLD0041
UACC-257	FLD0042
UACC-62	FLD0043
IGR-OV1	FLD0044
OVCAR-3	FLD0045
OVCAR-4	FLD0046
OVCAR-5	FLD0047
OVCAR-8	FLD0048
K562	FLD0049
MOLT-4	FLD0050
CCRF-CEM	FLD0051
RPMI-8226	FLD0052
HL-60(TB)	FLD0053
SR	FLD0054
SF-268	FLD0055
SF-295	FLD0056
SF-539	FLD0057
SNB-19	FLD0058
SNB-75	FLD0059
U251	FLD0060
BT-549	FLD0061
HS-578T	FLD0062
MCF7	FLD0063
NCI/ADR-RES	FLD0064
MBA-MB-231/ATCC	FLD0065
MDA-MB-435	FLD0066
T-47D	FLD0067
COLO 205	FLD0068
HCC-2998	FLD0069
HCT-116	FLD0070
HCT-15	FLD0071
HT-29	FLD0072
KM12	FLD0073
SW-620	FLD0074
A549/ATCC	FLD0075
EKVX	FLD0076
HOP-62	FLD0077
HOP-92	FLD0078
NCI-H322M	FLD0079
NCI-H226	FLD0080
NCI-H23	FLD0081

Sample	BC
NCI-H460	FLD0082
NCI-H522	FLD0083
LOX IMVI	FLD0084
M14	FLD0085
MALME-3M	FLD0086
SK-MEL-2	FLD0087
SK-MEL-28	FLD0088
SK-MEL-5	FLD0089
UACC-257	FLD0090
UACC-62	FLD0091
IGR-OV1	FLD0092
OVCAR-3	FLD0093
OVCAR-4	FLD0094
OVCAR-5	FLD0095
OVCAR-8	FLD0096

Table 8 Barcode Primers

Primer	Sequence
CS1-TS-F	5'-ACACTGACGACATGGTTCTACA-[TS-F ₀₁]-3'
CS2-TS-R	5'-TACGGTAGCAGAGACTTGGTCT-[TS-Rev]-3'
PE1-CS1	5'-AATGATACGGCGACCAACGAGATCTACACTGACGACATGGTTCTACA-3'
PE2-BC-CS2	5'-CAAGCAGAAGACGGCATACGAGAT-[BC]-TACGGTAGCAGAGACTTGGTCT-3'
PE1-CS2	5'-AATGATACGGCGACCAACGAGATCTTACGGTAGCAGAGACTTGGTCT-3'
PE2-BC-CS1	5'-CAAGCAGAAGACGGCATACGAGAT-[BC]-ACACTGACGACATGGTTCTACA-3'
CS1	5'-A+CA+CTG+ACGACATGGTTCTACA-3'
CS2	5'-T+AC+GGT+AGCAGAGACTTGGTCT-3'
CS1rc	5'-T+GT+AG+AACCATGTCGTCAGTGT-3'
CS2rc	5'-A+GAC+CA+AGTCTCTGCTACCGTA-3'
LNA nucleotides preceded by a "+"	

Example 11

Bidirectional DNA Sequencing Amplicon Tagging for Illumina Sequencers using the 48.48 ACCESS ARRAY™ IFC – Protocol 2

[0400] This Example provides a modified version of the protocol in Example 9.

5 The Introduction to Example 9 also applies to this Example.

Preparing Amplicons

[0401] The following documents may be consulted as references for this protocol:
Fluidigm® IFC Controller for ACCESS ARRAY™ System User Guide (PN 68000157);
Fluidigm® Control Line Fluid Loading Procedure Quick Reference (PN68000132); and
10 Agilent DNA 1000 Kit Guide.

[0402] The following Reagents were used for this protocol and were stored at -20°C:
FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001); 20X
ACCESS ARRAY™ Loading Reagent (Fluidigm, PN 100-0883); 1X ACCESS ARRAY™
Harvest Solution (Fluidigm, PN 100-1031); ACCESS ARRAY™ Barcode Library for
15 Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771); target-specific primer
pairs tagged with universal tags (CS1 forward tag, CS2 reverse tag), including 50 µM CS1-
Tagged TS Forward Primer and 50 µM CS2-Tagged TS Reverse Primer; and template DNA
at 50ng/µL. (The 1X ACCESS ARRAY™ Harvest Solution (Fluidigm, PN 100-1031) is
not packaged for individual sale. It can be purchased in units of 10, under the name
20 ACCESS ARRAY™ Harvest Pack, PN 100-3155, or as a component in the 48.48 ACCESS
ARRAY™ Loading Reagent Kit, PN 100-1032.) Also used were the Agilent DNA 1000
Kit Reagents (Agilent, PN 5067-1504), which are Stored at 4°C. Additionally, PCR
Certified Water (Teknova, PN W330) was used; this was stored at room temperature.

[0403] Multiplex PCR on the 48.48 ACCESS ARRAY™ IFC was performed
25 according to the instructions as detailed in Chapter 6 - Multiplex Amplicon Tagging on the
48.48 ACCESS ARRAY™ IFC of the *ACCESS ARRAY™ System for Illumina Platform
User Guide*. Alternatively, 2-Primer Target-Specific PCR on the 48.48 ACCESS
ARRAY™ IFC was performed to achieve bidirectional amplicon tagging without
multiplexing, according to the instructions as detailed in Appendix C of the *ACCESS*

ARRAY™ System for Illumina Platform User Guide. The harvested PCR products were then barcoded following the instructions below.

[0404] PCR products were barcoded in two 96-well plates for bidirectional amplicon tagging following the instructions as detailed in Chapter 6 - Attaching Sequence Tags and Sample Barcodes of the Fluidigm *ACCESS ARRAY™ System for Illumina Platform User Guide*. The 100-fold dilution of the harvested PCR product pool served as template in two (rather than one) barcoding PCR reactions: one reaction generated PCR product A that allowed for sequencing of the 5' end of the target region in one 96-well plate, and the other reaction generated PCR product B that allowed for sequencing of the 3' end of the target region in a second 96-well plate. The setup of the reaction is identical to "Attaching Sequence Tags and Sample Barcodes" in the Fluidigm *ACCESS ARRAY™ System for Illumina Platform User Guide*. However, the quantities in the Sample Pre-Mix Solution were doubled to compensate for the increase in the number of reactions, and ACCESS ARRAY™ Barcode Library for Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771) was used in the preparation of the Sample Mix Solution (Tables 9 and 10).

Table 9 Sample Mix Solutions – PCR Product A

Component	Volume (μL)
Sample Pre-Mix	15.0
ACCESS ARRAY™ Barcode Library for Illumina Sequencers - 384 (Bidirectional) A	4.0
Diluted Harvested PCR Product Pool	1.0
Total	20.0

Table 10 Sample Mix Solutions – PCR Product B

Component	Volume (μL)
Sample Pre-Mix	15.0
ACCESS ARRAY™ Barcode Library for Illumina Sequencer - 384 (Bidirectional) B	4.0
Diluted Harvested PCR Product Pool	1.0
Total	20.0

[0405] After the second PCR had finished, PCR Product A and PCR Product B pools were combined prior to sequencing. Chapter 8 of the Fluidigm *ACCESS ARRAY™ System for Illumina Platform User Guide* provides for methods describing post-PCR product library purification and quantitation. It was essential to use ACCESS ARRAY™ Barcode Library for Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771) to generate bidirectional amplicons for sequencing.

Sequencing Workflow Using Fluidigm FL1 and FL2 Sequencing Primers

[0406] The following instructions are intended for use with Illumina TruSeq sequencing reagents on the Illumina GAI and HiSeq systems. The Fluidigm sequencing reagents FL1 and FL2, contain equimolar mixtures of the CS1 and CS2 sequencing and indexing primers, respectively. FL1 is the custom sequencing primer and contains 50 μ M each of the CS1 and CS2 primers. FL2 is the custom indexing primer and contains 50 μ M each of the CS1rc and CS2rc primers. For single-read sequencing, reagents were prepared for Read 1 and the Indexing primers. For paired-end sequencing, reagents were prepared for Read 1, the Indexing, and Read 2 primers.

[0407] Results from PCR experiments to test for cross talk between Fluidigm Sequencing Primers and TruSeq Sequencing Primers are shown in Figures 32 and 33.

[0408] The following documents may be consulted as references for sequencing: Illumina cBot™ User Guide; Illumina Genome Analyzer II™ User Guide; and Illumina HiSeq™ User Guide. The Illumina Genome Analyzer II User Guide or the Illumina HiSeq User Guide should be referred to for instructions on how to perform a sequencing run. Technical Support at Illumina may also be contacted.

Preparing Reagents for Sequencing on the Illumina GAI and HiSeq Sequencing Systems

[0409] The Read 1 Sequencing Primer HT1/FL1 was prepared by first diluting the FL1 stock to a final concentration of 500 nM with Hybridization Buffer (HT1) in a DNase-, RNase-free 1.5 mL microcentrifuge tube (Table 11). The tube was vortexed for a minimum of 20 seconds, and centrifuged for 30 seconds to spin down all components. The following instructions outline preparation of the HT1/FL1 sequencing primer mix for Read 1 (per mL). Approximately 300 μ L was used per lane, using the cBot Custom Primers Reagent Stage. The custom primer orientation in the tube strip was aligned with the lanes of the GAI or HiSeq flow cell.

Table 11 Instructions for Preparing HT1/FL1 (per mL)

Reagent	Volume (μ L)
HT1 Buffer	990 μ L
FL1 Stock (from Appendix E)	10 μ L
Total	1.0 mL

[0410] The Indexing Primer HT1/FL2 was prepared by first diluting the FL2 stock to a final concentration of 500 nM with Hybridization Buffer (HT1) in a DNase-, RNase-free 1.5 mL microcentrifuge tube (Table 12). The tube was vortexed for a minimum of 20 seconds, and centrifuged for 30 seconds to spin down all components. The following instructions outline preparation of the HT1/FL2 indexing primer mix for the Index Read. Approximately 3 mL of Index Sequencing Primer Mix (HP8) was used for the Index Read. 1.5 mL of TruSeq Reagent HP8 was substituted for 1.5 mL of HT1/FL2.

Table 12 Instructions for Preparing HT1/FL2

Reagent	Volume
HT1 Buffer	1,485 μ L
FL1 Stock (from Appendix E)	15 μ L
Total	1.5 mL

[0411] The Read 2 Sequencing Primer HT1/FL1 (for Paired-End Sequencing) was prepared by first diluting the FL1 stock to a final concentration of 500 nM with Hybridization Buffer (HT1) in a DNase-, RNase-free 1.5 mL microcentrifuge tube (Table 13). The tube was vortexed for a minimum of 20 seconds, and centrifuged for 30 seconds to spin down all components. The following instructions outline preparation of the HT1/FL1 sequencing primer mix for Read 2. Approximately 3.2 mL of Read 2 Sequencing Primer (HP7) was used for Read 2. 1.6 mL of TruSeq Reagent HP7 was substituted for 1.6 mL of HT1/FL1.

Table 13 Instructions for preparing Read 2 Sequencing Primer HT1/FL1

Reagent	Volume
HT1 Buffer	1,584 μ L
FL1 Stock (from Appendix E)	16 μ L
Total	1.6 mL

Performing a Sequencing Run

[0412] The Illumina Genome Analyzer II or HiSeq user guides provide instructions on how to perform a sequencing run. Alternatively, Technical Support at Illumina may be contacted.

[0413] For the Index Read, 1.5 mL of TruSeq Reagent HP8 was substituted for 1.5 mL of the Indexing Primer HT1/FL2 for GAII and HiSeq sequencing runs. The barcode sequences used in the ACCESS ARRAY™ Barcode Library for Illumina have been

designed so that they can be distinguished even when sequencing errors are present. As more samples are run in parallel, the length of the index read required to distinguish the barcode sequences unambiguously increases. Recommendations for index reads are described in Table 14.

5

Table 14 Index Read Recommendations

Number of samples per lane	1-384	385-1920
Length of index read	8 bases	10 bases

[0414] When preparing the sequencing run, the length of the index read was adjusted according to the guidelines in Table 14. The volumes of the sequencing reagents loaded onto the sequencer were ensured to be sufficient for the index cycles. The *Illumina*

10 *Sequencer User Guide* was consulted, or Technical Support at Illumina was contacted, for detailed instructions on how to implement these changes.

CLAIMS

What is claimed is:

1. A method of adding adaptor molecules to each end of a plurality of target nucleic acids that comprise sticky ends, the method comprising:

5 (a) annealing adaptor molecules to the sticky ends of double-stranded target nucleic acid molecules to produce annealed adaptor-target nucleic acid molecules, wherein the adaptor molecules are:

(i) hairpin structures each comprising:

10 an adaptor nucleotide sequence, which is linked to
a nucleotide linker, which is linked to
a nucleotide sequence that is capable of annealing to
the adaptor nucleotide sequence and is linked to

a degenerate tail sequence; or

15 (ii) double-stranded or single-stranded molecules each comprising:

a first adaptor nucleotide sequence, which is linked to
a nucleotide linker, which is linked to
a second adaptor nucleotide sequence; and
a degenerate tail sequence on each strand, wherein

20 double-stranded molecules each comprise two degenerate tail sequences as sticky end(s);

(b) filling any gaps in the resulting annealed adaptor-target nucleic acid molecules; and

(c) ligating any adjacent nucleotide sequences in the annealed adaptor-target nucleic acid molecules to produce adaptor-modified target nucleic acid
25 molecules.

2. The method of claim 1, wherein the plurality of DNA molecules comprises a DNA library to be sequenced.

3. The method of any preceding claim, wherein the method comprises producing the plurality of target nucleic acid molecules that comprise sticky ends by fragmenting DNA molecules.

4. The method of claim 3, wherein the DNA molecules are fragmented
5 by digesting them with a DNase enzyme.

5. The method of claim 3, wherein said fragmenting of DNA molecules is followed by digesting the fragmented DNA molecules with an enzyme to produce sticky ends.

6. The method of claim 5, wherein the enzyme employed to produce
10 sticky ends comprises a strand-specific endonuclease that does not have polymerase activity under the conditions employed in the digestion.

7. The method of any preceding claim, wherein the sticky ends of the double-stranded target nucleic acid molecules are 3' extensions.

8. The method of any preceding claim, wherein the degenerate tail
15 sequence(s) is/are at the 3' ends of the adaptor molecules.

9. The method of any preceding claim, wherein the adaptor molecules are hairpin structures each comprising:

an adaptor nucleotide sequence, which is linked to

a nucleotide linker, which is linked to

20 a nucleotide sequence that is capable of annealing to the first adaptor nucleotide sequence and is linked to

a degenerate tail sequence.

10. The method of claim 9, wherein the adaptor molecules comprise:
a first type of adaptor molecule comprising a first adaptor nucleotide
25 sequence; and

a second type of adaptor molecule comprising a second adaptor nucleotide sequence that is different from the first adaptor nucleotide sequence.

11. The method of claim 9, wherein said ligating converts the annealed adaptor-target nucleic acid molecules to single-stranded circular DNA molecules.

12. The method of any of claims 1-8, wherein the adaptor molecules annealed in (a) are double-stranded molecules, each comprising:

- 5 a first adaptor nucleotide sequence, which is linked to
 a nucleotide linker, which is linked to
 a second adaptor nucleotide sequence that is different from the first
adaptor nucleotide sequence; and
 sticky ends comprising degenerate tail sequences.

10 13. The method of any of claims 1-8, wherein the adaptor molecules annealed in (a) are single-stranded molecules each comprising:

- a first adaptor nucleotide sequence, which is linked to
 a nucleotide linker, which is linked to
 a second adaptor nucleotide sequence that is different from the first
15 adaptor nucleotide sequence; and
 a degenerate tail sequence.

14. The method of claims 10, 12, or 13, wherein the first and second adaptor sequences comprise primer binding sites that are capable of being specifically bound by DNA sequencing primers.

20 15. The method of claims 12 or 13, wherein said ligating converts the annealed adaptor-target nucleic acid molecules to double-stranded circular DNA molecules.

16. The method of any preceding claim, wherein the nucleotide linker comprises a nucleotide sequence selected from the group consisting of an endonuclease site, a barcode nucleotide sequence, an affinity tag, and any combination thereof.

25 17. The method of any preceding claim, wherein the nucleotide linker comprises a restriction enzyme site.

18. The method of any preceding claim, wherein the nucleotide linker comprises a restriction enzyme site and at least one barcode nucleotide sequence.

19. The method of claim 16, wherein the nucleotide linker comprises an endonuclease site, and the method additionally comprises digesting the single-stranded or double-stranded circular DNA molecules to produce linear DNA molecules.

20. The method of claim 16, wherein the nucleotide linker comprises a
5 restriction enzyme site, and the method additionally comprises digesting double-stranded circular DNA molecules to produce linear DNA molecules.

21. The method of claim 19, wherein the linear DNA molecules comprise
5'-a first portion of a nucleotide linker-second adaptor nucleotide sequence-a first
degenerate tail sequence-target nucleic acid molecule-a second degenerate tail sequence-
10 first adaptor nucleotide sequence-a second portion of a nucleotide linker-3'.

22. The method of any preceding claim, wherein the method comprises:
producing the plurality of target nucleic acid molecules that comprise
sticky ends by:

digesting DNA molecules with DNase I to produce
15 fragmented DNA molecules, and then heat inactivating the DNase I;
digesting the fragmented DNA molecules with a nuclease
having 5' to 3' exonuclease activity in the absence of deoxynucleotides to produce a
plurality sticky-ended target nucleic acid molecules;
annealing the adaptors to the sticky ends of the plurality of target
20 nucleic acid molecules, wherein the nucleotide linker of the adaptors comprises an
endonuclease site;
filling any gaps and ligating any adjacent nucleotide sequences in the
annealed adaptor-target nucleic acid molecules in a single reaction comprising a polymerase
and a ligase to produce circular DNA molecules; and
25 digesting the circular DNA molecules with an endonuclease that cuts
at the endonuclease site to produce linear DNA molecules.

23. The method of claim 22, wherein the nuclease having 5' to 3'
exonuclease activity is Exonuclease III.

24. The method of any preceding claim, wherein the method additionally comprises sequencing the adaptor-modified target nucleic acid molecules.

25. A plurality of adaptor molecules, wherein the adaptor molecules are:

(i) hairpin structures each comprising:

5 an adaptor nucleotide sequence, which is linked to
a nucleotide linker, which is linked to
a nucleotide sequence that is capable of annealing to
the adaptor nucleotide sequence and is linked to

a degenerate tail sequence; or

10 (ii) double-stranded or single-stranded molecules each
comprising:

a first adaptor nucleotide sequence, which is linked to
a nucleotide linker, which is linked to
a second adaptor nucleotide sequence; and

15 a degenerate tail sequence on each strand, wherein
double-stranded molecules each comprise two degenerate tail sequences as sticky end(s).

26. The plurality of adaptor molecules of claim 25, wherein the adaptor molecules are hairpin structures each comprising:

20 an adaptor nucleotide sequence, which is linked to
a nucleotide linker, which is linked to
a nucleotide sequence that is capable of annealing to the first adaptor
nucleotide sequence and is linked to
a degenerate tail sequence.

25 27. The plurality of adaptor molecules of claim 26, wherein the adaptor molecules comprise:

a first type of adaptor molecule comprising a first adaptor nucleotide
sequence; and

a second type of adaptor molecule comprising a second adaptor
nucleotide sequence that is different from the first adaptor nucleotide sequence.

28. The plurality of adaptor molecules of claim 25, wherein the adaptor molecules annealed in (a) are double-stranded molecules, each comprising:

a first adaptor nucleotide sequence, which is linked to

a nucleotide linker, which is linked to

5 a second adaptor nucleotide sequence that is different from the first adaptor nucleotide sequence; and

sticky ends comprising degenerate tail sequences.

29. The plurality of adaptor molecules of claim 25, wherein the adaptor molecules annealed in (a) are single-stranded molecules each comprising:

10 a first adaptor nucleotide sequence, which is linked to

a nucleotide linker, which is linked to

a second adaptor nucleotide sequence that is different from the first adaptor nucleotide sequence; and

a degenerate tail sequence.

15 30. The plurality of adaptor molecules of claims 27-29, wherein the first and second adaptor sequences comprise primer binding sites that are capable of being specifically bound by DNA sequencing primers.

31. The plurality of adaptor molecules of any of claims 25-30, wherein the nucleotide linker comprises a nucleotide sequence selected from the group consisting of
20 an endonuclease site, a barcode nucleotide sequence, an affinity tag, and any combination thereof.

32. The plurality of adaptor molecules of any of claims 25-30, wherein the nucleotide linker comprises a restriction enzyme site.

33. The plurality of adaptor molecules of any of claims 25-30, wherein
25 the nucleotide linker comprises a restriction enzyme site and at least one barcode nucleotide sequence.

34. A kit comprising the plurality of adaptor molecules of any of claims 25-33 and one or more components selected from the group consisting of a DNase enzyme, an exonuclease, an endonuclease, a polymerase, and a ligase.

35. A method for tagging a plurality of target nucleic acids with nucleotide sequences, the method comprising:

(a) preparing a first reaction mixture for each target nucleic acid, the first reaction mixture comprising a pair of inner primers and a pair of outer primers,

5 wherein:

(i) the inner primers comprise:

a forward, inner primer comprising a first nucleotide tag, a first barcode nucleotide sequence, and a target-specific portion; and

10 a reverse, inner primer comprising a target-specific portion, a first barcode nucleotide sequence, and a second nucleotide tag; and

(ii) the outer primers comprise:

a forward, outer primer comprising a second barcode nucleotide sequence and a first nucleotide tag-specific portion; and

15 a reverse, outer primer comprising a second nucleotide tag-specific portion and a second barcode nucleotide sequence;

wherein the outer primers are in excess of the inner primers;

and

(b) subjecting each first reaction mixture to a reaction to produce a plurality of tagged target nucleotide sequences, each comprising 5'-second barcode

20 nucleotide sequence-first nucleotide tag sequence-first barcode nucleotide sequence-target nucleotide sequence-first barcode nucleotide sequence-second nucleotide tag sequence-second barcode nucleotide sequence-3'.

36. The method of claim 35, wherein the reaction comprises amplification.

25 37. The method of claim 35, wherein the reaction comprises ligation.

38. The method of claim 35, wherein the outer primers additionally comprise first and second primer binding sites that are capable of being bound by DNA sequencing primers.

39. The method of claim 38, wherein the reaction produces tagged target
30 nucleotide sequences comprising 5'-first primer binding site-second barcode nucleotide

sequence-first nucleotide tag sequence-first barcode nucleotide sequence-target nucleotide sequence-first barcode nucleotide sequence-second nucleotide tag sequence-second barcode nucleotide sequence-second primer binding site-3'.

40. A kit for performing the method of claim 35, wherein the kit
5 comprises a polymerase and:
(i) inner primers comprising:
a forward, inner primer comprising a first nucleotide tag, a
first barcode nucleotide sequence, and a target-specific portion; and
a reverse, inner primer comprising a target-specific portion, a
10 first barcode nucleotide sequence, and a second nucleotide tag; and
(ii) outer primers comprising:
a forward, outer primer comprising a second barcode
nucleotide sequence and a first nucleotide tag-specific portion; and
a reverse, outer primer comprising a second nucleotide tag-
15 specific portion and a second barcode nucleotide sequence;
wherein the outer primers are in excess of the inner primers.

41. A method for tagging a plurality of target nucleic acids with nucleotide sequences, the method comprising:

- (a) preparing a first reaction mixture for each target nucleic acid, the
20 first reaction mixture comprising a pair of inner primers, a pair of stuffer primers, and a pair of outer primers, wherein:
(i) the inner primers comprise:
a forward, inner primer comprising a first nucleotide
tag and a target-specific portion; and
25 a reverse, inner primer comprising a target-specific
portion and a second nucleotide tag;
(ii) the stuffer primers comprise:
a forward, stuffer primer comprising a third nucleotide
tag, a first barcode nucleotide sequence, and a first nucleotide tag-specific portion; and

a reverse, stuffer primer comprising a second nucleotide tag-specific portion, a first barcode nucleotide sequence, a fourth nucleotide tag; and

(iii) the outer primers comprise:

5 a forward, outer primer comprising a second barcode nucleotide sequence and a third nucleotide tag-specific portion; and

a reverse, outer primer comprising a fourth nucleotide tag-specific portion and a second barcode nucleotide sequence;

10 wherein the outer primers are in excess of the stuffer primers, which are in excess of the inner primers; and

(b) subjecting each first reaction mixture to a reaction to produce a plurality of tagged target nucleotide sequences, each comprising 5'-second barcode nucleotide sequence-third nucleotide tag sequence-first barcode nucleotide sequence-first nucleotide tag sequence-target nucleotide sequence-second nucleotide tag sequence-first
15 barcode nucleotide sequence-fourth nucleotide tag sequence-second barcode nucleotide sequence-3'.

42. The method of claim 41, wherein the reaction comprises amplification.

43. The method of claim 41, wherein the reaction comprises ligation.

20 44. The method of claim 41, wherein the outer primers additionally comprise first and second primer binding sites that are capable of being bound by DNA sequencing primers.

45. The method of claim 44, wherein the reaction produces tagged target nucleotide sequences comprising 5'- first primer binding site-second barcode nucleotide
25 sequence-third nucleotide tag sequence-first barcode nucleotide sequence-first nucleotide tag sequence-target nucleotide sequence-second nucleotide tag sequence-first barcode nucleotide sequence-fourth nucleotide tag sequence-second barcode nucleotide sequence-second primer binding site-3'.

46. A kit for performing the method of claim 41, wherein the kit comprises a polymerase and:

(i) inner primers comprising:

5 a forward, inner primer comprising a first nucleotide tag and a target-specific portion; and
a reverse, inner primer comprising a target-specific portion and a second nucleotide tag;

(ii) stuffer primers comprising:

10 a forward, stuffer primer comprising a third nucleotide tag, a first barcode nucleotide sequence, and a first nucleotide tag-specific portion; and
a reverse, stuffer primer comprising a second nucleotide tag-specific portion, a first barcode nucleotide sequence, a fourth nucleotide tag; and

(iii) outer primers comprising:

15 a forward, outer primer comprising a second barcode nucleotide sequence and a third nucleotide tag-specific portion; and
a reverse, outer primer comprising a fourth nucleotide tag-specific portion and a second barcode nucleotide sequence;

wherein the outer primers are in excess of the stuffer primers, which are in excess of the inner primers.

20 47. The kit of claims 40 or 46, wherein the outer primers additionally comprise first and second primer binding sites that are capable of being bound by DNA sequencing primers.

48. A method for combinatorial tagging of a plurality of target nucleotide sequences, the method comprising:

25 cutting a plurality of tagged target nucleotide sequences derived from target nucleic acids, each tagged target nucleotide sequence comprising an endonuclease site and a first barcode nucleotide sequence, wherein tagged target nucleotide sequences in the plurality comprise the same endonuclease site, but N different first barcode nucleotide sequences, wherein N is an integer greater than 1, with an endonuclease specific for the
30 endonuclease site to produce a plurality of sticky-ended, tagged target nucleotide sequences;

ligating a plurality of adaptors comprising a second barcode nucleotide sequence and complementary sticky ends to the plurality of sticky-ended, tagged target nucleotide sequences in a first reaction mixture, wherein the plurality of adaptors comprise M different second barcode nucleotide sequences, wherein M is an integer greater than 1, to produce a plurality of combinatorially tagged target nucleotide sequences, each comprising first and second barcode nucleotide sequences, wherein the plurality comprises $N \times M$ different first and second barcode combinations.

49. The method of claim 48, wherein the endonuclease site is adjacent to the first barcode nucleotide sequence in the tagged target nucleotide sequences.

50. The method of claim 49, wherein the second barcode nucleotide sequence is adjacent to the complementary sticky end in the adaptors.

51. The method of any of claims 50-52, wherein the combinatorially tagged target nucleotide sequences comprise the first and second barcode nucleotide sequences separated by fewer than 5 nucleotides.

52. The method of any of claims 50-53, wherein the tagged target nucleotide sequences comprise first and second primer binding site having an arrangement selected from:

5'-endonuclease site-first barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site; and

5'-first primer binding site-target nucleotide sequence-second primer binding site-first barcode nucleotide sequence-endonuclease site-3'.

53. The method of claim 52, wherein the first and second primer binding sites are binding sites for DNA sequencing primers.

54. The method of claims 52 or 53, wherein the combinatorially tagged target nucleotide sequences comprise:

5'-second barcode nucleotide sequence-first barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site; or

5'-first primer binding site-target nucleotide sequence-second primer binding site-first barcode nucleotide sequence- second barcode nucleotide sequence -3'.

55. The method of any of claims 48-54, wherein the tagged target nucleotide sequences are prepared by ligating adaptors onto a plurality of target nucleic acids, wherein the adaptors comprise:

- 5 a first adaptor comprising the endonuclease site, the first barcode nucleotide sequence, the first primer binding site, and a sticky end;
a second adaptor comprising a second primer binding site and a sticky end.

56. The method of claims 52 or 53, wherein the tagged target nucleotide sequences comprise a first additional nucleotide sequence having an arrangement selected from:

- 10 5'-endonuclease site-first barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site- first additional nucleotide sequence; and
5'- first additional nucleotide sequence-first primer binding site-target
15 nucleotide sequence-second primer binding site-first barcode nucleotide sequence-
endonuclease site-3'.

57. The method of claim 56, wherein the adaptors comprise a second additional nucleotide sequence and have the arrangement: 5'-second additional nucleotide sequence-second barcode nucleotide sequence-complementary sticky end-3'.

20 58. The method of claim 57, wherein the combinatorially tagged target nucleotide sequences comprise:

- 5'-second additional nucleotide sequence-second barcode nucleotide sequence-first barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-first additional nucleotide sequence; and
25 5'- second additional nucleotide sequence-first primer binding site-
target nucleotide sequence-second primer binding site-first barcode nucleotide sequence-
second barcode nucleotide sequence-first additional nucleotide sequence -3'.

59. The method of claims 56-58, wherein the first and/or second additional nucleotide sequences comprise a primer binding site.

60. The method of any of claims 56-59, wherein the tagged target nucleotide sequences are prepared by ligating adaptors onto a plurality of target nucleic acids, wherein the adaptors comprise:

- a first adaptor comprising the endonuclease site, the first barcode nucleotide sequence, the first primer binding site, and a sticky end; and
- a second adaptor comprising a first additional nucleotide sequence, a second primer binding site, and a sticky end.

61. A plurality of adaptors comprising:

- a plurality of first adaptors, each comprising the same endonuclease site, N different barcode nucleotide sequences, wherein M is an integer greater than 1, a first primer binding site and a sticky end;
- a second adaptor comprising a second primer binding site and a sticky end; and
- a plurality of third adaptors comprising a second barcode nucleotide sequence and sticky ends complementary to those produced upon cutting the first adaptors at the endonuclease site, wherein the plurality of third adaptors comprise M different second barcode nucleotide sequences, wherein M is an integer greater than 1.

62. A kit comprising the adaptors of claim 61 and one or more

- components selected from the group consisting of: an endonuclease specific for the endonuclease site in the first adaptors and a ligase.

63. A method for combinatorial tagging of a plurality of target nucleotide sequences, the method comprising:

- annealing a plurality of barcode primers to a plurality of tagged target nucleotide sequences derived from target nucleic acids, wherein:
 - each tagged target nucleotide sequence comprises a nucleotide tag at one end and a first barcode nucleotide sequence, wherein tagged target nucleotide sequences in the plurality comprise the same nucleotide tag, but N different first barcode nucleotide sequences, wherein N is an integer greater than one; and
 - each barcode primer comprises:
 - a first tag-specific portion linked to;

a second barcode nucleotide sequence linked to;
a second tag-specific portion;
wherein the barcode primers in the plurality each
comprise the same first and second tag-specific portions, but M different second barcode
5 nucleotide sequences, wherein M is an integer greater than one; and
amplifying the tagged target nucleotide sequences in a first reaction
mixture to produce a plurality of combinatorially tagged target nucleotide sequences, each
comprising first and second barcode nucleotide sequences, wherein the plurality comprises
 $N \times M$ different first and second barcode combinations.

10 64. The method of claim 63, wherein the first barcode nucleotide
sequence is separated from the nucleotide tag by the target nucleotide sequence.

65. The method of claims 63 or 64, wherein the first tag-specific portion
of the barcode primer anneals to a 5' portion of the nucleotide tag, and the second tag-
specific portion of the barcode primer anneals to an adjacent 3' portion of the nucleotide
15 tag, and the second barcode nucleotide sequence does not anneal to the nucleotide tag,
forming a loop between the annealed first and second tag-specific portions.

66. The method of any of claims 63-65, wherein the tagged nucleotide
sequences additionally comprise a primer binding site between the target nucleotide
sequence and the first barcode nucleotide sequence.

20 67. The method of any of claims 63-66, wherein the first and second tag-
specific portions of the barcode primer are sufficiently long to serve as primer binding sites.

68. The method of claims 66 or 67, wherein binding sites are binding
sites for DNA sequencing primers.

25 69. The method of any of claims 66-68, wherein the combinatorially
tagged target nucleotide sequences comprise 5'-first tag-specific portion-second barcode
nucleotide sequence-second tag-specific portion-target nucleotide sequence-primer binding
site-first barcode nucleotide sequence-3'.

70. The method of claims 66-69, wherein the tagged target nucleotide sequences comprise a first additional nucleotide sequence having the arrangement: 5'-nucleotide tag-target nucleotide sequence-primer binding site-first barcode nucleotide sequence-first additional nucleotide sequence-3'.

5 71. The method of claim 70, wherein the barcode primers additionally comprise a second additional nucleotide sequence and have the arrangement: 5'-second additional nucleotide sequence-first tag-specific portion-second barcode nucleotide sequence-second tag-specific portion-3'.

10 72. The method of claim 71, wherein the combinatorially tagged target nucleotide sequences comprise 5'-second additional nucleotide sequence-first tag-specific portion-second barcode nucleotide sequence-second tag-specific portion-target nucleotide sequence-primer binding site-first barcode nucleotide sequence-first additional nucleotide sequence-3'.

15 73. The method of claims 70-72, wherein the first and/or second additional nucleotide sequences comprise a primer binding site.

74. The method of any of claims 66-73, wherein the nucleotide tag comprises a transposon end, which is incorporated into the tagged target nucleotide sequences using a transposase.

20 75. The method of any of claims 35-74, wherein the reaction mixtures are prepared in separate compartments of a microfluidic device, the separate compartments being arranged as an array defined by rows and columns, wherein the combination of the first and second barcode nucleotide sequences in each tagged target nucleotide sequence identifies the row and column of the compartment that was the source of the tagged target nucleotide sequence.

25 76. The method of claim 75, wherein the method comprises recovering the tagged target nucleotide sequences from the microfluidic device.

77. The method of claim 76, wherein said recovering comprises pooling tagged target nucleotide sequences from compartments in a row or in a column to produce an assay pool.

78. The method of claim 77, wherein the method comprises further
5 analyzing the assay pool by nucleic acid amplification.

79. The method of claim 77, wherein the method comprises further analyzing the assay pool by nucleic acid sequencing.

80. The method of claims 78 or 79, wherein said further analysis comprises determining whether a tagged target nucleotide sequence comprising a selected
10 combination of first and second barcode nucleotide sequences is present in the assay pool, or aliquot thereof.

81. A kit for performing the method of claim 63, the kit comprising:
one or more nucleotide tag(s); and
a plurality of barcode primers, wherein each barcode primer
15 comprises:

a first portion that is specific for a first portion of the
nucleotide tag(s) linked to;

a barcode nucleotide sequence that does not anneal to the
nucleotide tag(s) linked to;

20 a second portion that is specific for a second portion of the
nucleotide tag(s);

wherein the barcode primers in the plurality each comprise the same first and second tag-specific portions, but M different second barcode nucleotide sequences, wherein M is an integer greater than one.

25 82. The kit of claim 81, wherein the nucleotide tag(s) comprise transposon ends, and the kit additionally comprises a transposase.

83. The kit of claims 81 or 82, wherein the kit additionally comprises a polymerase.

84. An assay method for detecting a plurality of target nucleic acids, the method comprising:

preparing M first reaction mixtures that will be pooled prior to assay, wherein M is an integer greater than 1, and each first reaction mixture comprises:

- 5 sample nucleic acid(s);
- a first, forward primer comprising a target-specific portion;
- a first, reverse primer comprising a target-specific portion,

wherein the first, forward primer or the first, reverse primer additionally comprises a barcode nucleotide sequence, wherein each barcode nucleotide sequence in each of said M
10 reaction mixtures is different;

subjecting each first reaction mixture to a first reaction to produce a plurality of barcoded target nucleotide sequences, each comprising a target nucleotide sequence linked to a barcode nucleotide sequence.

- 15 for each of the M first reaction mixtures, pooling the barcoded target nucleotide sequences to form an assay pool;

subjecting the assay pool, or one or more aliquots thereof, to a second reaction using unique pairs of second primers, wherein each second primer pair comprises:

a second, forward or a reverse primer that anneals to a target nucleotide sequence; and

- 20 a second, reverse or a forward primer, respectively, that anneals to a barcode nucleotide sequence; and

for each unique, second primer pair, determining whether a reaction product is present in the assay pool, or aliquot thereof, whereby the presence of a reaction product indicates the presence of a particular target nucleic acid in a particular first reaction
25 mixture.

85. An assay method for detecting a plurality of target nucleic acids, the method comprising:

preparing M first reaction mixtures that will be pooled prior to assay, wherein M is an integer greater than 1, and each first reaction mixture comprises:

- 30 sample nucleic acid(s)
- a first, forward primer comprising a target-specific portion;

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

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

subjecting each first reaction mixture to a first reaction to produce a plurality of barcoded target nucleotide sequences, each comprising a target nucleotide sequence linked to a nucleotide tag, which is linked to a barcode nucleotide sequence.

for each of the M reaction mixtures, pooling the barcoded target nucleotide sequences to form an assay pool;

subjecting the assay pool, or one or more aliquots thereof, to a second reaction using unique pairs of second primers, wherein each second primer pair comprises: a second, forward or a reverse primer that anneals to a target nucleotide sequence; and

a second, reverse or a forward primer, respectively, that anneals to a barcode nucleotide sequence; and

for each unique, second primer pair, determining whether a reaction product is present in the assay pool, or aliquot thereof, whereby the presence of a reaction product indicates the presence of a particular target nucleic acid in a particular first reaction mixture.

86. The method of any of claims 84 or 85, wherein the method additionally comprises preparing $M \times N$ first reaction mixtures, wherein N is an integer greater than 1, and:

each first reaction mixture comprises a pair of first, forward and reverse primers that is specific for a different target nucleic acid:

said pooling comprises preparing N assay pools of M first reaction mixtures, wherein each barcoded target nucleotide sequence in an assay pool comprises a different barcode nucleotide sequence; and

the second reaction is carried out in each of the N assay pools, with each assay pool being separate from every other assay pool.

87. The method of any of claims 84-86, wherein the first reaction comprises amplification.

88. The method of any of claims 84-87, wherein the second reaction comprises amplification.

5 89. The method of any of claims 35-86, wherein each first reaction mixture comprises target nucleic acids from a single particle.

90. The method of claim 89, wherein the single particle comprises a particle selected from the group consisting of a cell, an organelle, a vesicle, a chromosome, a chromosomal fragment, and a particle produced using a chemical crosslinker.

10 91. The method of claim 89, wherein the single particle comprises a single cell.

92. The method of any of claims 84-91, wherein the first reaction mixtures are prepared in separate compartments of a microfluidic device, the separate compartments being arranged as an array defined by rows and columns.

15 93. The method of claim 86, wherein:
the first reaction mixtures are prepared in separate compartments of a microfluidic device, the separate compartments being arranged as an array defined by rows and columns;

20 each of the N assay pools is obtained by pooling the first reaction mixtures in a row or a column of the device; and

the barcode nucleotide sequence in each barcoded target nucleotide sequence, taken with the identity of the assay pool, identifies the row and column of the compartment that was the source of the barcoded target nucleotide sequence.

25 94. The method of any of claims 84-93, wherein the second reaction mixtures are prepared in separate compartments of a microfluidic device, the separate compartments being arranged as an array defined by rows and columns.

95. The method of claim 94, wherein the first reaction mixtures are prepared in separate compartments of a first microfluidic device, the second reaction

mixtures are prepared in separate compartments of a second microfluidic device, wherein the second microfluidic device is different from the first.

96. The method of claims 92 or 93, wherein particles to be analyzed are distributed into the compartments of the device.

5 97. The method of claim 96, wherein the number of particles in each compartment is determined by brightfield microscopy or fluorescence microscopy.

98. The method of claim 96, wherein a stain, dye, or label is employed to detect the number of particles in each compartment.

10 99. The method of claim 98, wherein the particles are cells, and a cell membrane-permeant nucleic acid dye is employed to detect the number of cells in each compartment.

100. The method of claim 98, wherein the particles are cells, and a labeled antibody specific for a cell-surface marker is employed to detect the number of cells in each compartment.

15 101. The method of claim 96, wherein the method comprises:
determining whether any compartment comprises more than a single particle; and
not further analyzing, or disregarding, results from, any compartment that comprises more than a single particle.

20 102. The method of claim 96, wherein the method comprises:
determining whether any compartment comprises a particle having a particular characteristic; and
further analyzing, or only considering results from, any compartment that comprises a particle having the particular characteristic.

25 103. The method of claim 102, wherein the characteristic is selected from the group consisting of a specific genomic rearrangement, copy number variation, or polymorphism; expression of a specific gene; and expression of a specific protein.

104. The method of any of claims 89-103, wherein at least one reaction is performed with intact particles.

105. The method of any of claims 89-103, wherein at least one reaction is performed with disrupted particles.

5 106. The method of any of claims 89-105, wherein the particles are treated with an agent that elicits biological response prior to performing the plurality of first reactions.

107. The method of any of claims 84-106, wherein said determining whether a reaction product is present in the assay pool, or aliquot thereof, is carried out by
10 polymerase chain reaction (PCR).

108. The method of any of claims 84-106, wherein said determining whether a reaction product is present in the assay pool, or aliquot thereof, is carried out by ligase chain reaction (LCR).

109. The method of claims 84-108, wherein said determining whether a
15 reaction product is present in the assay pool, or aliquot thereof, is carried out by real-time detection.

110. The method of claim 109, wherein said determining whether a reaction product is present in the assay pool, or aliquot thereof, is carried out by an assay employing a labeled probe and an unlabeled probe, wherein the simultaneous hybridization
20 of the probes to a reaction product results in the formation of a flap at the 5' end of the labeled probe, and cleavage of the flap produces a signal.

111. The method of claim 108, wherein cleavage of the flap separates a fluorophore from a quencher.

112. The method of claim 108, wherein a portion of the labeled probe is
25 specific for a barcode nucleotide sequence in the reaction product.

113. The method of claim 108, wherein said determining whether a reaction product is present in the assay pool, or aliquot thereof, is carried out by melting temperature analysis comprising detection at a temperature at which:

- 5 reaction products are substantially double-stranded and capable of producing signal in the presence of a double-stranded DNA-binding dye; and primers are substantially single-stranded and incapable of producing signal in the presence of a double-stranded DNA-binding dye.

114. The method of any of claims 84-113, wherein the method comprises sequencing the reaction products.

- 10 115. The method of any of claims 35-114, wherein the method comprises determining the amount of at least one target nucleic acid in the first reaction mixtures.

116. The method of any of claims 35-115, wherein the method comprises determining the copy number(s) of one or more DNA molecule(s) in the first reaction mixtures.

- 15 117. The method of claim 116, wherein tagged or barcoded target nucleotide sequences are produced by fewer than 20 cycles of PCR.

118. The method of any of claims 35-117, wherein the method comprises determining the genotype(s) at one or more loci in the first reaction mixtures.

- 20 119. The method of any of claims 35-118, wherein the method comprises determining a haplotype for a plurality of loci in the first reaction mixtures.

120. The method of claim 119, wherein the method comprises:
condensing chromosomes; and
distributing chromosomes into first reaction mixtures to produce a plurality of first reaction mixtures that comprise a single chromosome;
25 wherein the reaction comprises sequencing a plurality of loci in each of the first reaction mixtures.

121. The method of any of claims 35-120, wherein the method comprises determining the expression level(s) of one or more RNA molecule(s) in the first reaction mixtures.

122. The method of claim 121, wherein tagged or barcoded target
5 nucleotide sequences are produced by fewer than 20 cycles of RT-PCR.

123. The method of any of claims 35-122, wherein the method comprises determining the nucleotide sequence(s) of one or more RNA molecule(s) in the first reaction mixtures.

124. The method of any of claims 35-123, wherein the method comprises:
10 performing a plurality of reactions in each first reaction mixture,
wherein one of the plurality of reactions comprises amplification to produce a tagged or barcoded target nucleotide sequence;
analyzing the results of the plurality of reactions; and
associating the results of the analysis with each first reaction mixture.

125. The method of claim 124, wherein the results of the analysis indicate
15 the presence of two or more of a variation selected from the group consisting of:
a copy number variation;
a mutation;
an expression level variation;
20 a splice variant;
wherein the presence of said two or more variations is correlated with
a phenotype.

126. The method of claim 125, wherein the phenotype is selected from the risk, presence, severity, prognosis, and responsiveness to a specific therapy of a disease.

127. The method of claim 126, wherein the phenotype comprises
25 resistance to a drug.

128. The method of claim 124, where the results of analysis indicate the occurrence of genomic recombination.

129. The method of claim 124, where the results of analysis indicate co-expression of particular splice variants.

130. The method of claim 124, where the results of analysis indicate co-expression of particular light and heavy chains in B cells.

5 131. The method of claim 124, where the results of analysis indicate the presence of a particular pathogen in a particular host cell.

132. A method for amplifying a target nucleic acid, the method comprising:

amplifying a target nucleic acid using:

10 a set of inner primers, wherein the set comprises:

an inner, forward primer comprising a target-specific portion and a first primer binding site;

an inner, reverse primer comprising a target-specific portion and a second primer binding site, wherein the first and second primer binding sites
15 are different;

a first set of outer primers, wherein the set comprises:

a first outer, forward primer comprising a portion specific for the first primer binding site; and

20 a first outer, reverse primer comprising a barcode nucleotide sequence and a portion specific for the second primer binding site;

a second set of outer primers, wherein the set comprises:

a second outer, forward primer comprising a barcode nucleotide sequence and a portion specific for the first primer binding site; and

25 a second outer, reverse primer comprising a portion specific for the second primer binding site;

to produce two target amplicons, wherein:

a first target amplicon comprises 5'-first primer binding site-target nucleotide sequence-second primer binding site-barcode nucleotide sequence-3'; and

30 a second target amplicon comprises 5'-barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-3'.

133. The method of claim 132, wherein the barcode nucleotide sequence in each target amplicon is the same, and wherein each target amplicon comprises only one barcode nucleotide sequence.

134. The method of claim 132, wherein the first and second primer
5 binding sites are binding sites for DNA sequencing primers.

135. The method of any of claims 132-134, wherein the outer primers each additionally comprise an additional nucleotide sequence, wherein:

the first outer, forward primer comprises a first additional nucleotide sequence, and the first outer, reverse primer comprises a second additional nucleotide
10 sequence; and

the second outer, forward primer comprises the second additional nucleotide sequence, and the second outer, reverse primer comprises the first additional nucleotide sequence; and

the first and second additional nucleotide sequences are different; and
15 said amplifying produces two target amplicons, wherein:

a first target amplicon comprises 5'-first additional nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-barcode nucleotide sequence-second additional nucleotide sequence-3'; and

a second target amplicon comprises 5'-second additional
20 nucleotide sequence-barcode nucleotide sequence-first primer binding site-target nucleotide sequence-second primer binding site-first additional nucleotide sequence 3'.

136. The method of claim 135, wherein the first or second additional nucleotide sequence comprises a primer binding site.

137. The method of claim 136, wherein the first and second additional
25 nucleotide sequences comprise a primer binding site.

138. The method of claims 135-137, wherein the first set of outer primers comprises PE1-CS1 and PE2-BC-CS2, and the second set of outer primers comprises PE1-CS2 and PE2-BC-CS1 (Table, Example 9).

139. The method of claim 132, wherein said amplifying is carried out in a single amplification reaction.

140. The method of claim 132, wherein said amplifying comprises employing the inner primers in a first amplification reaction and employing the outer
5 primers in a second amplification reaction, wherein said second amplification reaction is separate from the first.

141. The method of claim 140, wherein the second amplification reaction comprises two separate amplification reactions, wherein one employs the first set of outer primers and the other employs the second set of outer primers.

10 142. The method of claim 141, wherein the target amplicons produced in the two separate second amplification reactions are pooled.

143. The method of any of claims 132-142, wherein the method comprises amplifying a plurality of target nucleic acids.

144. The method of claim 143, wherein the plurality of target nucleic acids
15 is selected from the group consisting of genomic DNA, cDNA, fragmented DNA, DNA reverse-transcribed from RNA, a DNA library, and nucleic acids is extracted or amplified from a cell, a bodily fluid or a tissue sample.

145. The method of claim 144, wherein the plurality of target nucleic acids is amplified from a formalin-fixed, paraffin-embedded tissue sample.

20 146. The method of any of claims 132-145, wherein the method additionally comprises sequencing the target amplicons.

147. The method of claim 143, wherein the method comprises amplifying the target amplicons using primers that bind to the first and second additional nucleotide sequences to produce templates for DNA sequencing.

25 148. The method of claim 147, wherein one or both primers that bind to the first and second additional nucleotide sequences are immobilized on a substrate.

149. The method of claims 143 or 147, wherein the amplification is carried out by isothermal nucleic acid amplification.

150. The method of claim 147, wherein the method comprises performing DNA sequencing using the templates and primers that bind to the first and second primer
5 binding sites and prime sequencing of the target nucleotide sequence(s).

151. The method of claim 150, wherein the primers that bind to the first and second primer binding sites and prime sequencing of the target nucleotide sequence(s) are present in substantially equal amounts.

152. The method of claim 147, wherein the method comprises performing
10 DNA sequencing using the templates and primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequences(s).

153. The method of claim 152, wherein the primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequences(s) are present in substantially equal amounts.

154. The method of claim 150, wherein the method comprises performing DNA sequencing using the templates and primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequences(s), wherein said primers are reverse complements of the primers that prime sequencing of the target nucleotide sequences.

155. The method of claim 154, wherein the primers employed to prime sequencing of the target nucleotide sequence(s) and barcode nucleotide sequence(s) comprise CS1, CS2, CS1rc, and CS2rc (Table 4, Example 9).

156. The method of any of claims 132-155, wherein the barcode nucleotide sequence is selected so as to avoid substantial annealing to the target nucleic
25 acids.

157. The method any of claims 132-156, wherein the barcode nucleotide sequence identifies a particular sample.

158. The method of claim 147, wherein at least 50 percent of the sequences determined from DNA sequencing are present at greater than 50 percent of the average number of copies of sequences and less than 2-fold the average number of copies of sequences.

5 159. The method of claim 147, wherein at least 70 percent of the sequences determined from DNA sequencing are present at greater than 50 percent of the average number of copies of sequences and less than 2-fold the average number of copies of sequences.

10 160. The method of claim 147, wherein at least 90 percent of the sequences determined from DNA sequencing are present at greater than 50 percent of the average number of copies of sequences and less than 2-fold the average number of copies of sequences.

161. The method of any of claims 132-160, wherein the average length of the target amplicons is less than 200 bases.

15 162. The method of any of claims 132-161, wherein the first amplification reaction is carried out in a volume in the range of about 1 picoliter to about 50 nanoliters.

163. The method of any of claims 132-162, wherein the first amplification reaction is carried out in a volume in the range of about 5 picoliters to about 25 nanoliters.

20 164. The method of any of claims 132-163, wherein the first amplification reaction(s) is/are formed in, or distributed into, separate compartments of a microfluidic device prior to amplification.

165. The method of claim 164, wherein the microfluidic device is fabricated, at least in part, from an elastomeric material.

25 166. The method of any of claims 132-163, wherein the first amplification reaction(s) is/are carried out in (a) fluid droplet(s).

167. The method of claim 166, wherein a plurality of first amplification reactions are carried out in fluid droplets in an emulsion.

168. A kit for use in amplifying a target nucleic acid, the kit comprising:
a first set of outer primers, wherein the set comprises:

a first outer, forward primer comprising a portion
specific for a first primer binding site; and

5 a first outer, reverse primer comprising a barcode
nucleotide sequence and a portion specific for a second primer binding site, wherein the first
and second primer binding sites are different;

a second set of outer primers, wherein the set comprises:

10 a second outer, forward primer comprising a barcode
nucleotide sequence and a portion specific for the first primer binding site; and

a second outer, reverse primer comprising a portion
specific for the second primer binding site.

169. The kit of claim 168, wherein the first and second primer binding
sites are binding sites for DNA sequencing primers.

15 170. The kit of claim 168 or 169, wherein the outer primers each
additionally comprise an additional nucleotide sequence, wherein:

the first outer, forward primer comprises a first additional nucleotide
sequence, and the first outer, reverse primer comprises a second additional nucleotide
sequence; and

20 the second outer, forward primer comprises the second additional
nucleotide sequence, and the second outer, reverse primer comprises the first additional
nucleotide sequence; and the first and second additional nucleotide sequences are different.

171. The kit of claim 170, wherein the first set of outer primers comprises
PE1-CS1 and PE2-BC-CS2, and the second set of outer primers comprises PE1-CS2 and
25 PE2-BC-CS1 (Table, Example 9).

172. The kit of claim 168, additionally comprising:

a set of inner primers, wherein the set comprises:

an inner, forward primer comprising a target-specific portion
and the first primer binding site; and

an inner, reverse primer comprising a target-specific portion and the second primer binding site.

173. The kit of claim 172, comprising a plurality of sets of inner primers, each specific for a different target nucleic acid.

5 174. The kit of any of claims 168-172, additionally comprising DNA sequencing primers that bind to the first and second primer binding sites and prime sequencing of the target nucleotide sequence(s).

175. The kit of any of claims 168-174, additionally comprising DNA sequencing primers that bind to the first and second primer binding sites and prime
10 sequencing of the barcode nucleotide sequence(s).

176. The kit of claim 175, wherein the primers that bind to the first and second primer binding sites and prime sequencing of the barcode nucleotide sequences(s) are reverse complements of the primers that prime sequencing of the target nucleotide sequences.

15 177. The kit of claim 176, wherein the primers employed to prime sequencing of the target nucleotide sequence(s) and barcode nucleotide sequence(s) comprise CS1, CS2, CS1rc, and CS2rc (Table 4, Example 9).

178. A method for detecting, and/or quantifying the relative amounts of, at least two different target nucleic acids in a nucleic acid sample, the method comprising:

20 producing first and second tagged target nucleotide sequences from first and second target nucleic acids in the sample,

the first tagged target nucleotide sequence comprising a first nucleotide tag; and

the second tagged target nucleotide sequence comprising a
25 second nucleotide tag, wherein the first and second nucleotide tags are different;

subjecting the tagged target nucleotide sequences to:

a first primer extension reaction using a first primer that anneals to the first nucleotide tag; and

a second primer extension reaction using a second primer that anneals to the second nucleotide tag; and

detecting and/or quantifying:

a signal that indicates extension of the first primer; and

5 a signal that indicates extension of the second primer

wherein the a signal for a given primer indicates the presence, and/or relative amount of, the corresponding target nucleic acid.

179. The method of claim 178, wherein the first and second tagged target nucleotide sequences comprise adaptors for DNA sequencing at the ends of each molecule.

10 180. The method of claim 178 or 179, wherein the first and second tagged target nucleotide sequences are produced by amplifying first and second target nucleic acids with first and second primer pairs, respectively, wherein at least one primer in the first primer pair comprises a first nucleotide tag and at least one primer in the second primer pair comprises a second nucleotide tag.

15 181. The method of claim 180, wherein one primer in each primer pair comprises 5'-(DNA sequencing adaptor)-(nucleotide tag)-(target-specific portion)-3' and the other primer in each primer pair comprises 5'-(DNA sequencing adaptor)-(target-specific portion)-3'.

20 182. The method of claim 180, wherein the tagged target nucleotide sequences are further amplified prior to primer extension.

183. The method of claim 182, wherein said further amplification comprises emulsion amplification or bridge amplification.

25 184. The method of any of claims 178-183, wherein the first and second primer extension reactions are carried out sequentially in at least two cycles of primer extension, wherein:

a first cycle of primer extension is carried out using the first primer that anneals to the first nucleotide tag;

a second cycle of primer extension is carried out using the second primer that anneals to the second nucleotide tag;

all deoxynucleoside triphosphates are provided in each cycle of primer extension;

the incorporation of any deoxynucleoside triphosphate into a DNA molecule produces a detectable signal; and

5 the signal detected in the first cycle indicates the presence, and/or relative amount, of the first target nucleic acid in the nucleic acid sample, and

the signal detected in the second cycle indicates the presence, and/or relative amount, of the second target nucleic acid in the nucleic acid sample.

10 185. The method of any of claims 178-184, wherein the detectable signal comprises pyrophosphate release.

186. The method of claims 184 or 185, wherein the tagged target nucleotide sequences are further amplified by emulsion PCR prior to primer extension.

187. The method of claims 178 or 182, wherein the first and second primer extension reactions are carried out by oligonucleotide ligation and detection, and wherein:
15 the ligation of a labeled di-base oligonucleotide to the first and/or second primer(s) produces a detectable signal; and
the total signal detected for a particular primer indicates the presence, and/or relative amount, of the corresponding target nucleic acid in the nucleic acid sample.

20 188. The method of claim 187, wherein the ligation of a labeled di-base oligonucleotide to the first primer produces the same detectable signal as the ligation of a labeled di-base oligonucleotide to the second primer, and the first and second primer extension reactions are carried out separately.

189. The method of claim 188, wherein the first and second primer extension reactions are carried out in sequential cycles.

25 190. The method of claim 187, wherein the ligation of a labeled di-base oligonucleotide to the first primer produces a different detectable signal than the ligation of a labeled di-base oligonucleotide to the second primer.

191. The method of claim 190, wherein the first and second primer extension reactions are carried out simultaneously, in one reaction mixture.

192. The method of any of claims 187-191, wherein the detectable signal is a fluorescent signal.

5 193. The method of any of claims 187-192, wherein the tagged target nucleotide sequences are further amplified by emulsion PCR prior to primer extension.

194. The method of claims 178 or 182, wherein the first and second primer extension reactions comprise sequencing-by-synthesis, wherein:

10 each deoxynucleoside triphosphates is labeled with a distinct, base-specific label;

the incorporation of a deoxynucleoside triphosphate into a DNA molecule produces a base-specific detectable signal; and

the total signal detected for a particular primer indicates the presence, and/or relative amount, of the corresponding target nucleic acid in the nucleic acid sample.

15 195. The method of claim 194, wherein the extension of the first primer produces the same detectable signal as the extension of the second primer, and the first and second primer extension reactions are carried out separately.

196. The method of claim 195, wherein the first and second primer extension reactions are carried out in sequential cycles.

20 197. The method of claim 194, wherein the extension of the first primer produces a different detectable signal than the extension of the second primer.

198. The method of claim 197, wherein the first and second primer extension reactions are carried out simultaneously, in one reaction mixture.

25 199. The method of any of claims 194-197, wherein the detectable signal is a fluorescent signal.

200. The method of any of claims 194-199, wherein the tagged target nucleotide sequences are further amplified by bridge PCR prior to primer extension.

201. The method of claim 182, wherein amplification produces clonal populations of tagged target nucleotide sequences that are, or become, located at discrete reaction sites.

202. The method of claim 201, wherein the number of reaction sites comprising the first nucleotide tag relative to the number of reaction sites comprising the second nucleotide tag indicates the amount of the first target nucleic acid relative to the second target nucleic acid in the sample.

203. The method of claims 201 or 202, wherein said detecting and/or quantifying comprises:

10 detecting and comparing the total signal from all reaction sites comprising the first nucleotide tag with the total signal from all reaction sites comprising the second nucleotide tag; or

detecting and comparing the number of reaction sites comprising the first nucleotide tag with the number of reaction sites comprising the second nucleotide tag.

15 204. The method of claim 203, wherein said comparing comprises determining a ratio.

205. The method of claims 179-187, 189-194, and 196-204, wherein the first nucleotide tag comprises a poly-(first nucleotide) sequence, and the second nucleotide tag comprises a poly-(second nucleotide) sequence, wherein the first and second nucleotides are different.

206. The method of any of claims 178-205, wherein the first and second target nucleic acids are selected from:

two different alleles of a polymorphic site;

25 a target nucleic acid that may be present in the nucleic acid sample at an altered copy number and a reference target nucleic acid that is expected to be present in the sample at a normal copy number;

target nucleic acids on a single chromosome; and

target nucleic acids on different chromosomes.

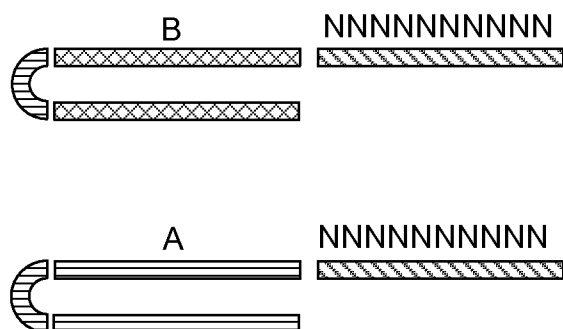
207. The method of claim 206, wherein the first and second target nucleic acids comprise a mutant allele and a wild type allele.

208. The method of any of claims 178-207, the method comprising:
producing three or more tagged target nucleotide sequences from
5 three or more target nucleic acids in the sample
the tagged target nucleotide sequences are subjected to three or more
primer extension reactions, each using a primer that anneals to a distinct nucleotide tag; and
a signal is detected and/or quantified for the extension of each primer.

209. The kit of any of claims 34, 40, 47, 62, 81-83, and 168-177, wherein
10 the kit additionally comprises a matrix-type microfluidic device.

1 / 43

Degenerate-tailed hairpin sequencing linkers



A- Sequencer specific tag1

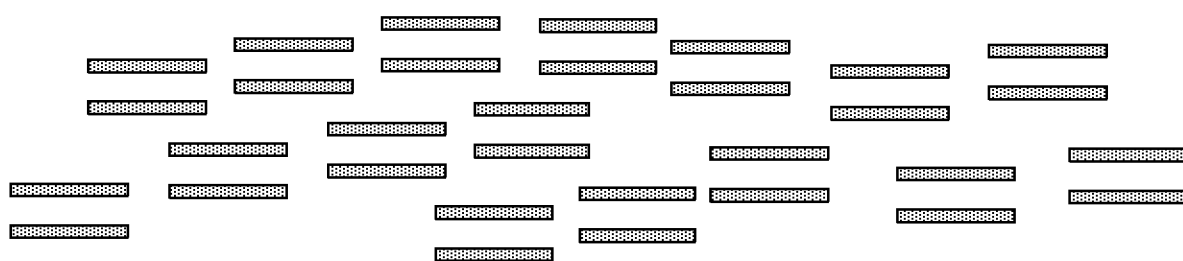
B- Sequencer specific tag2

Rest. Site: Optional restriction site/specific enzyme cutting site
(dUTP, phosphothiorate possible)

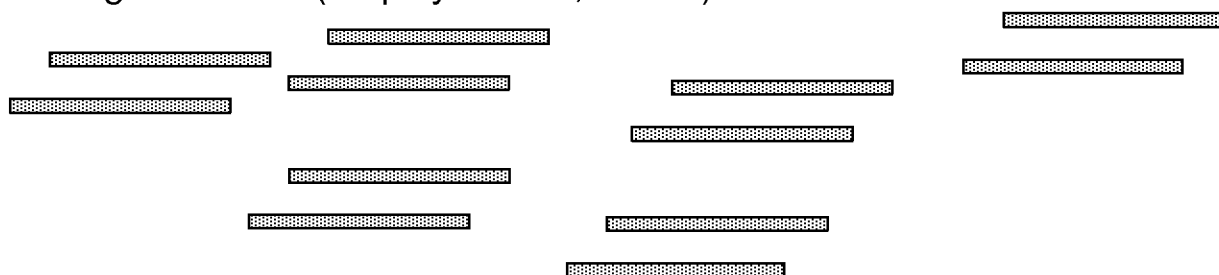
NNNNNNNNNN- Degenerate primer region-10mer, 20mer, 3' end protected

FIG. 1A

Fragment DNA (general nuclease)

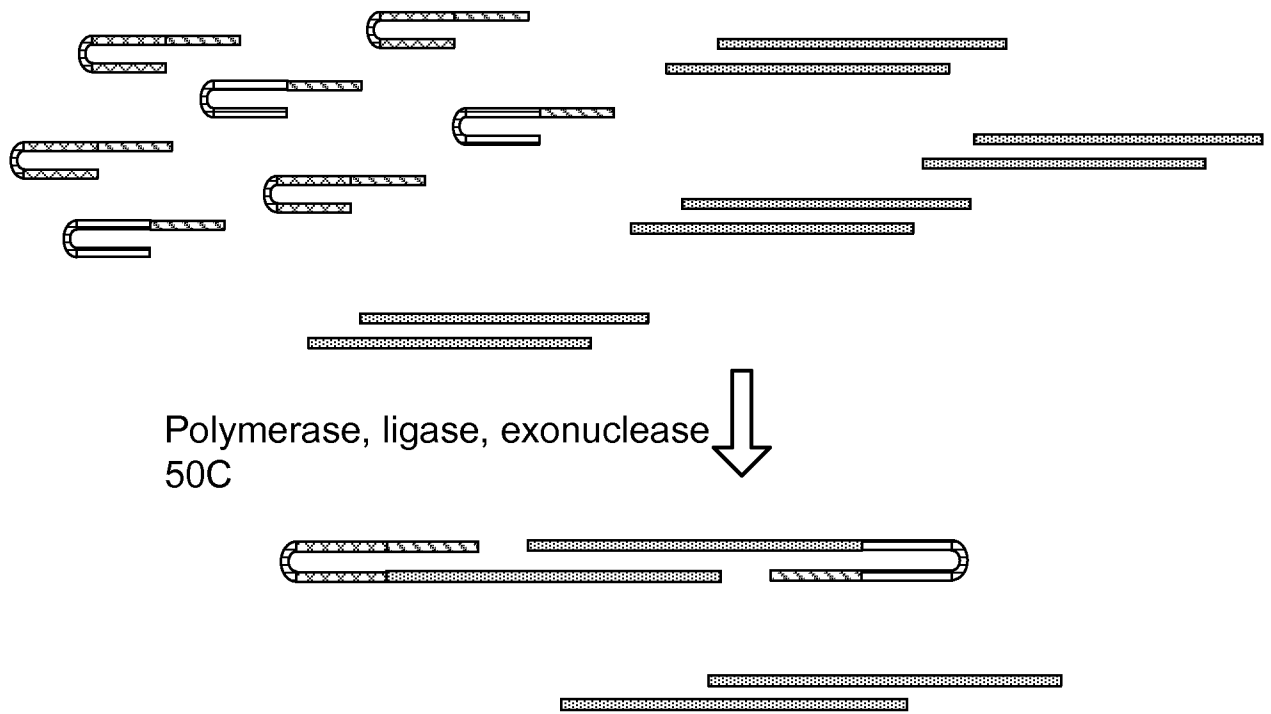


Digest 5' ends (T4 polymerase, Exo III)

**FIG. 1B**

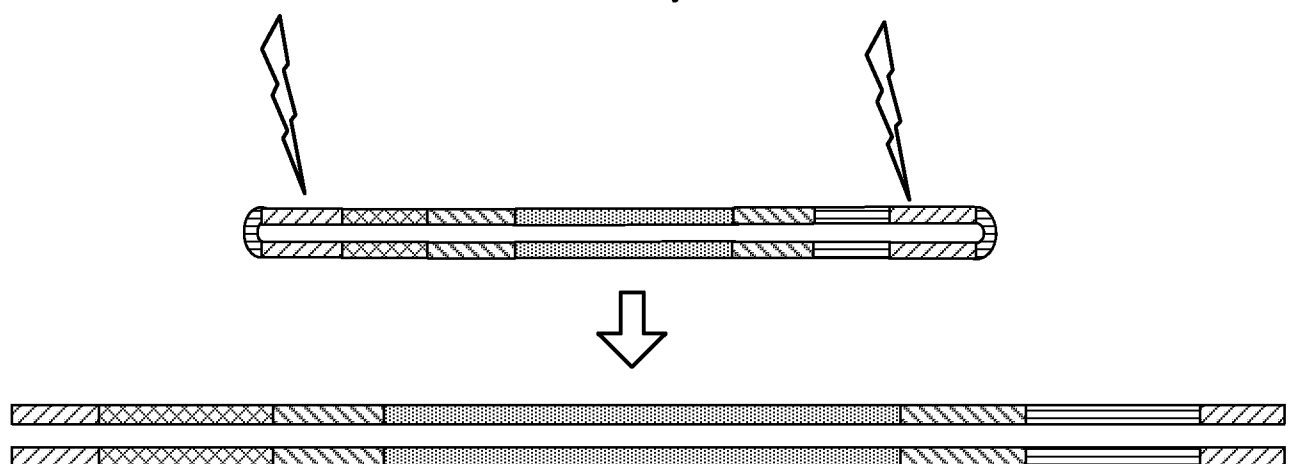
2 / 43

Add degenerate-hairpin sequencing linker

**FIG. 1C**

Linearize circular DNA with restriction enzyme

Restriction Enzyme

**FIG. 1D**

3 / 43

Degenerate-tailed sequencing linker



A- Sequencer specific tag1

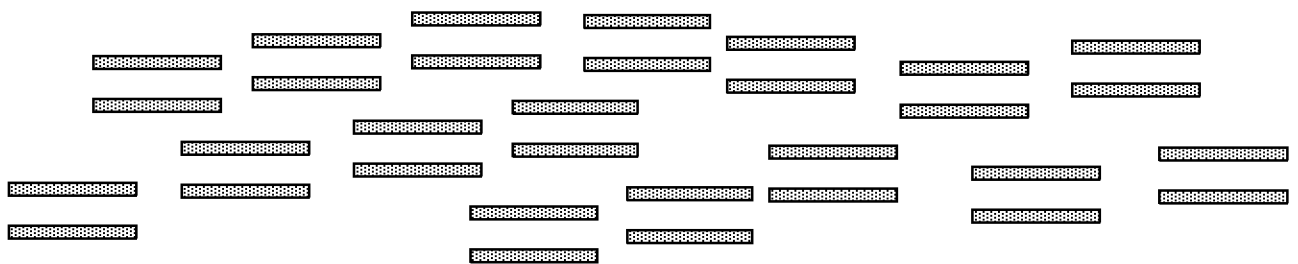
B- Sequencer specific tag2

Rest. Site: restriction site/specific enzyme cutting site (dUTP possible)

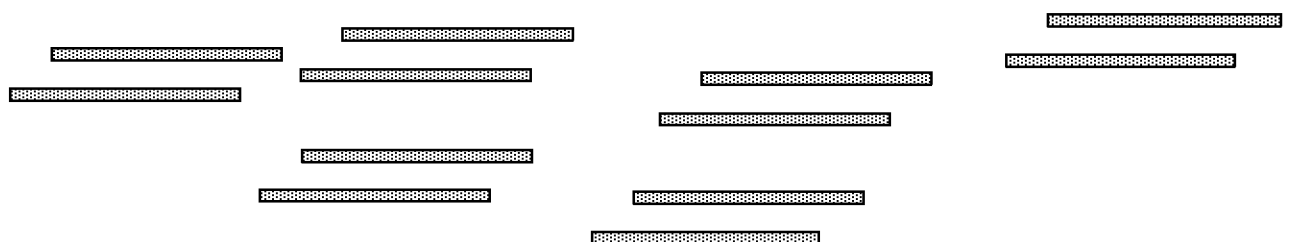
NNNNNNNNNN- Degenerate primer region-10mer, 20mer

FIG. 2A

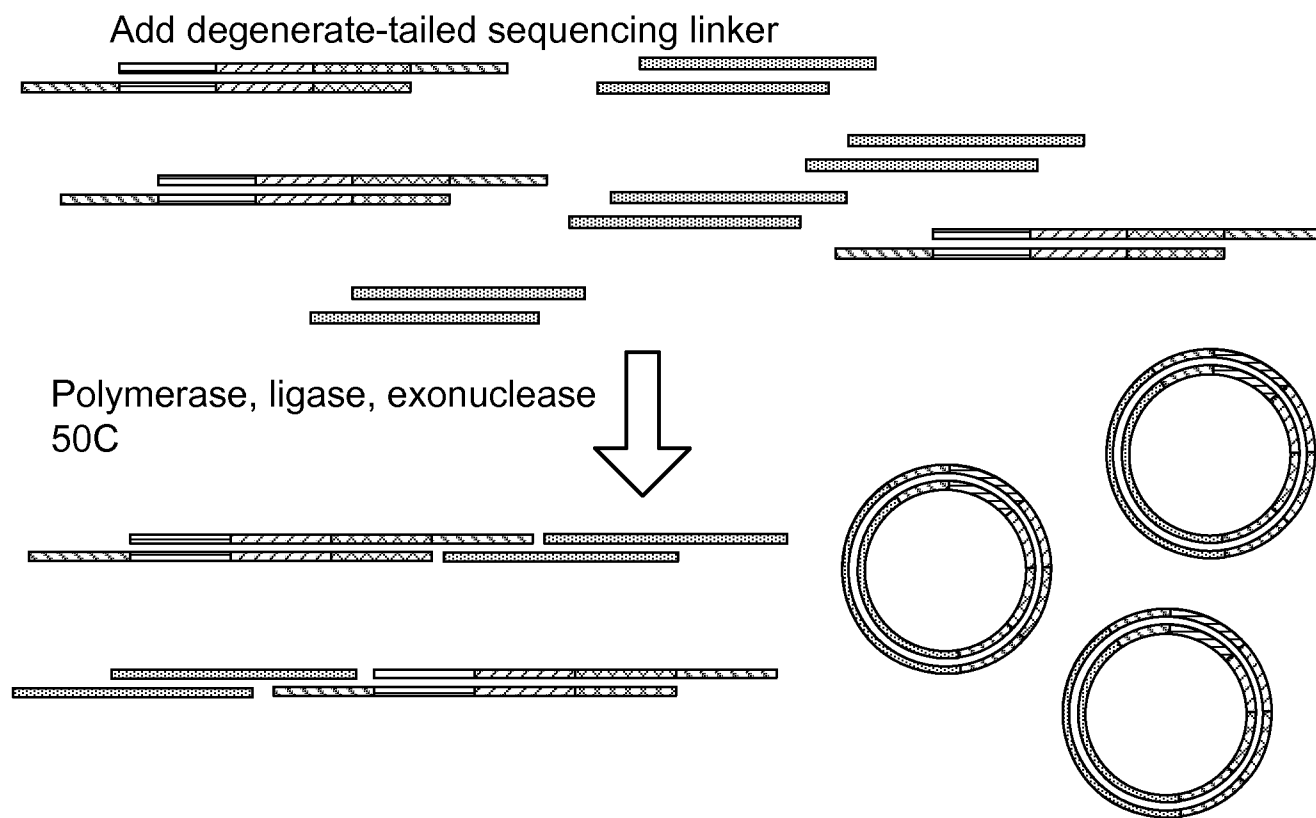
Fragment DNA (general nuclease)



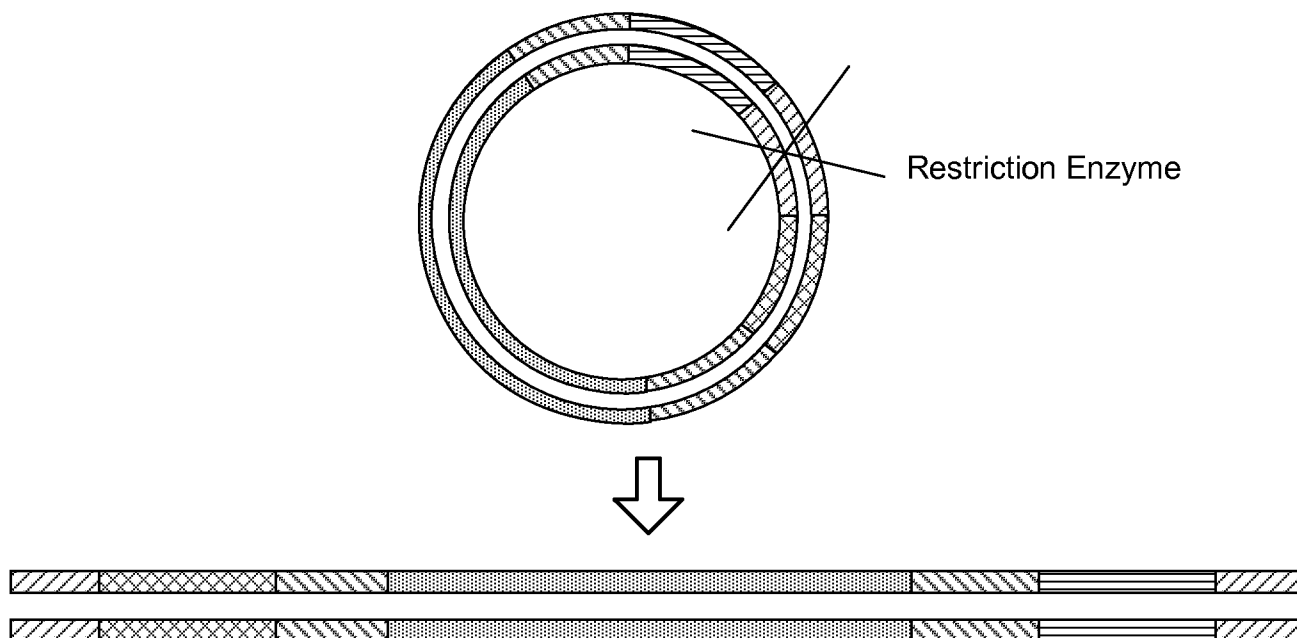
Digest 5' ends (T4 polymerase, Exo III)

**FIG. 2B**

4 / 43

**FIG. 2C**

Linear circular DNA with restriction enzyme

**FIG. 2D**

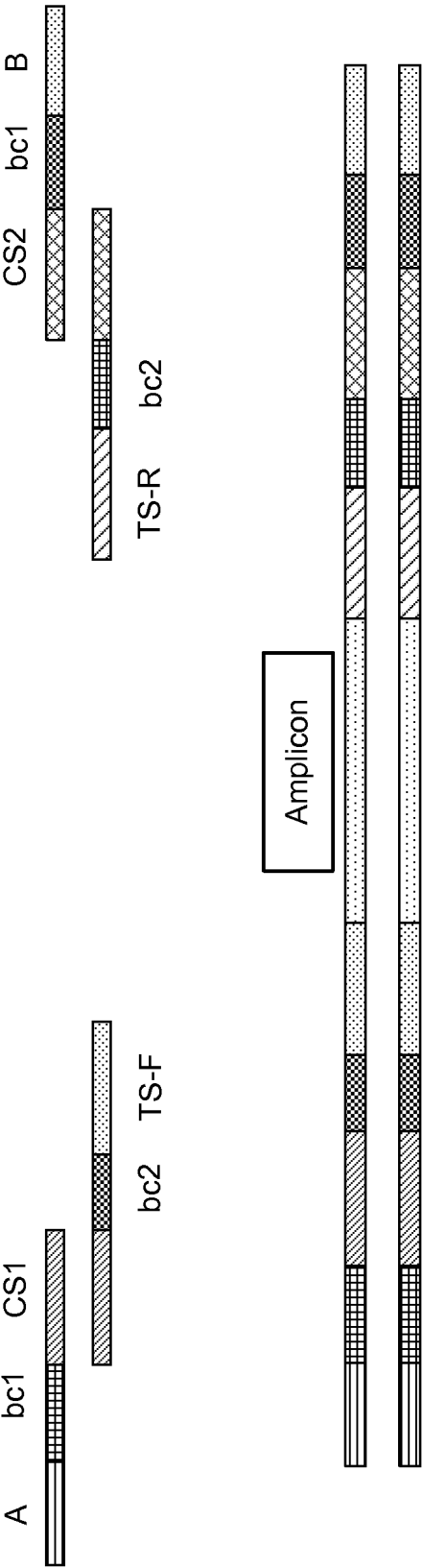


FIG. 3

5 / 43

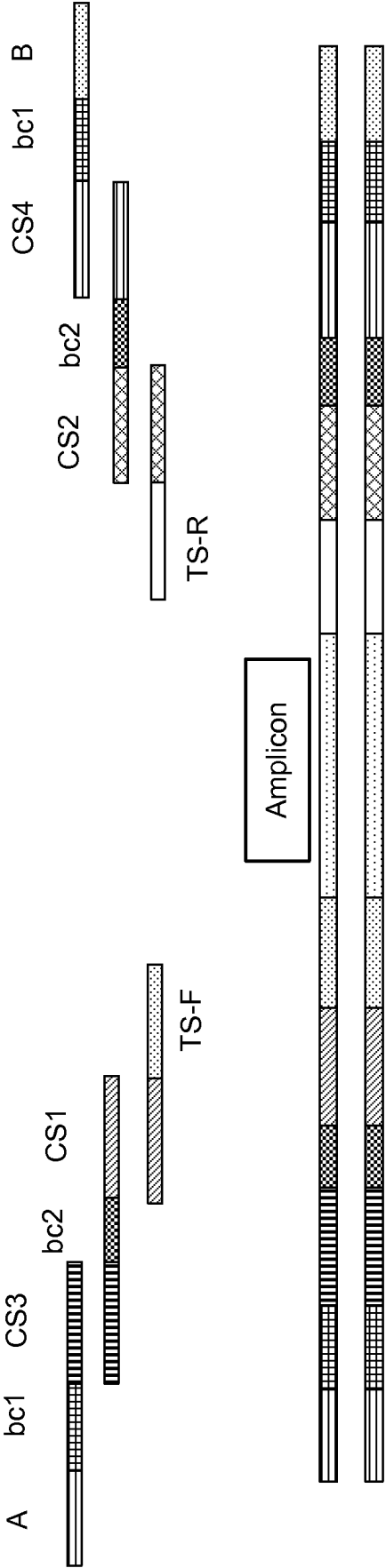


FIG. 4

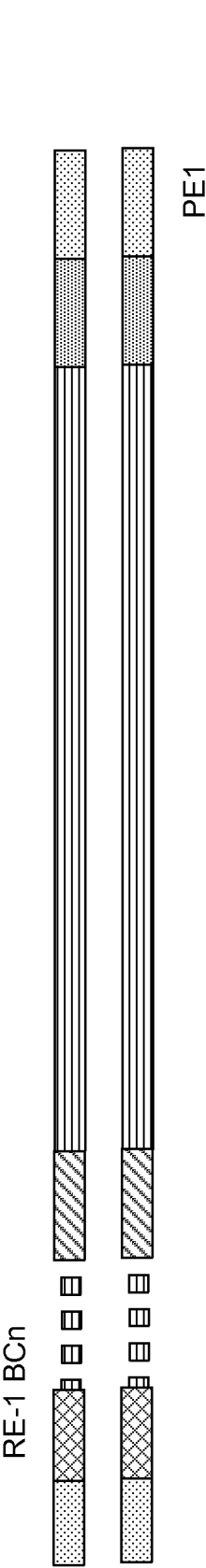


FIG. 5A

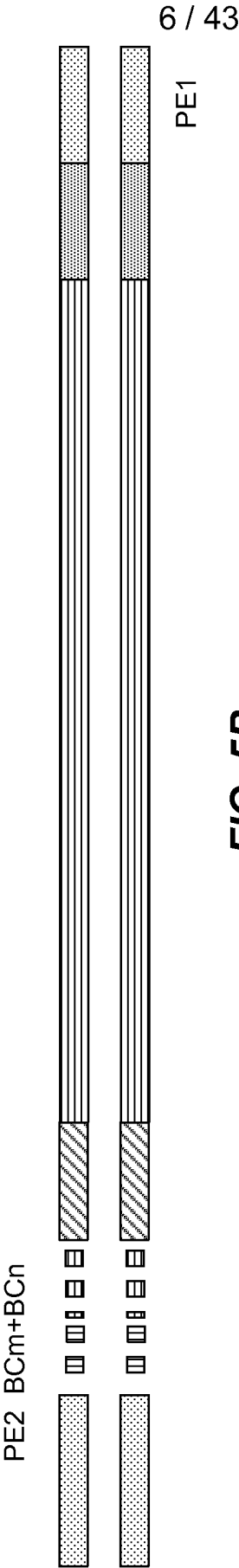


FIG. 5B

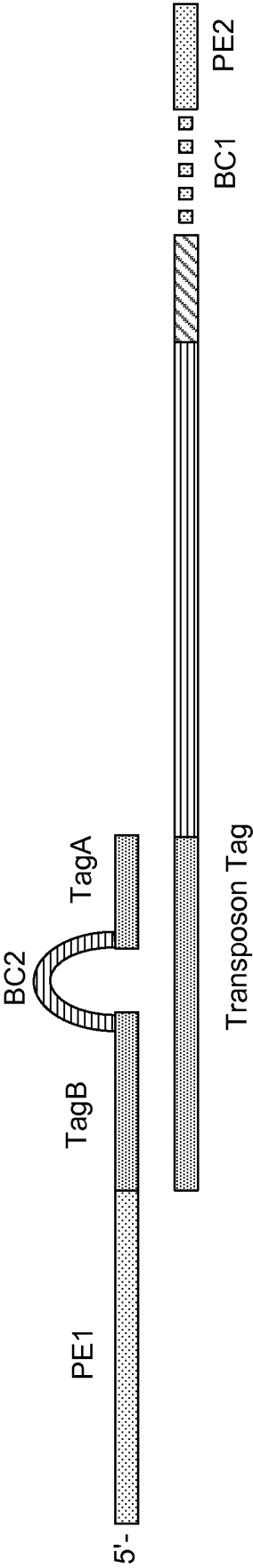


FIG. 6

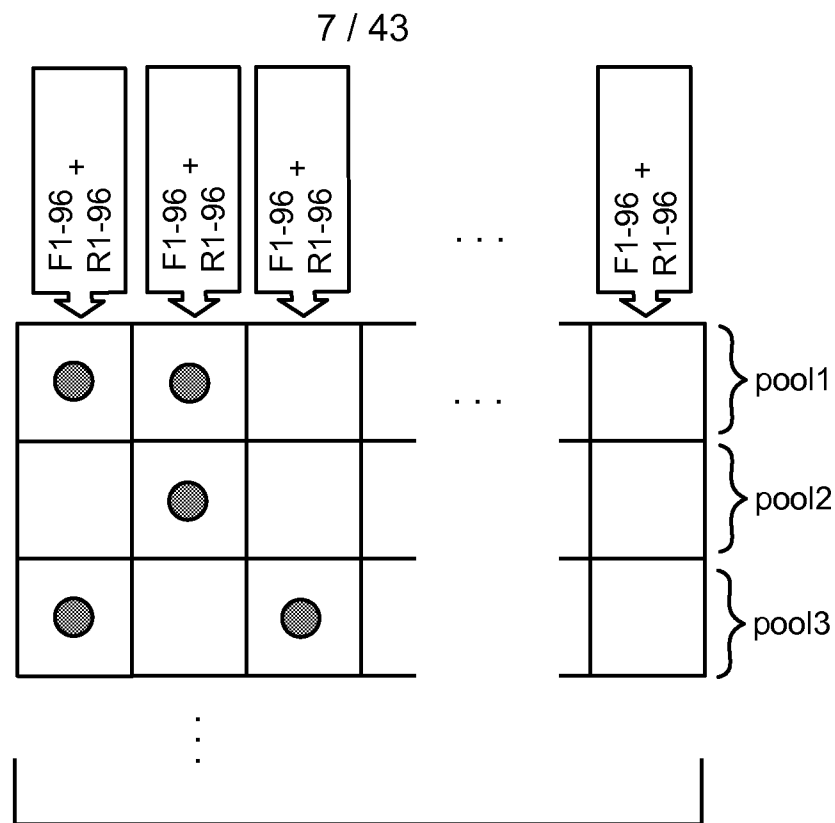


FIG. 7A

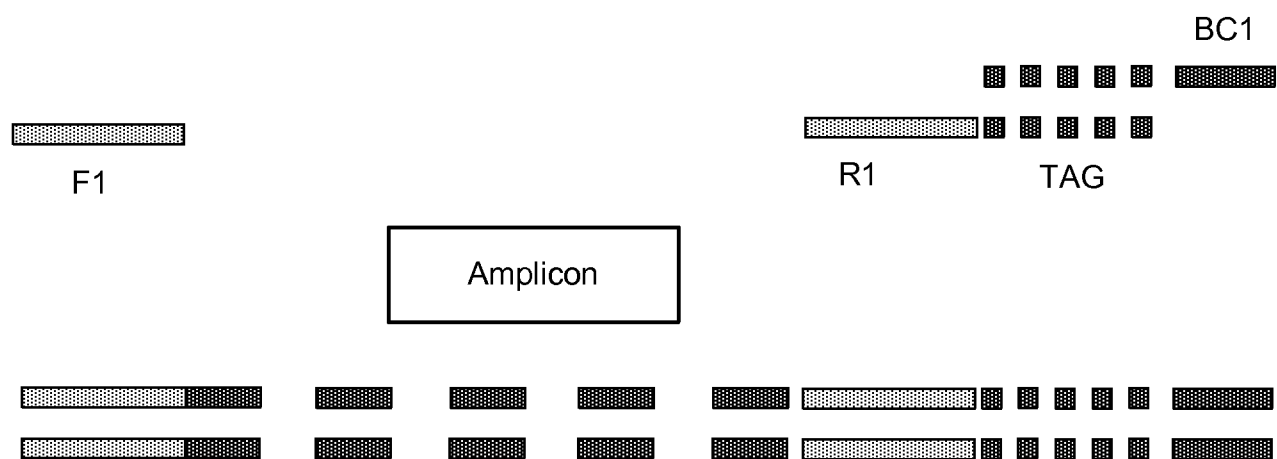
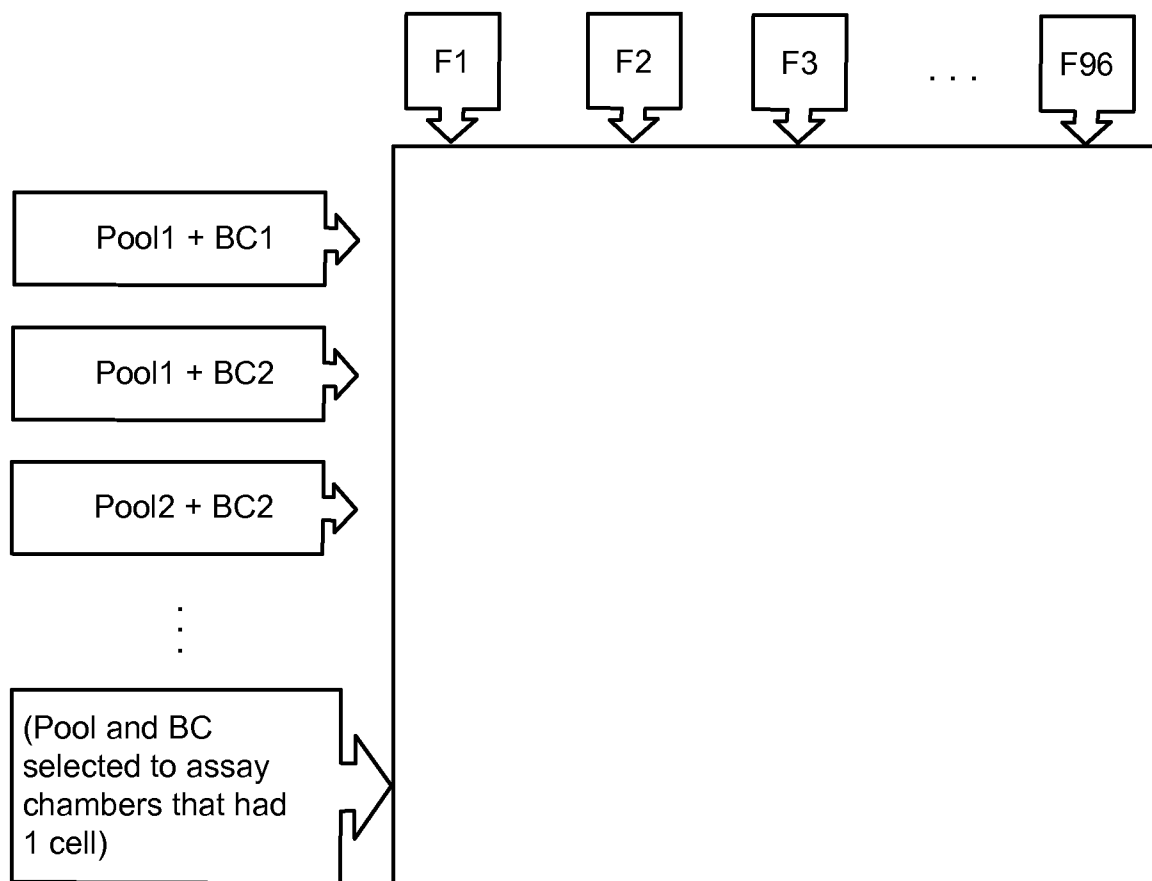


FIG. 7B

8 / 43

**FIG. 7C**

9 / 43

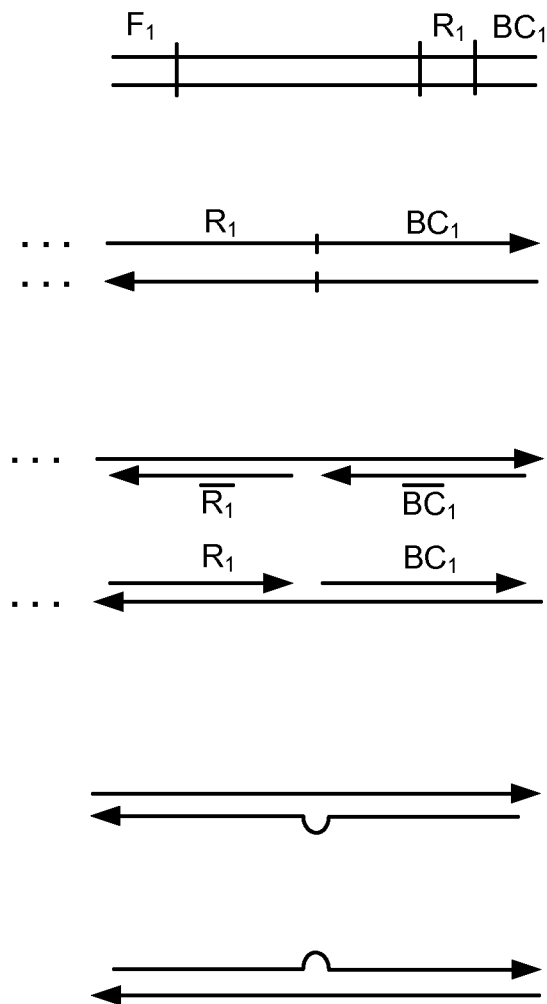


FIG. 8A

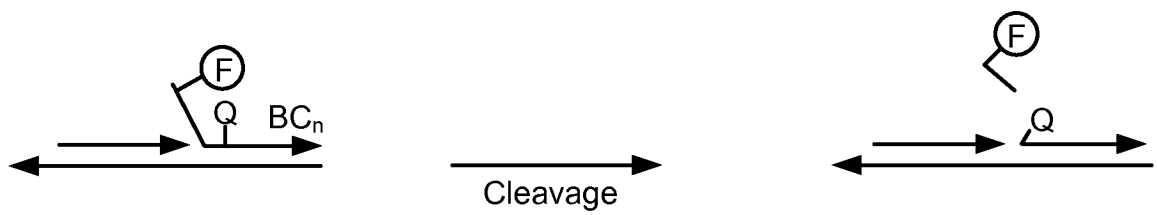


FIG. 8B

10 / 43

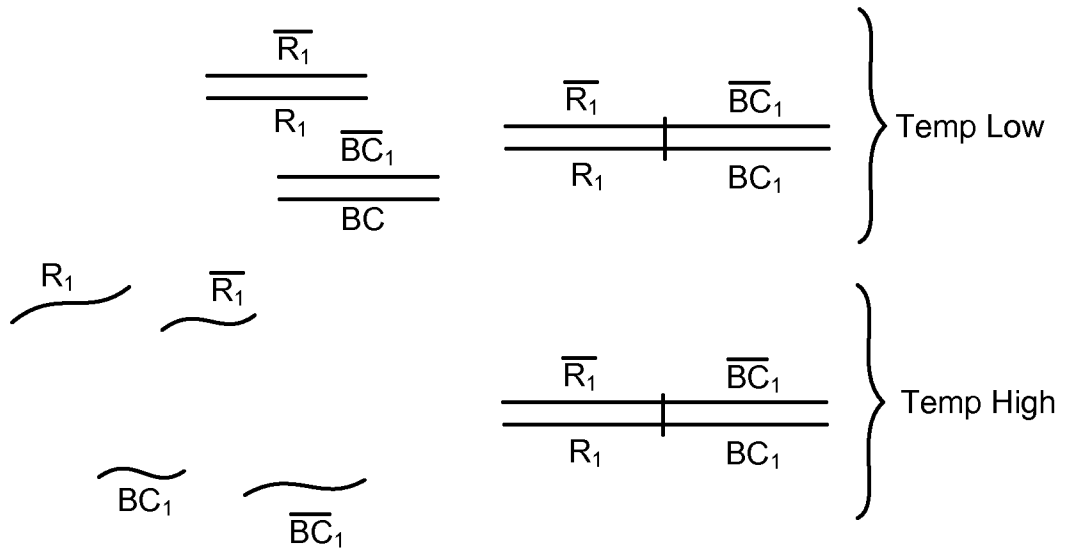


FIG. 8C

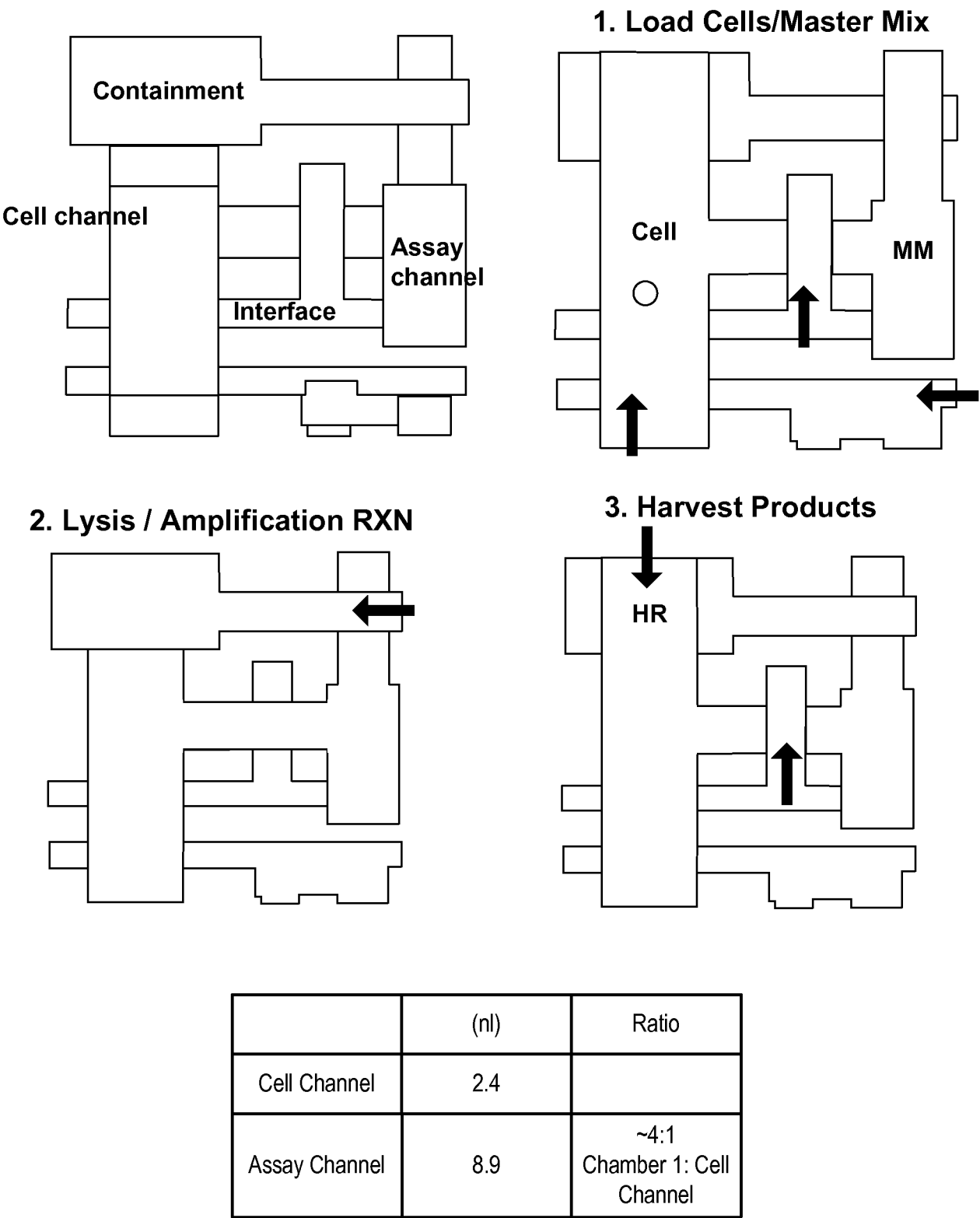
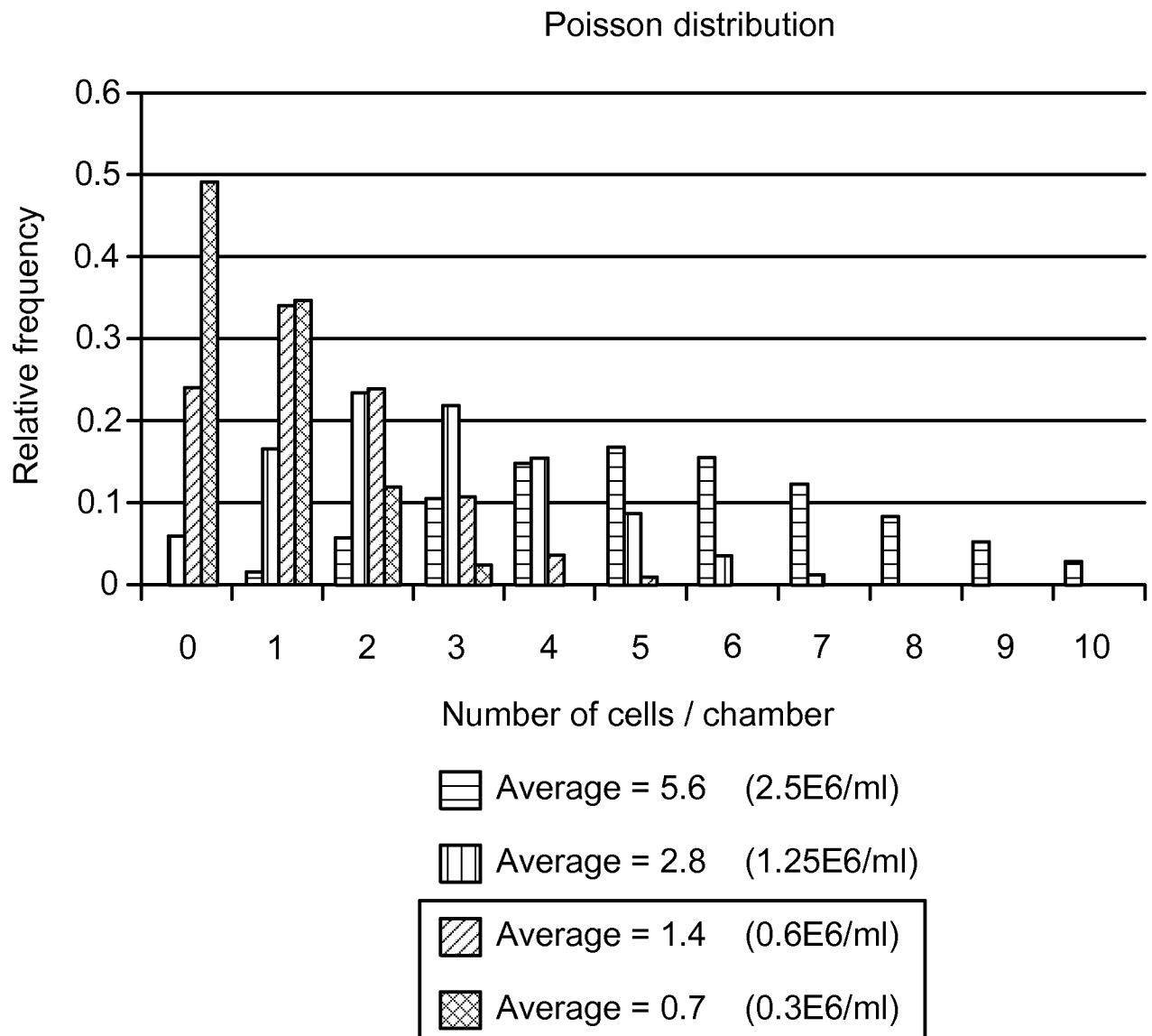


FIG. 9

12 / 43

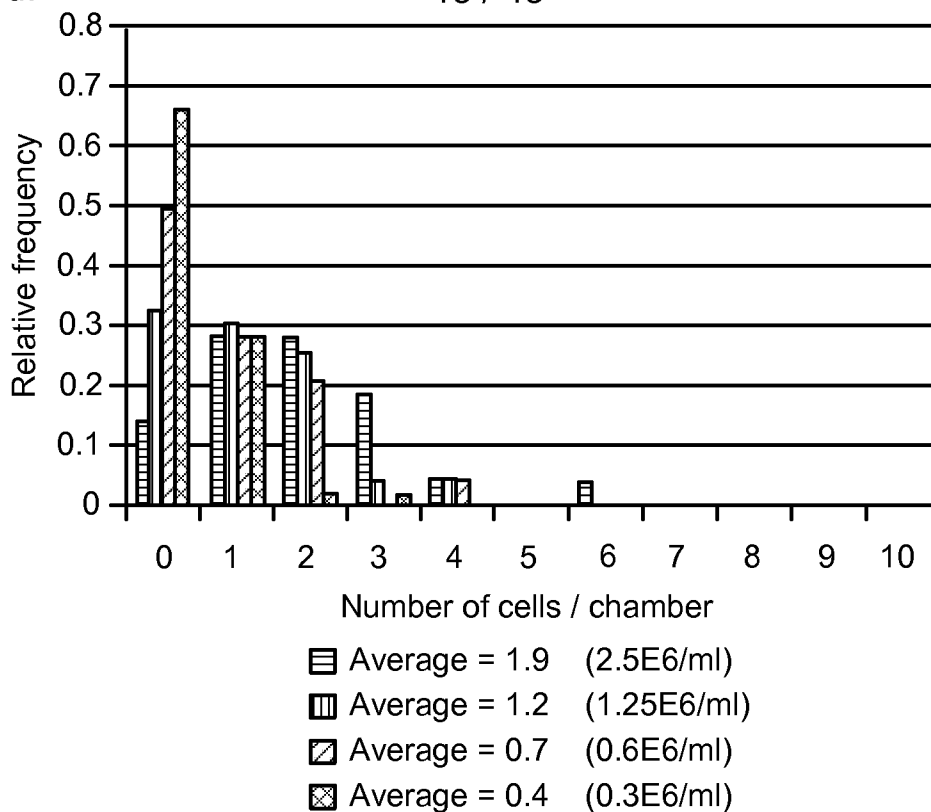


The “best” theoretical cell density for single cells:
~1 per chamber on average = $1 / 2.4 \text{ nl} = \sim 0.5\text{E}6/\text{ml}$

FIG. 10

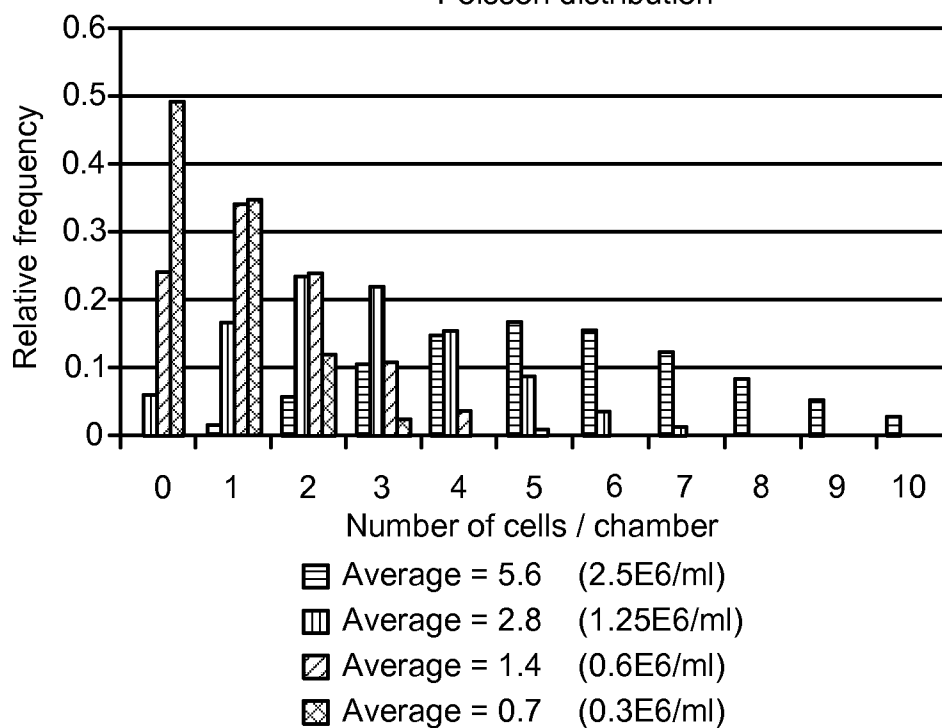
Measured:

13 / 43

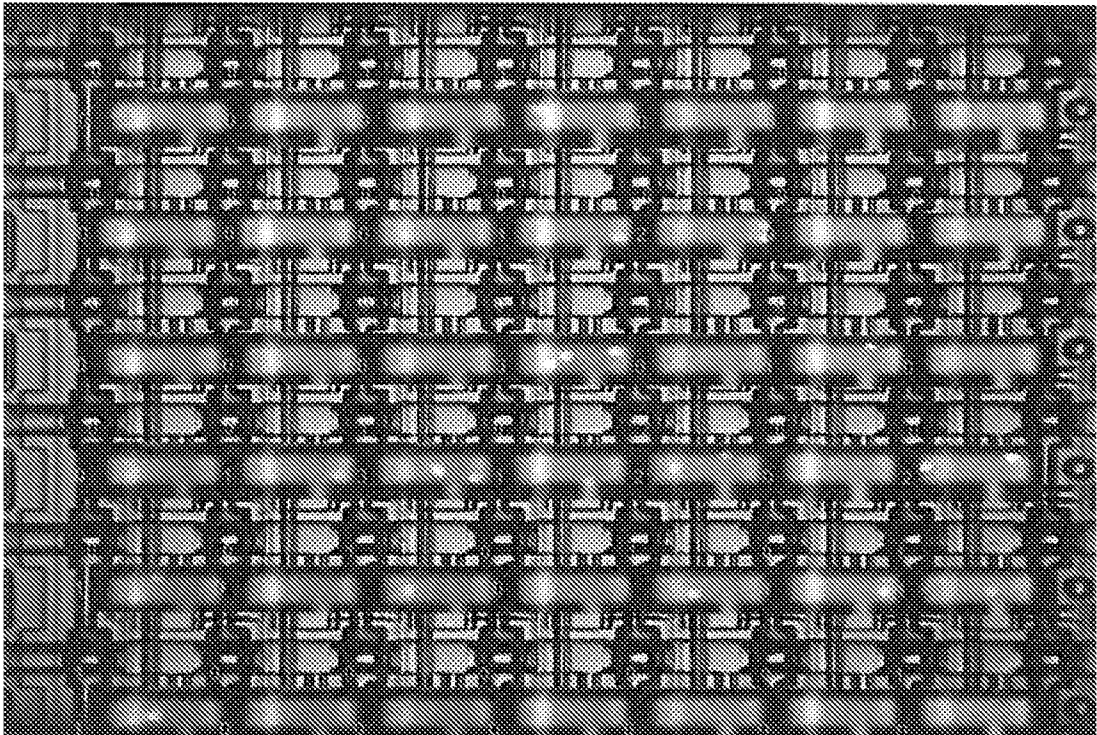
**FIG. 11A**

Theoretical:

Poisson distribution

**FIG. 11B**

0.15E6 /ml
Expect
0-1
chamber

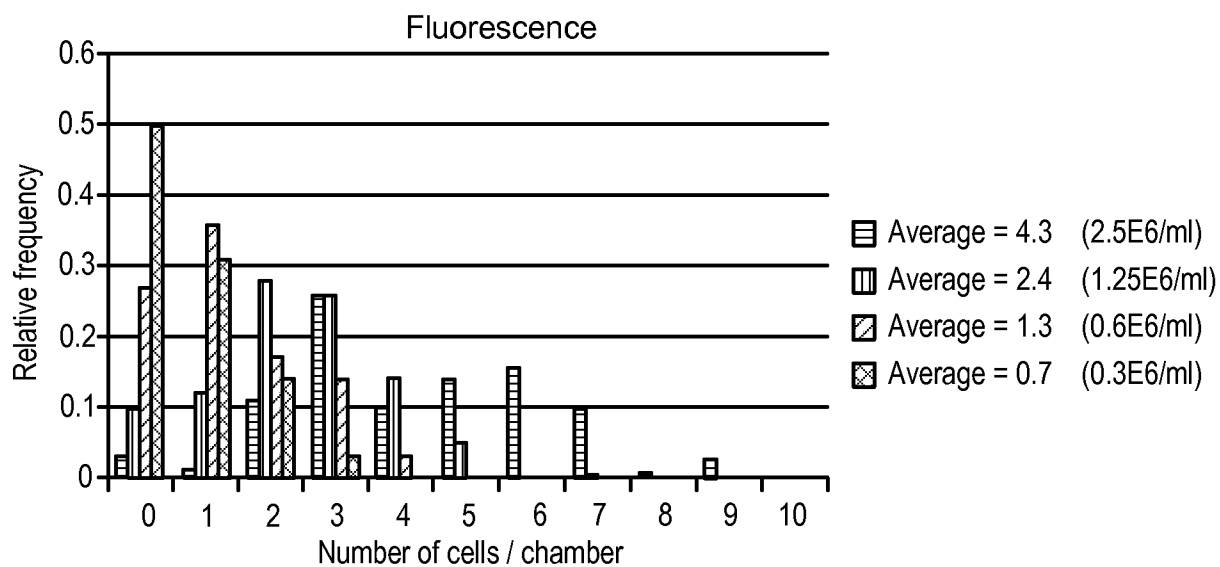
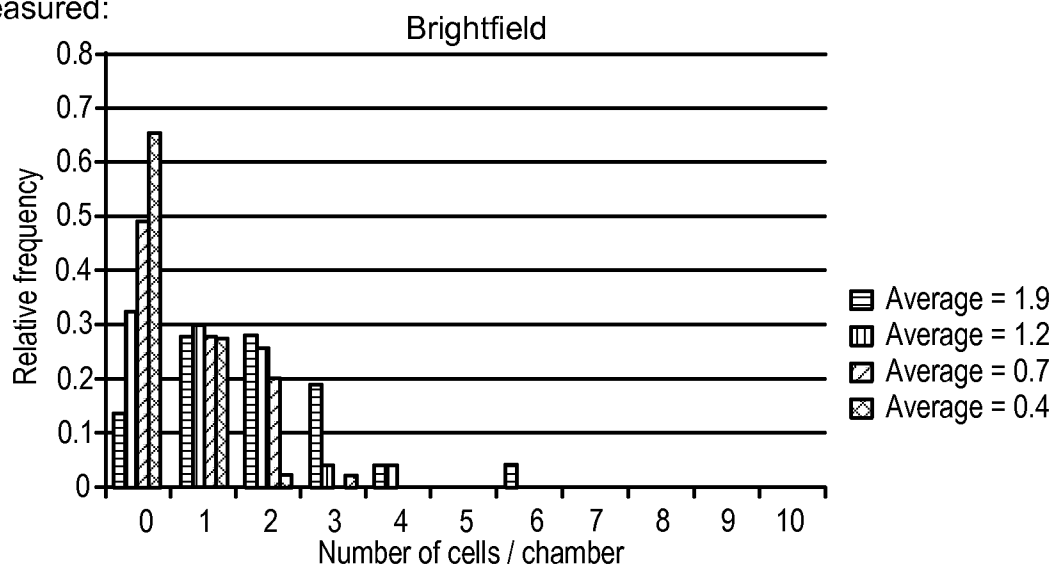


	Brightfield	Fluorescence
Average cells/ chamber	0.29	0.52

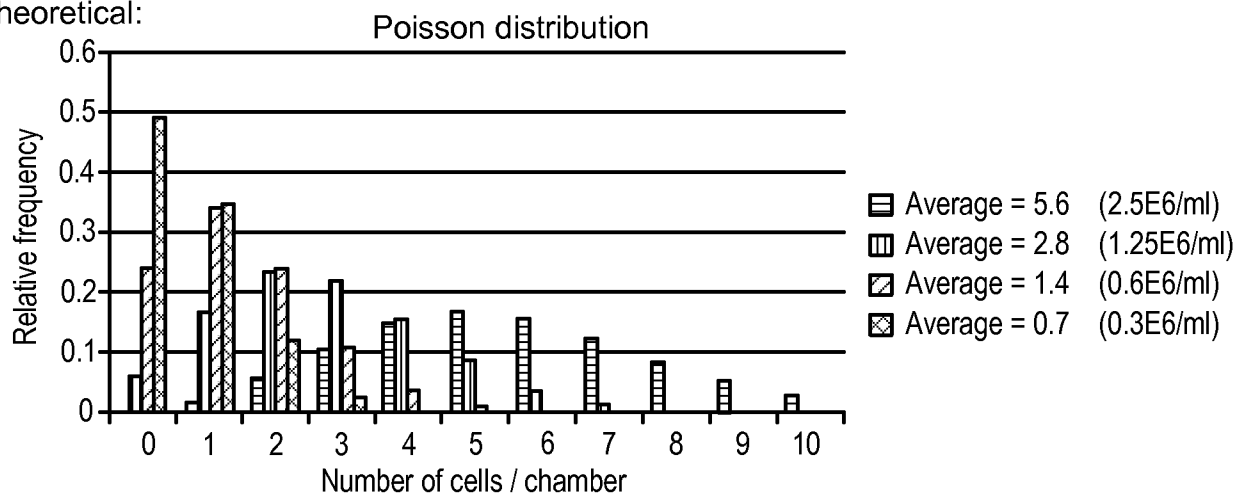
FIG. 12A

15 / 43

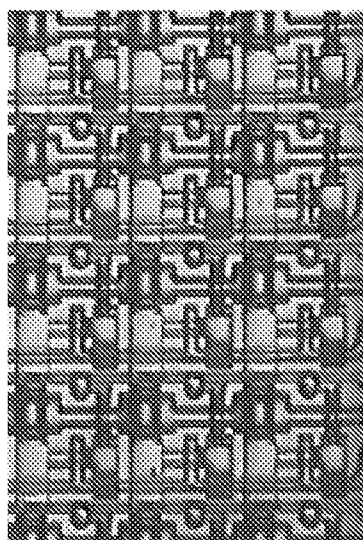
Measured:



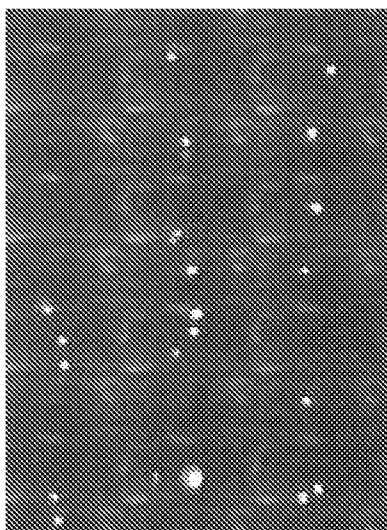
Theoretical:

**FIG. 12B**

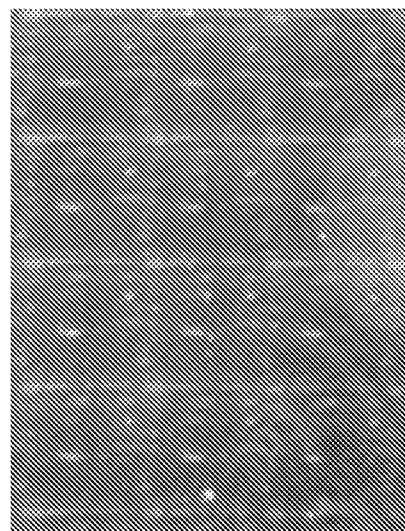
16 / 43



Brightfield



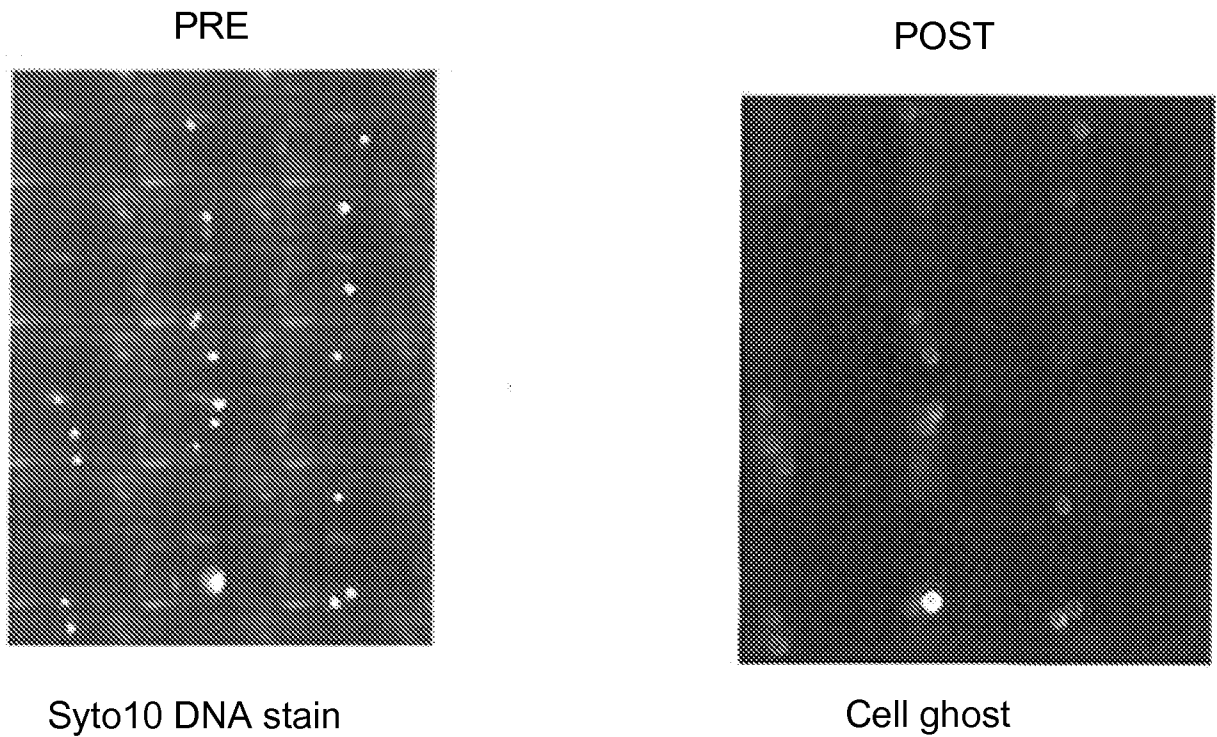
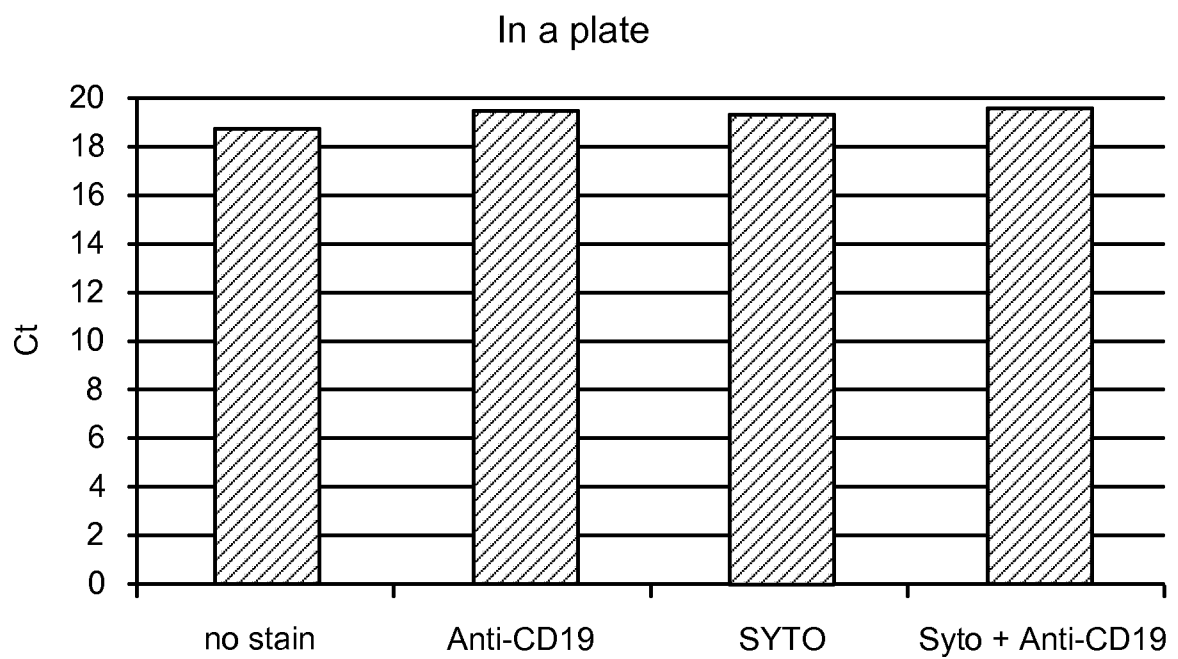
Syto10 DNA stain



Cy5 antibody

FIG. 13

17 / 43

**FIG. 14A****FIG. 14B**

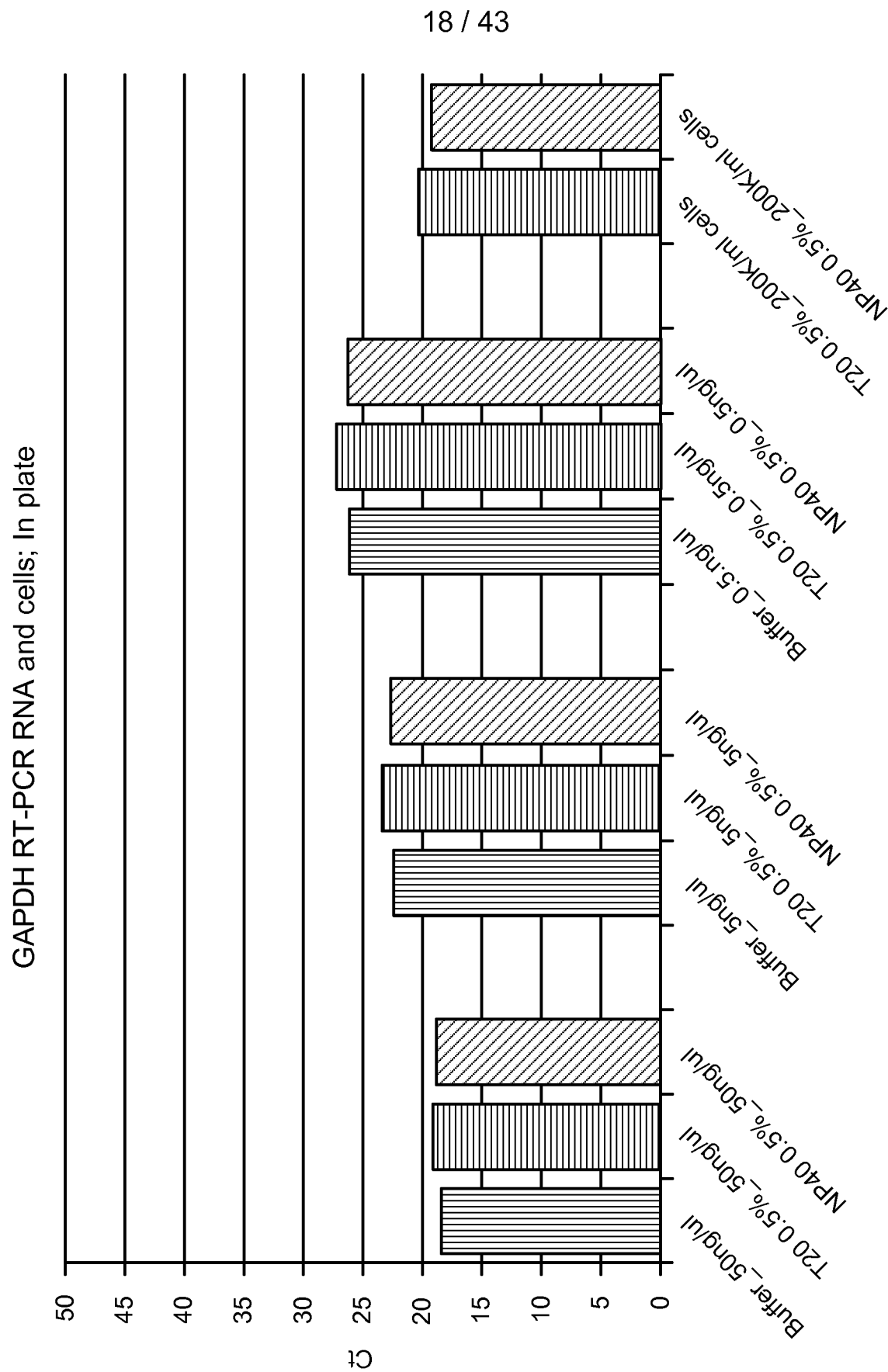


FIG. 15

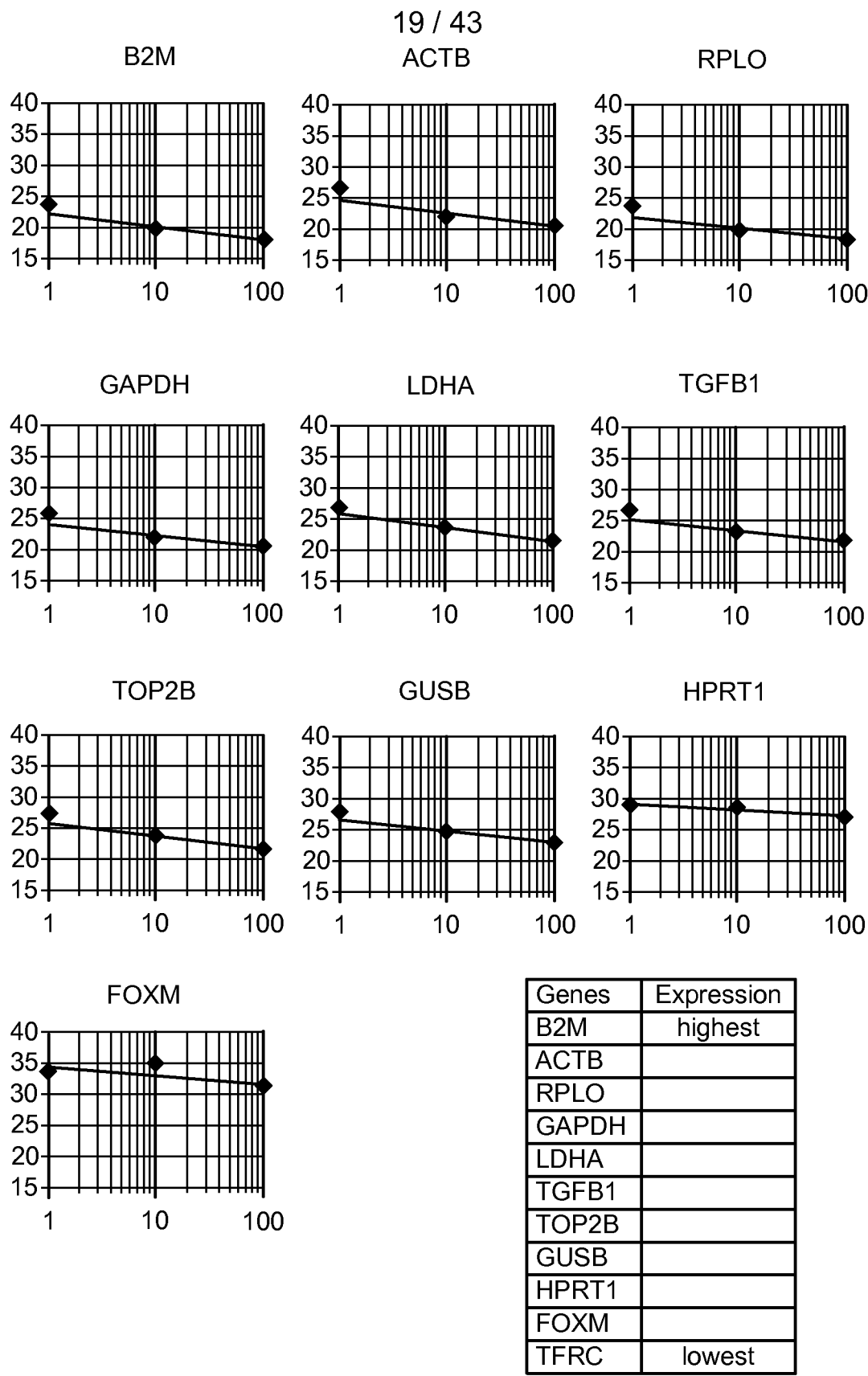


FIG. 16

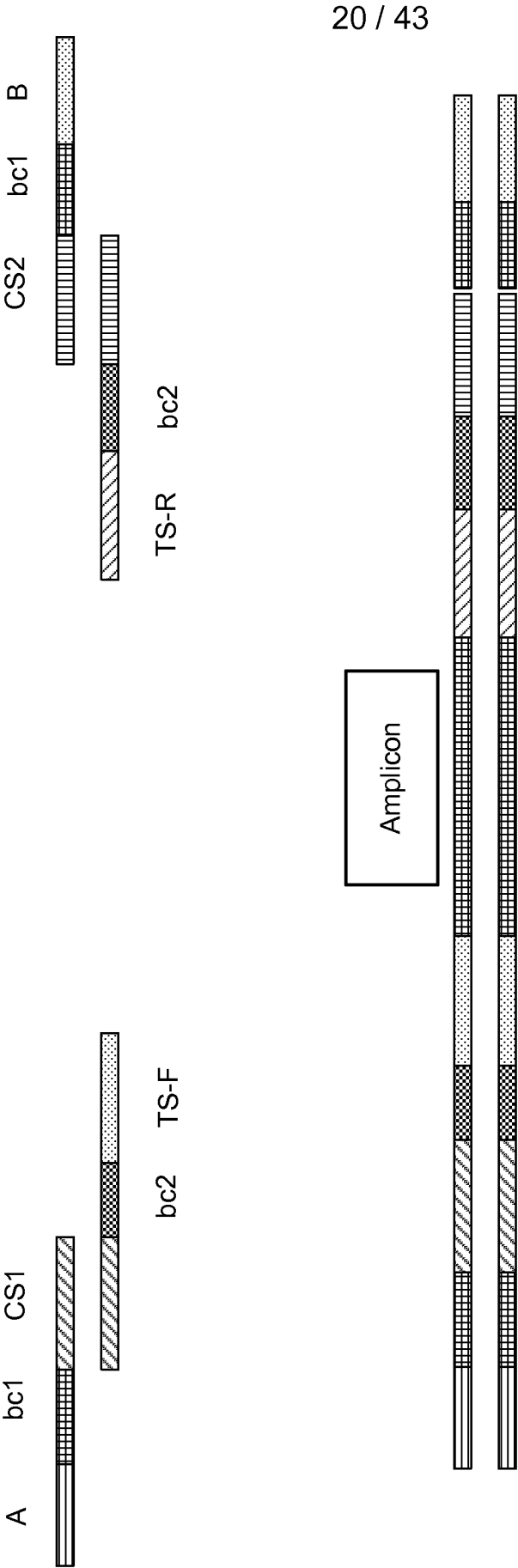


FIG. 17

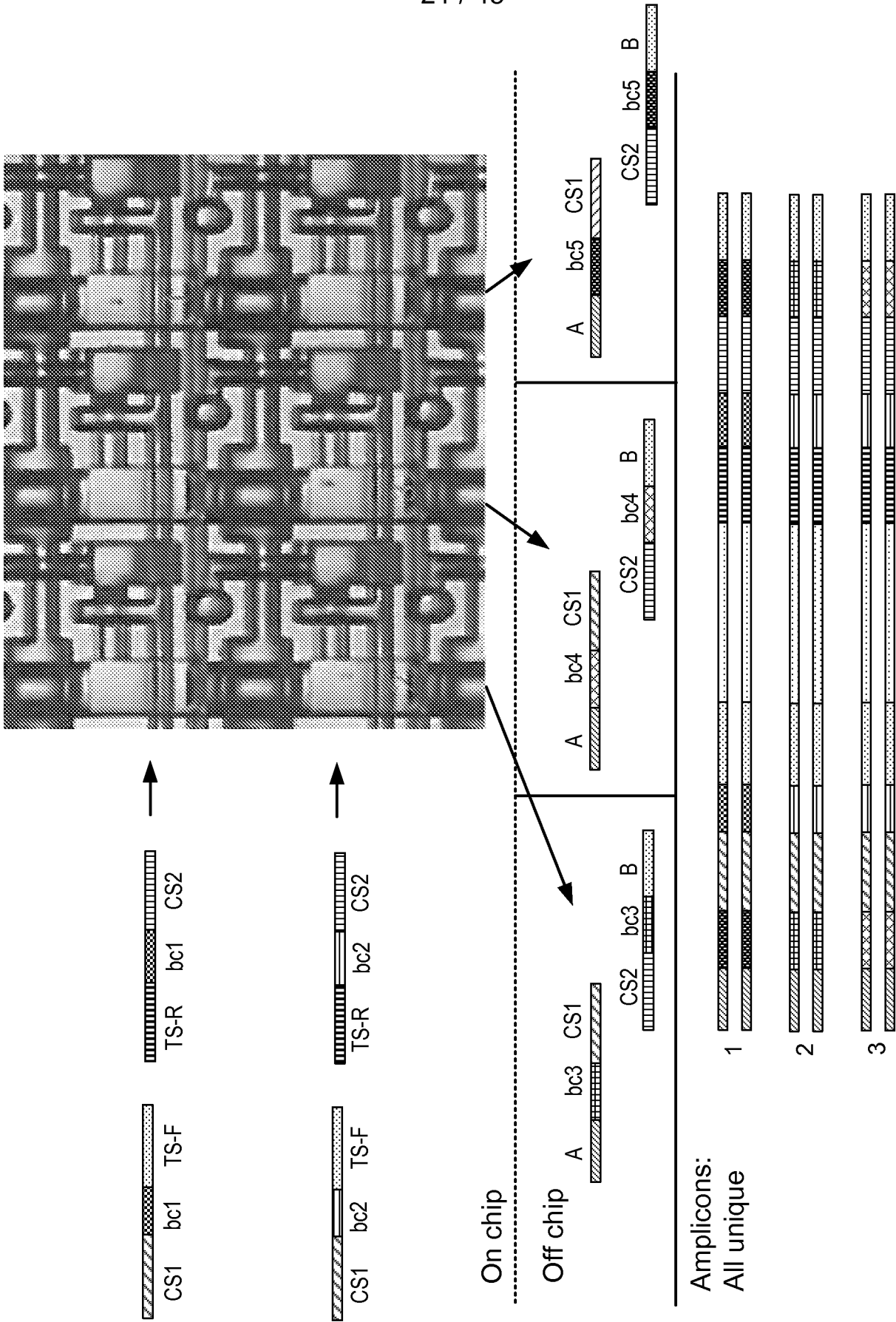
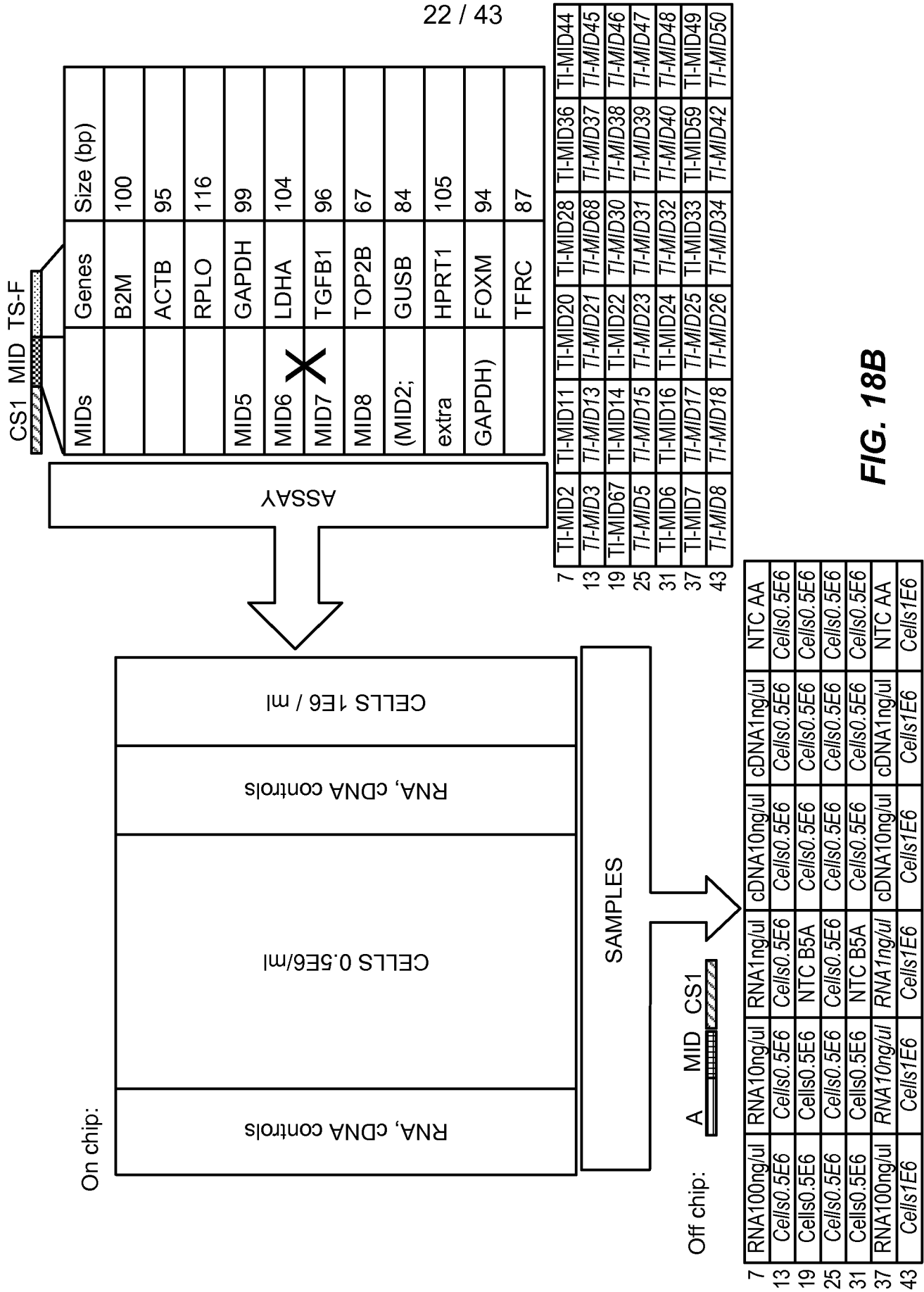


FIG. 18A



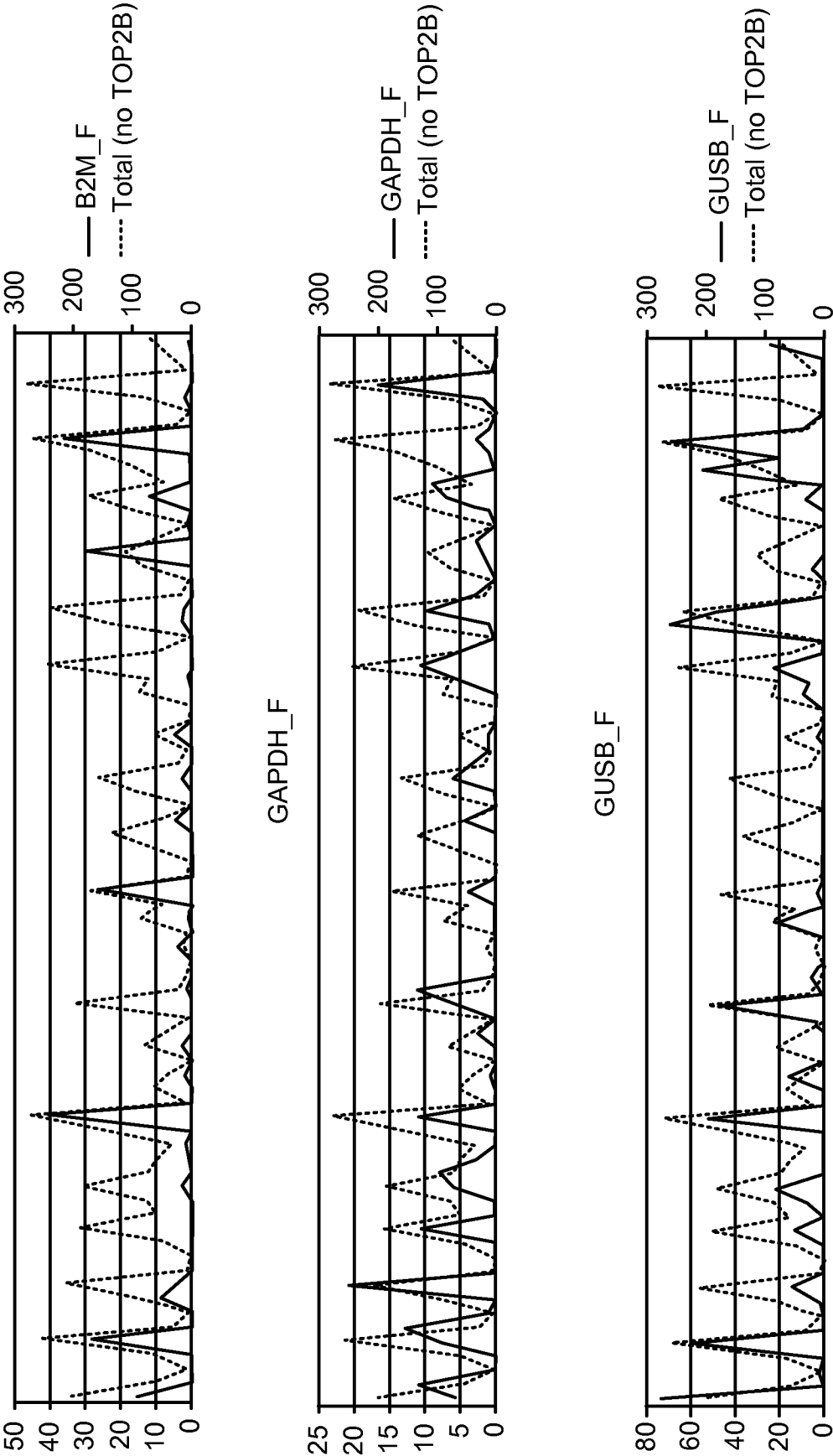


FIG. 19

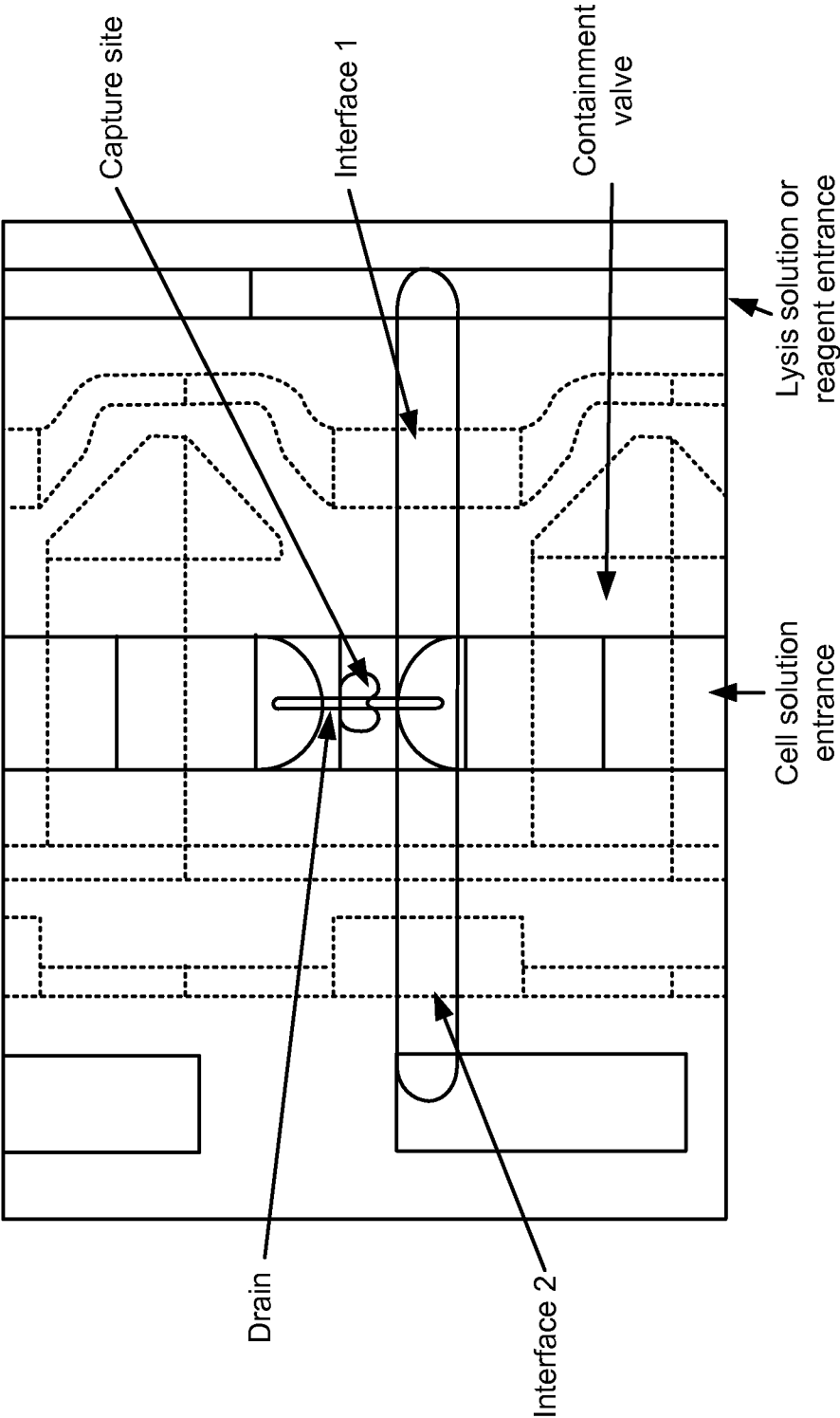


FIG. 20A

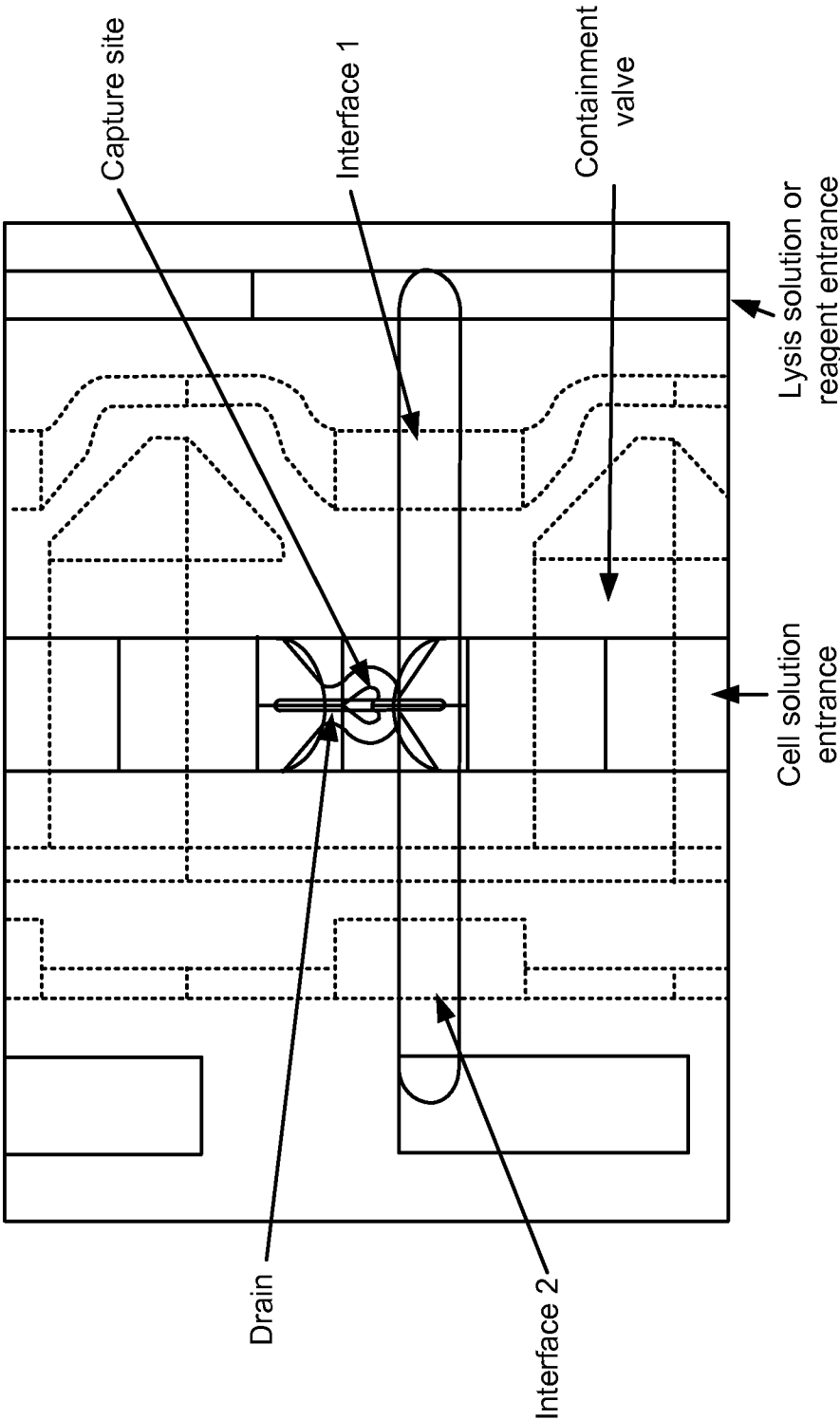


FIG. 20B

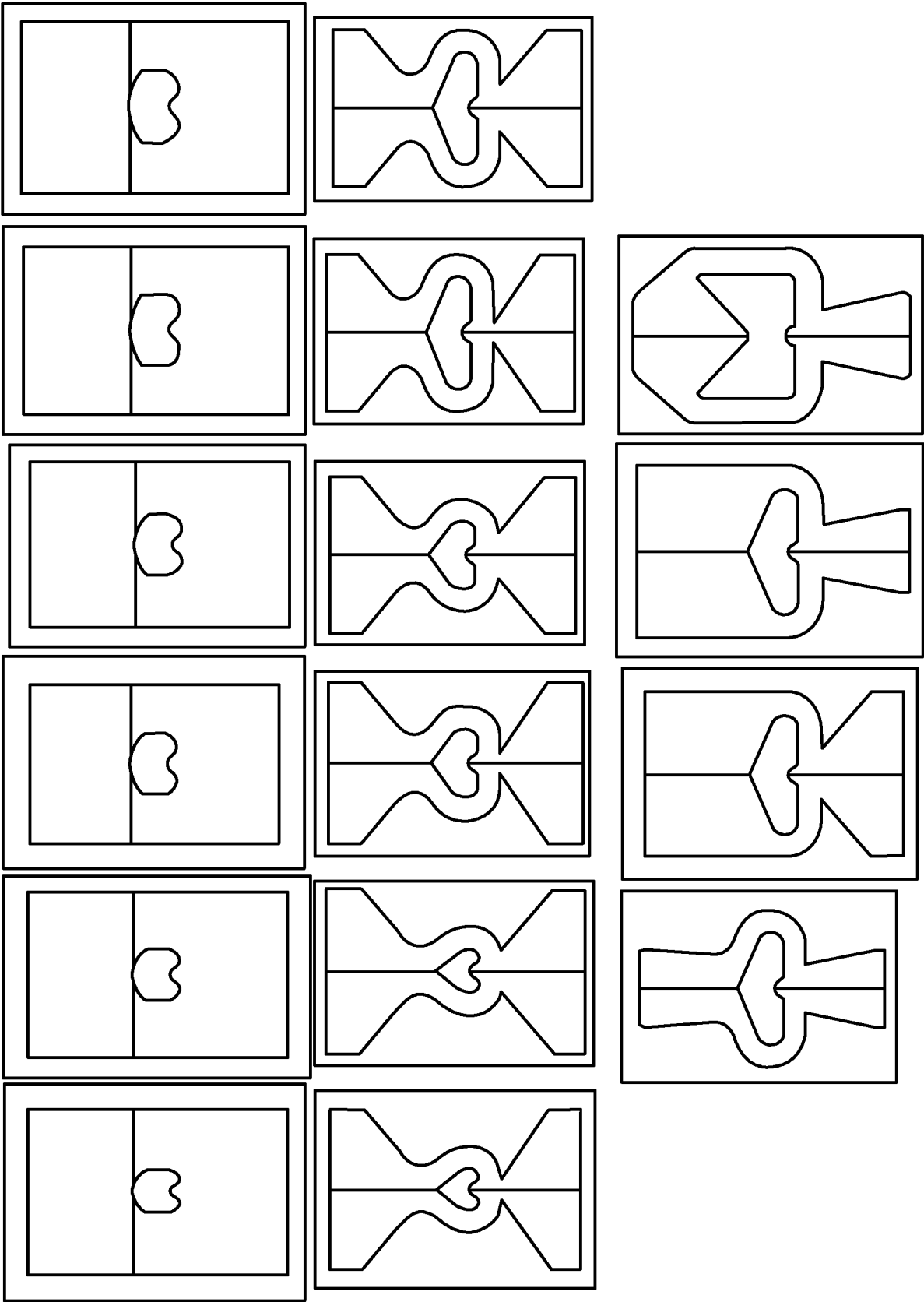


FIG. 21

27 / 43

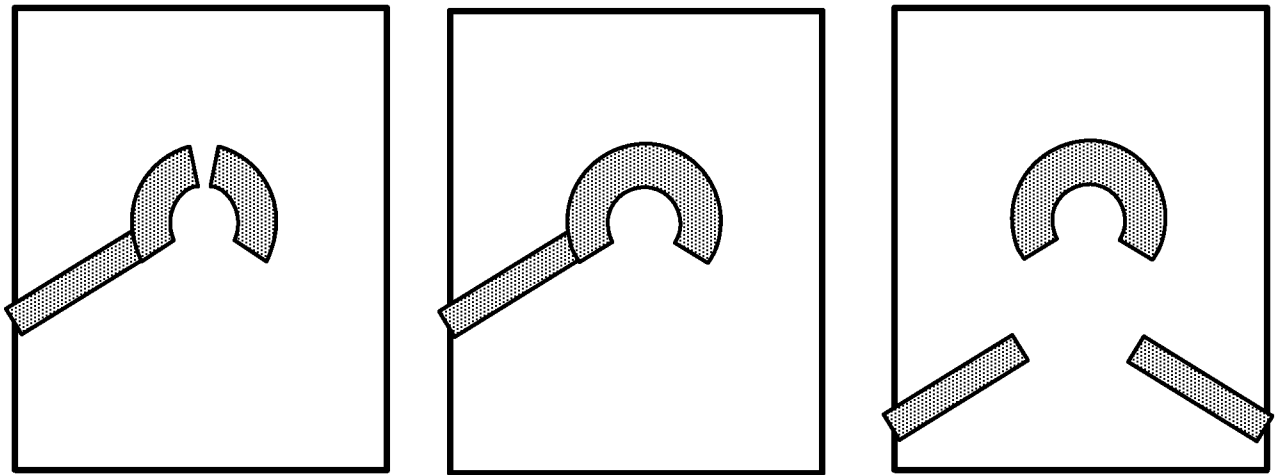


FIG. 22A

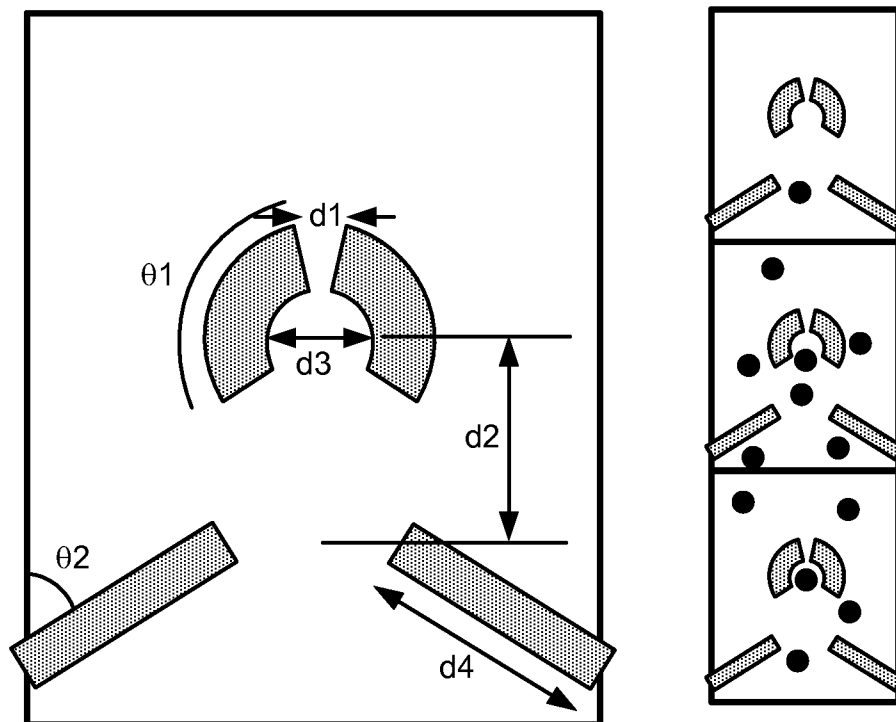


FIG. 22B

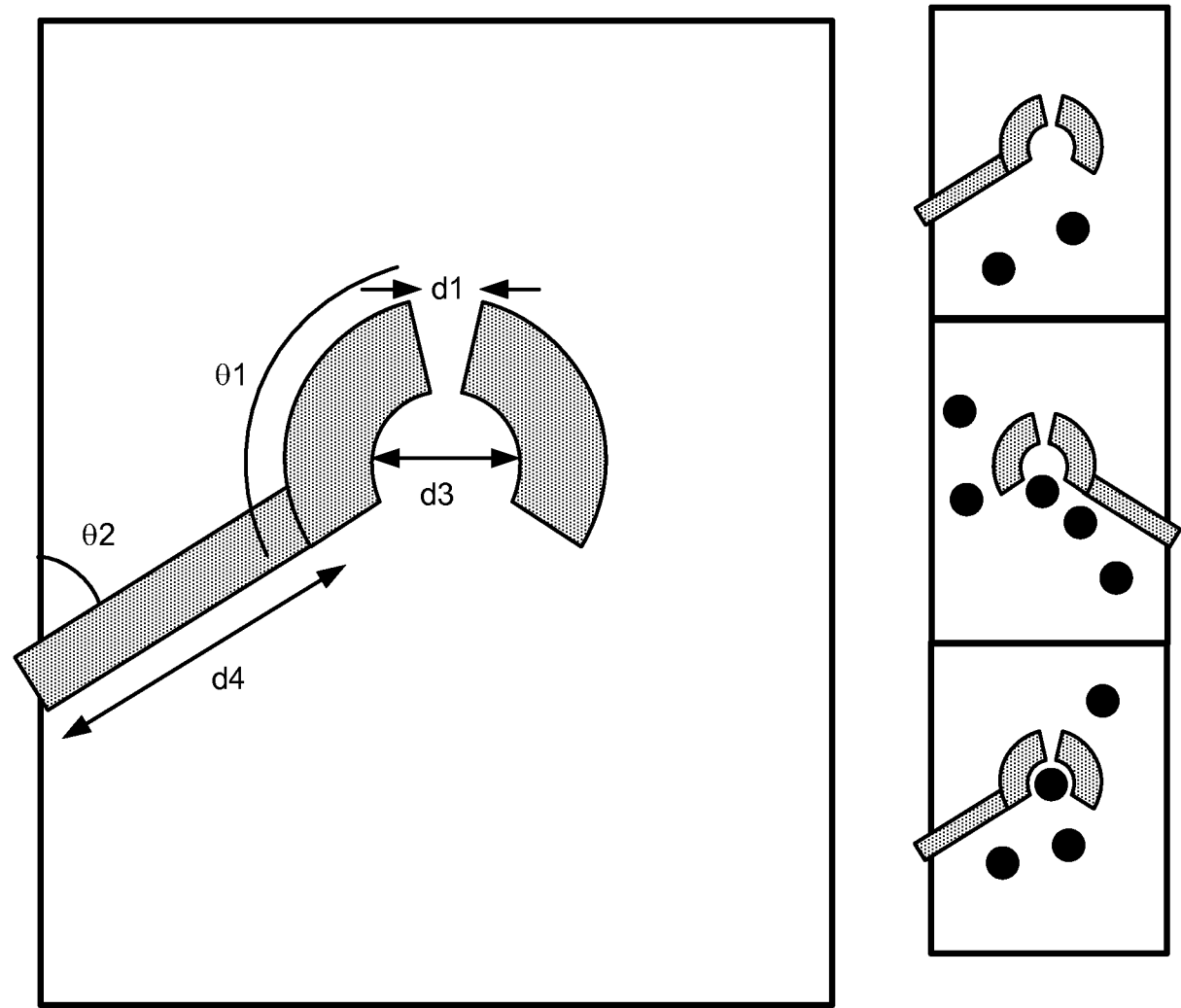


FIG. 22C

29 / 43

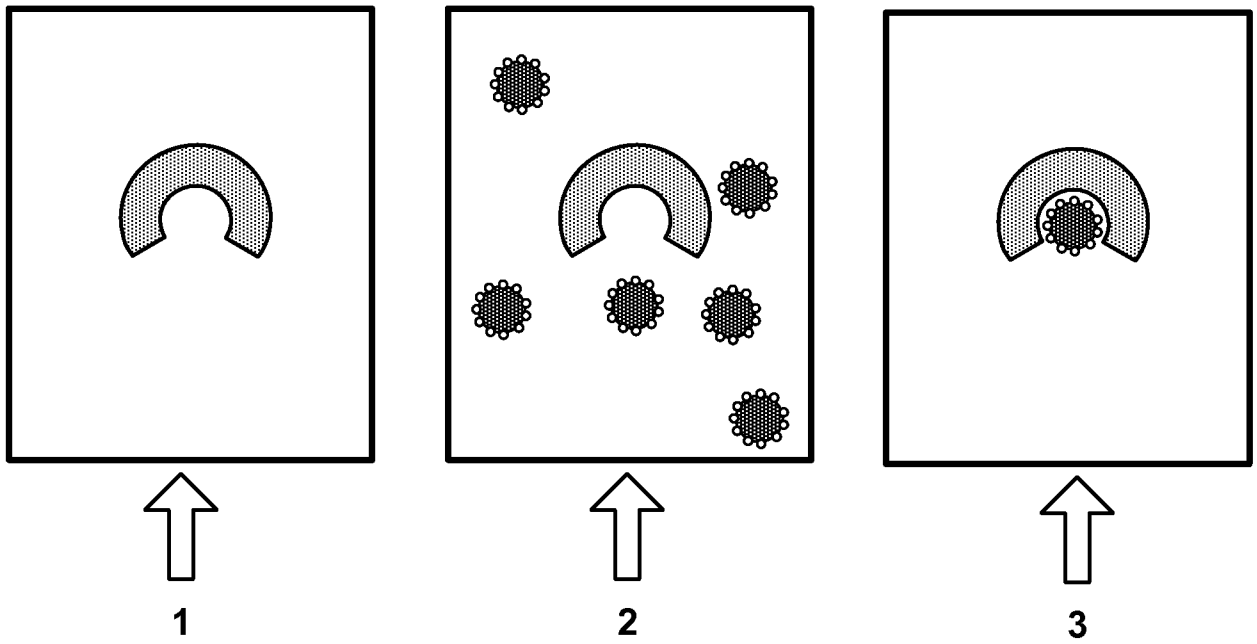


FIG. 23A

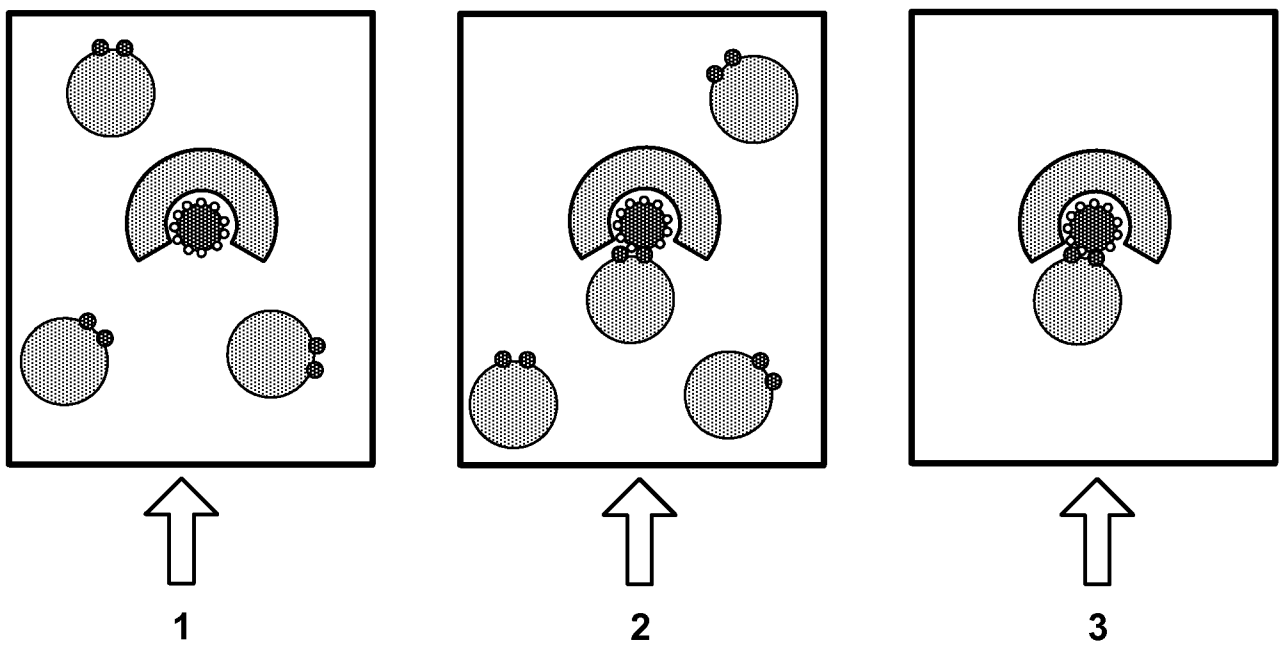


FIG. 23B

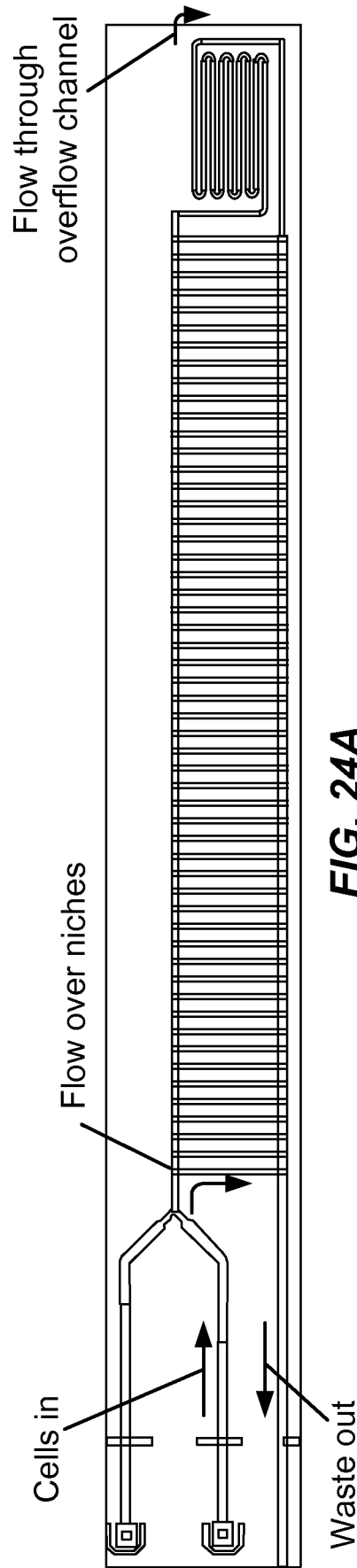


FIG. 24A

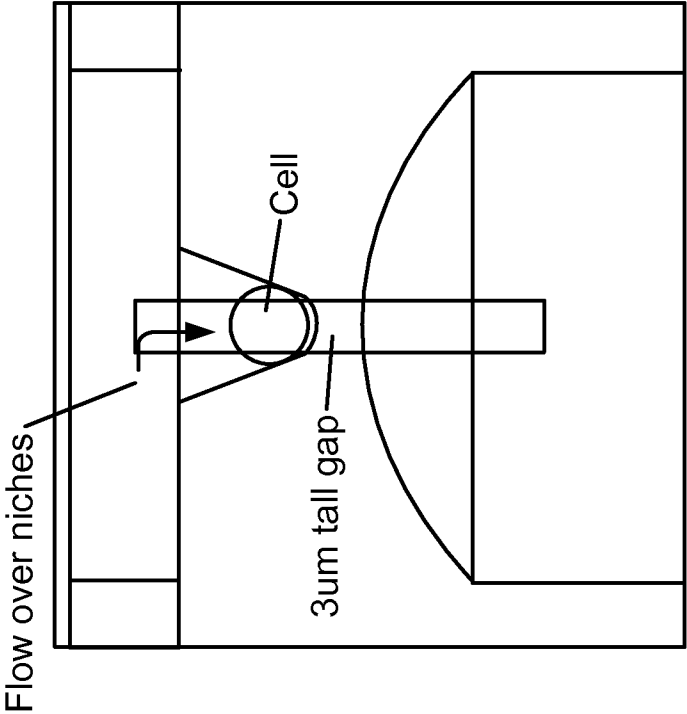


FIG. 24B

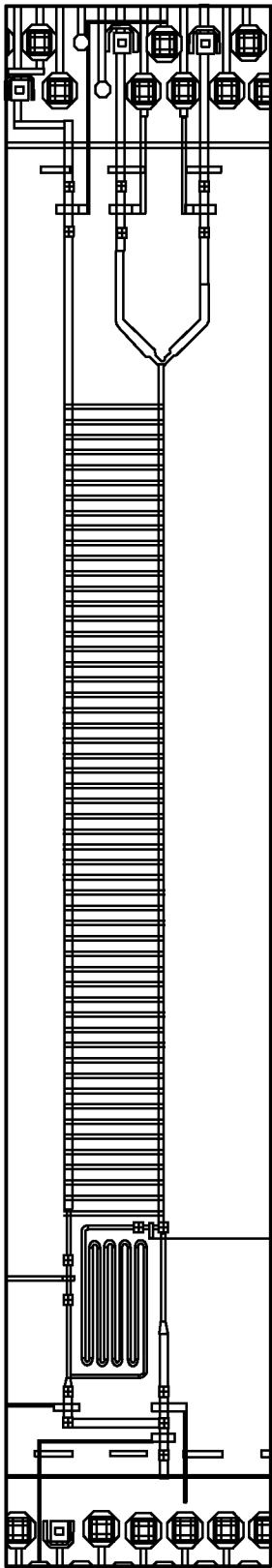


FIG. 24C

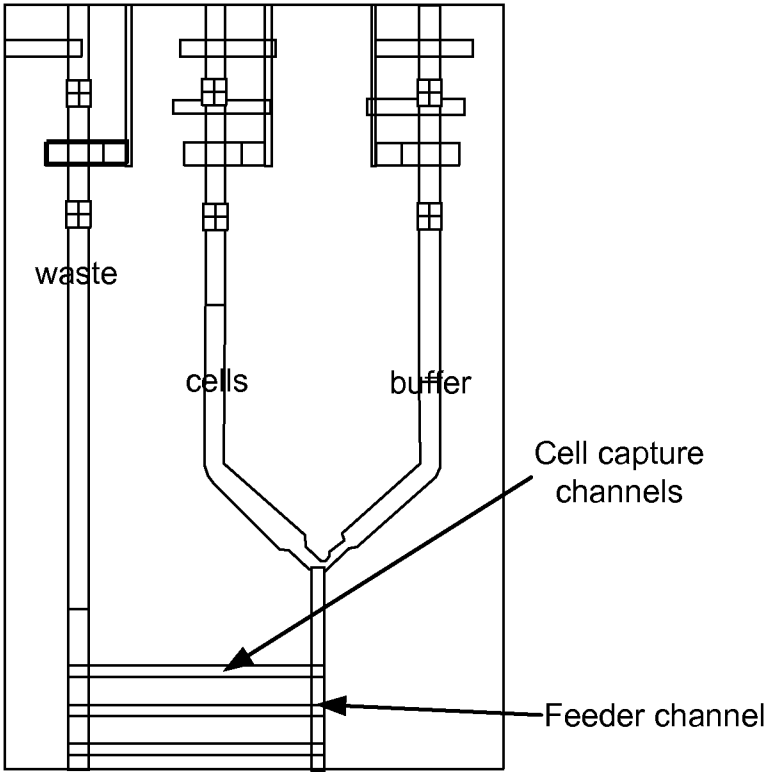


FIG. 24D

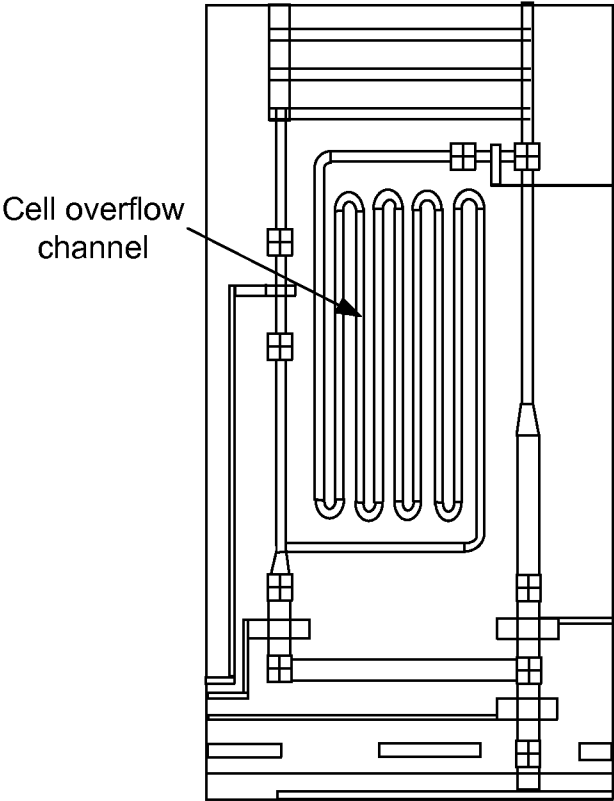


FIG. 24E

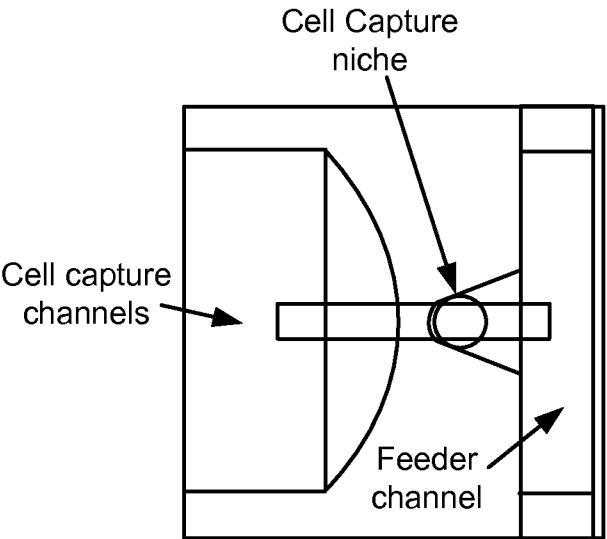


FIG. 24F

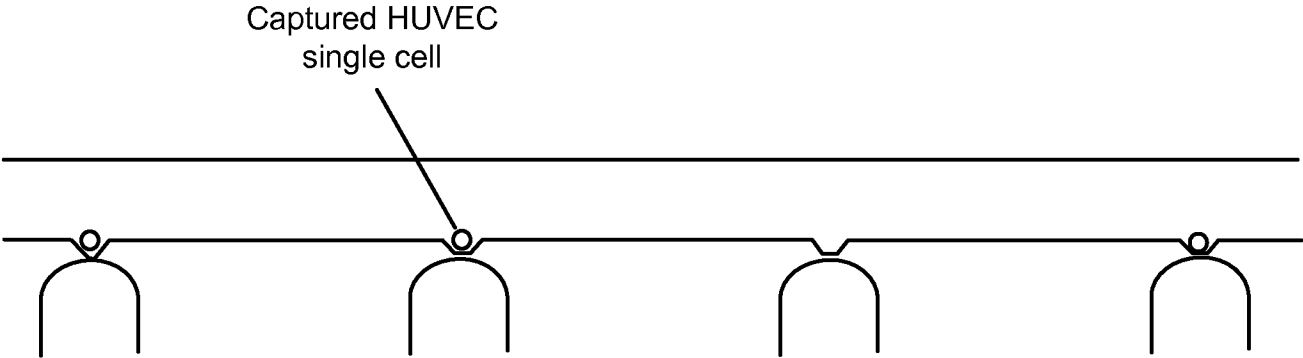
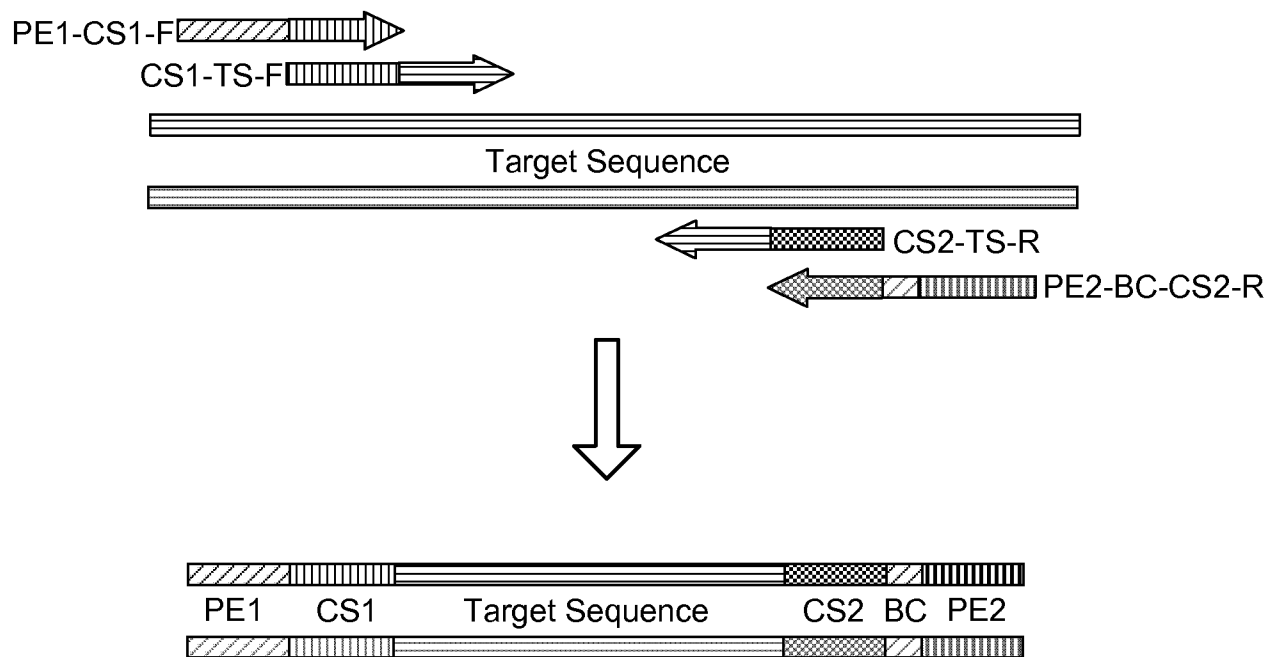
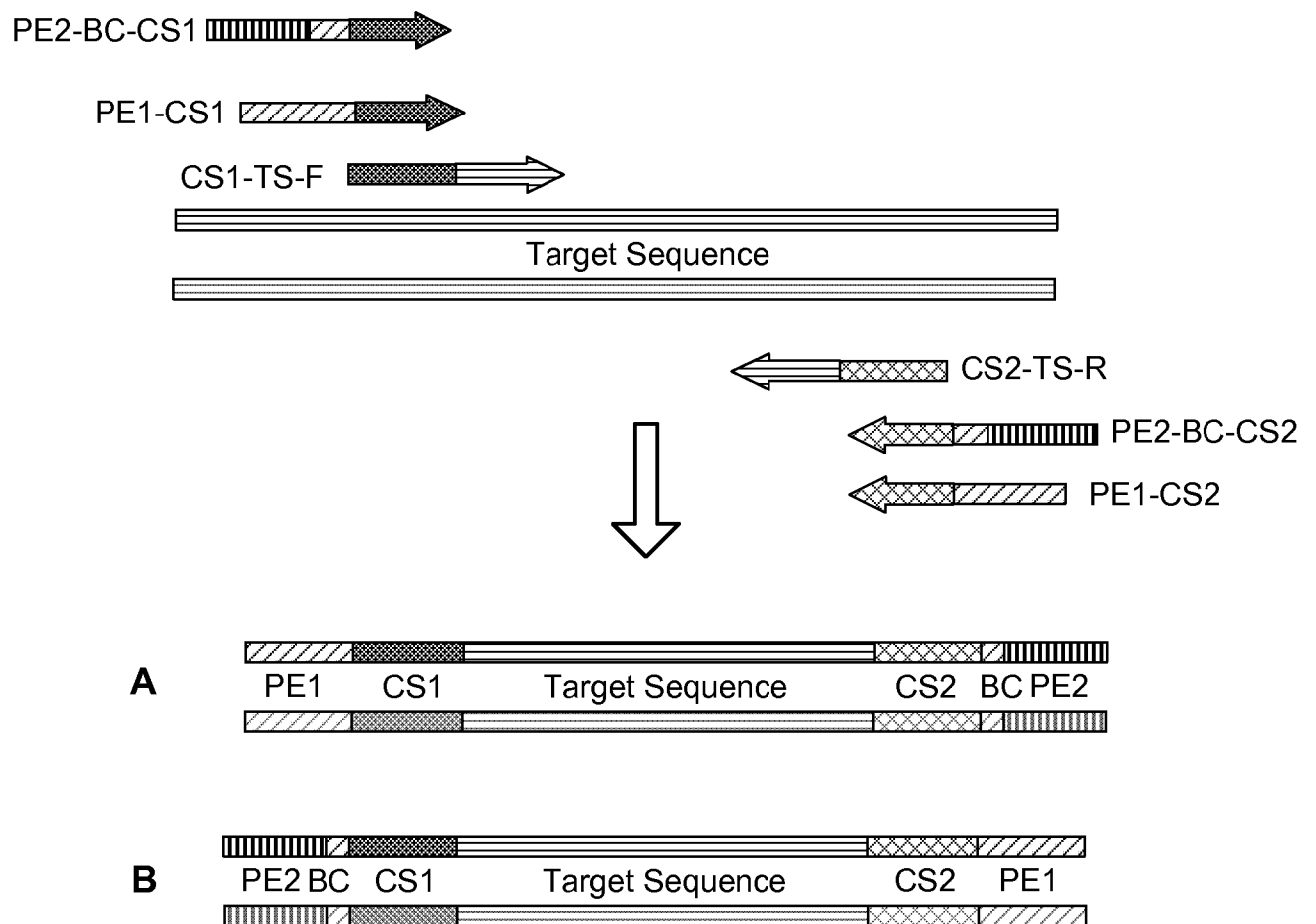
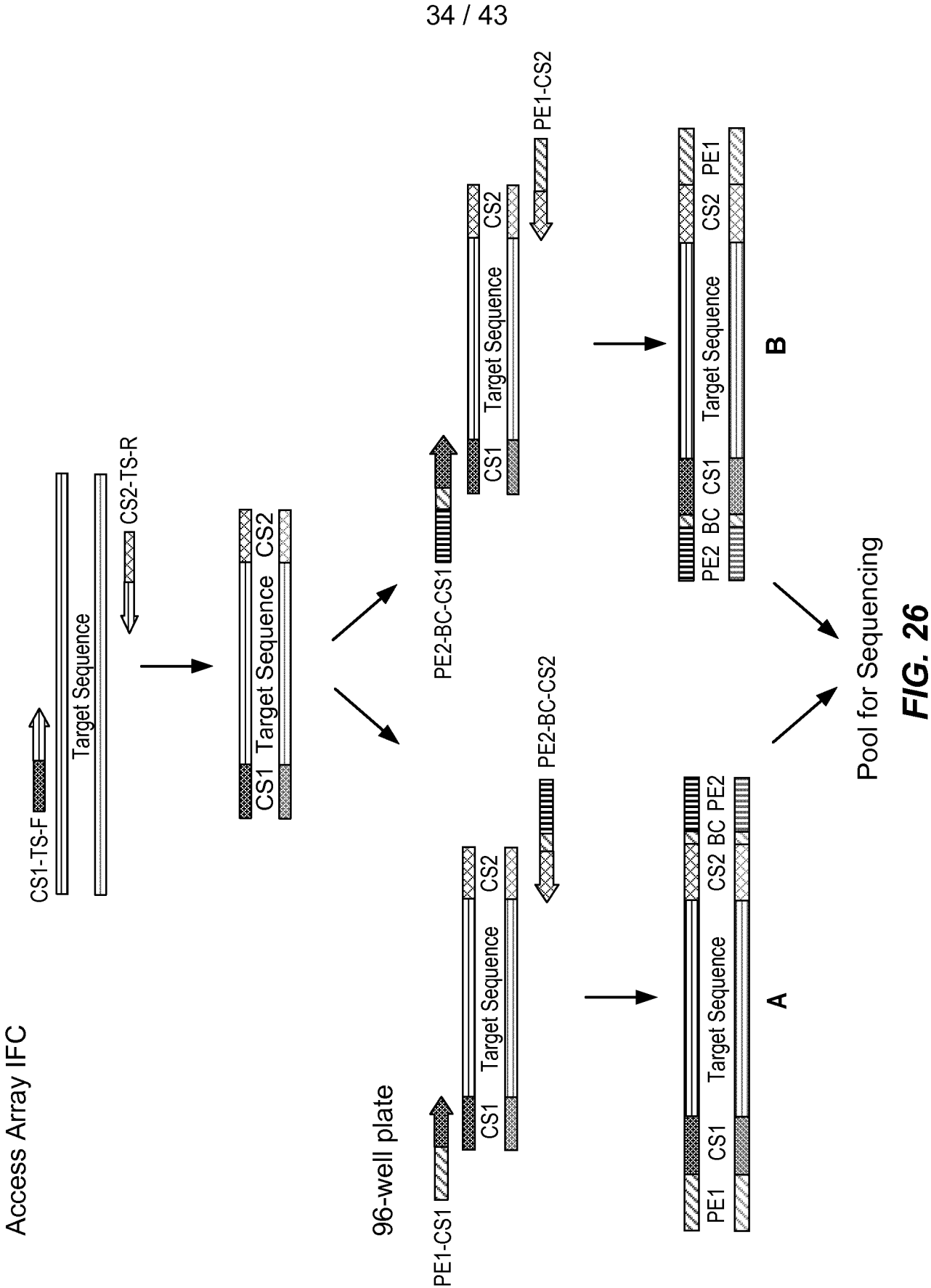


FIG. 24G

33 / 43

A**B****FIG. 25**



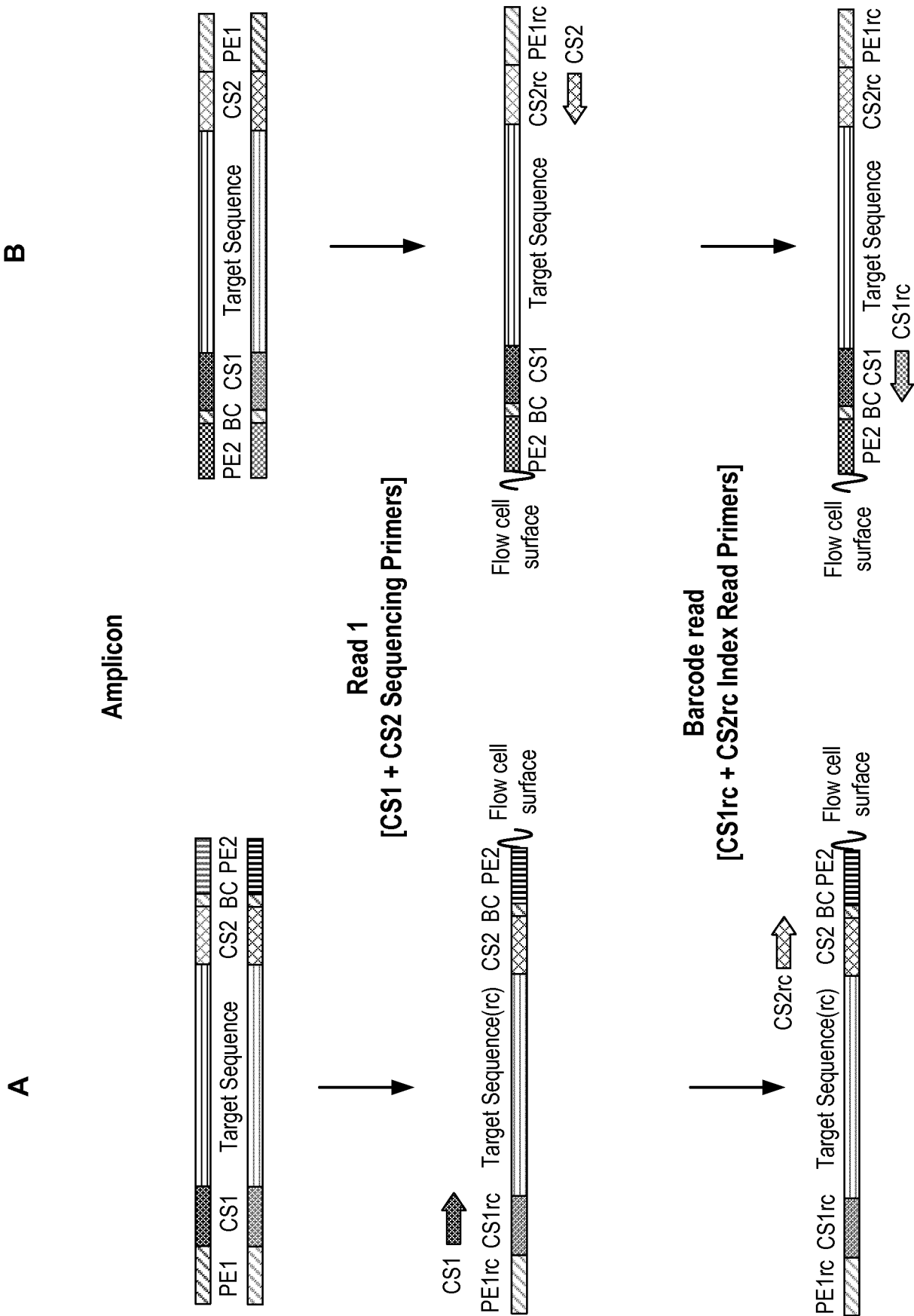


FIG. 27

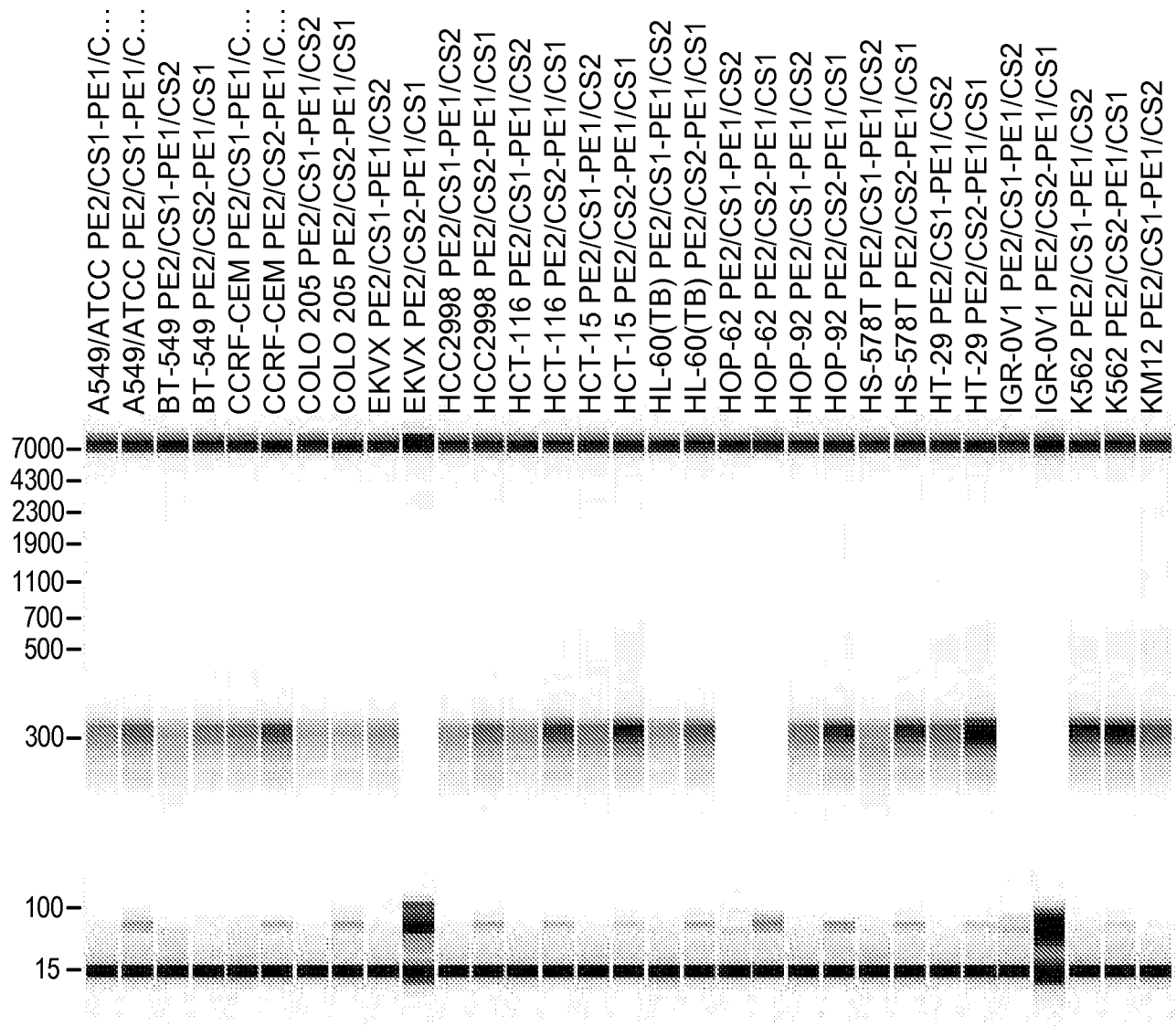


FIG. 28

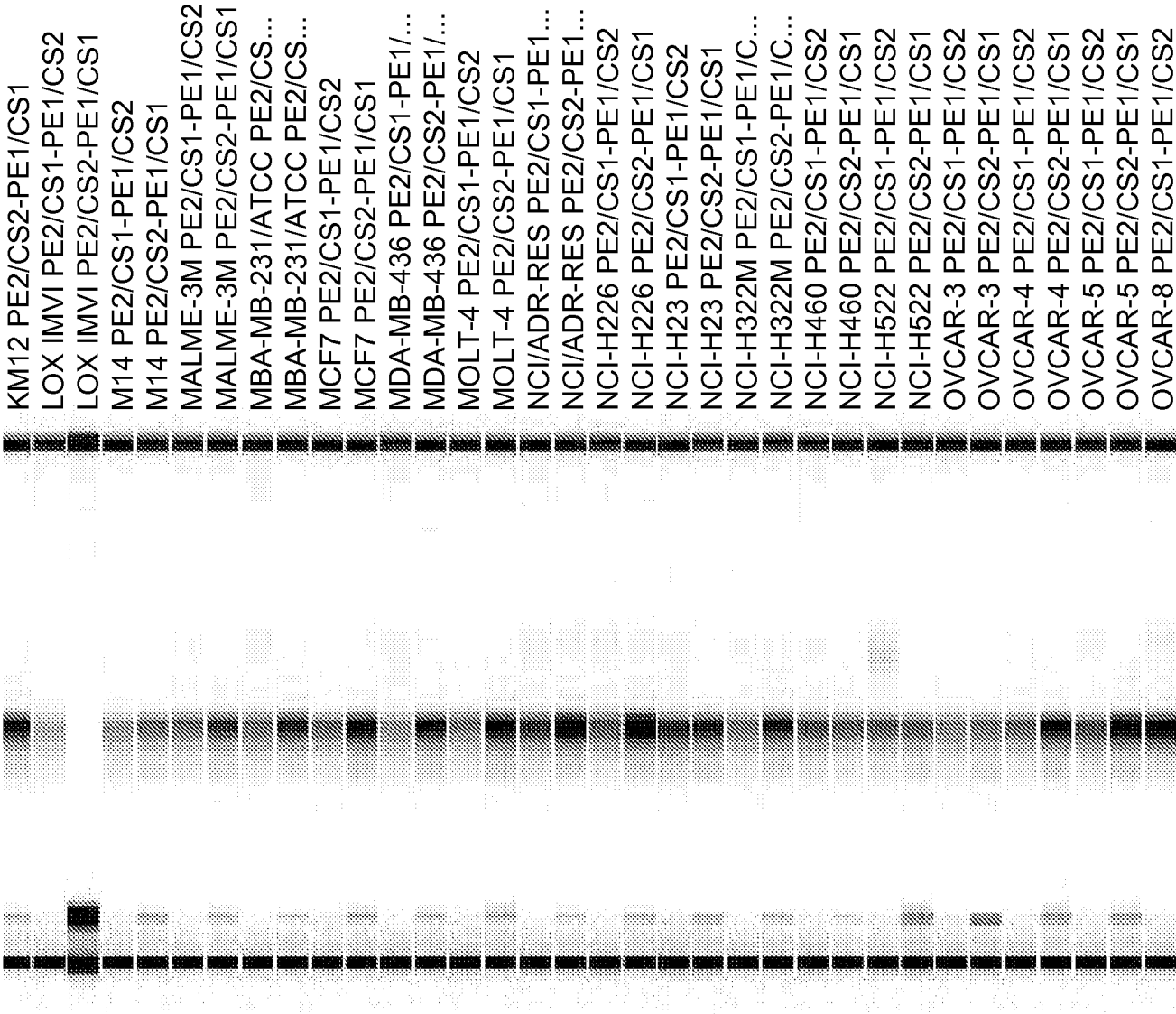


FIG. 28
(Continued)

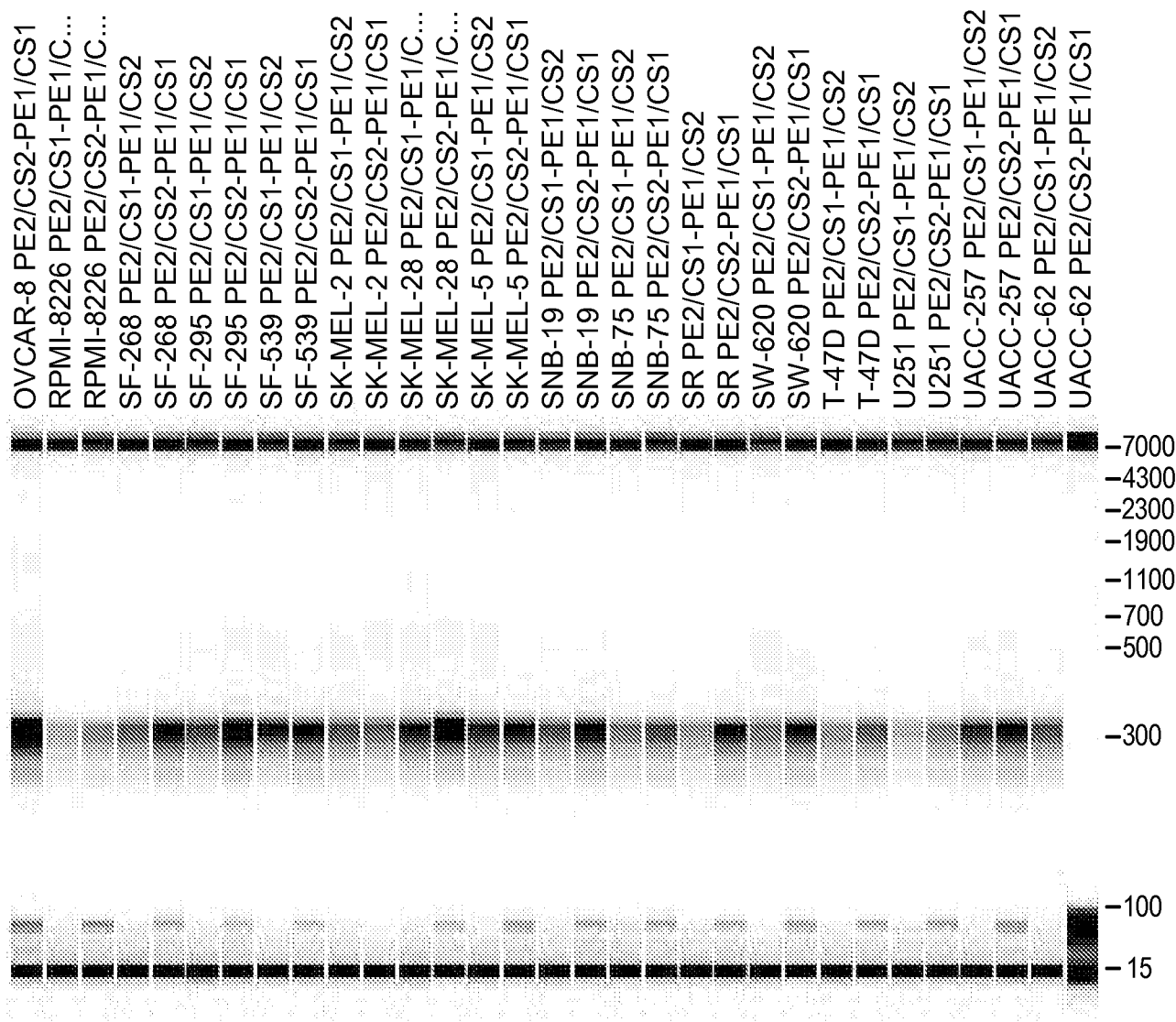


FIG. 28
(Continued)

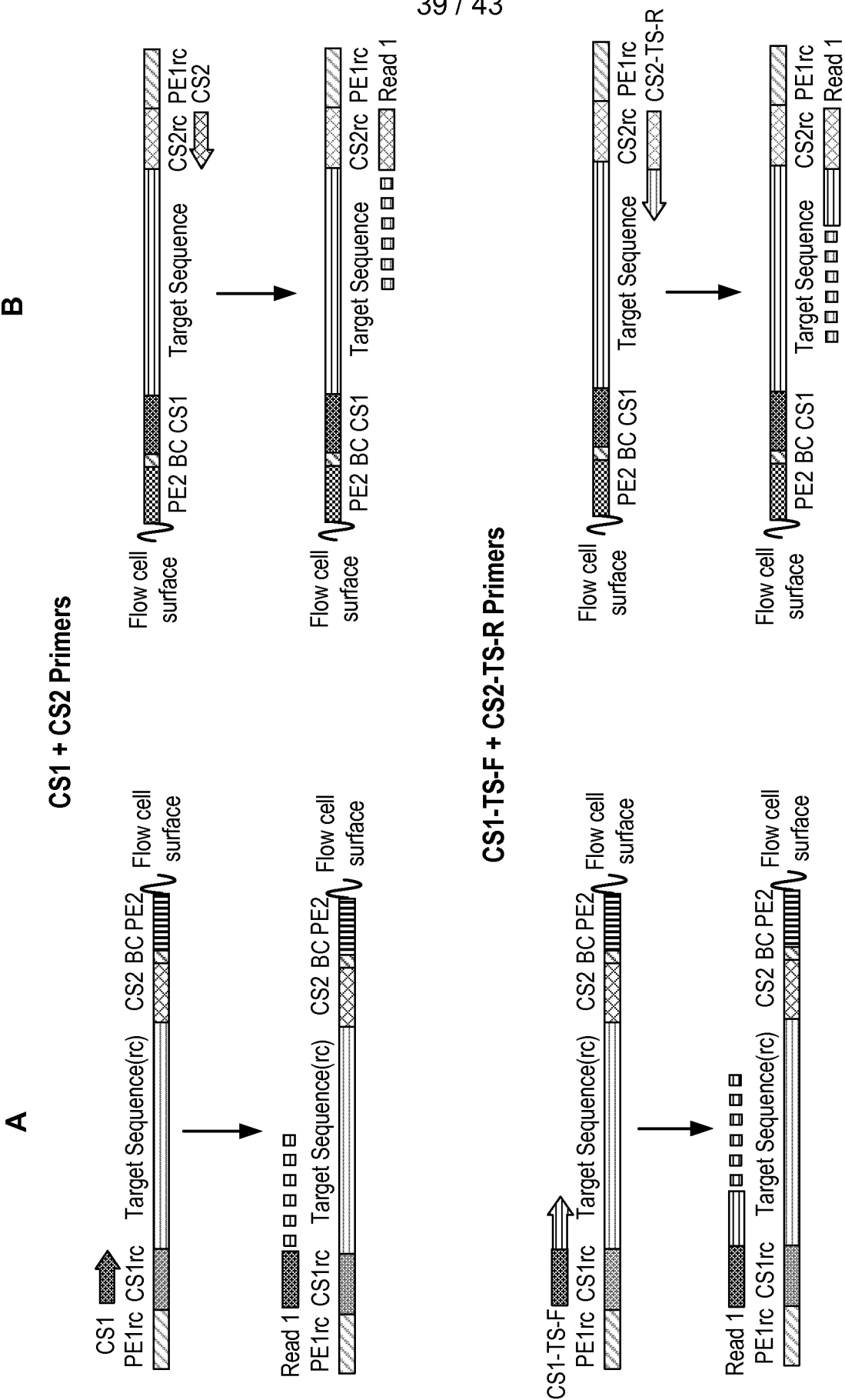


FIG. 29

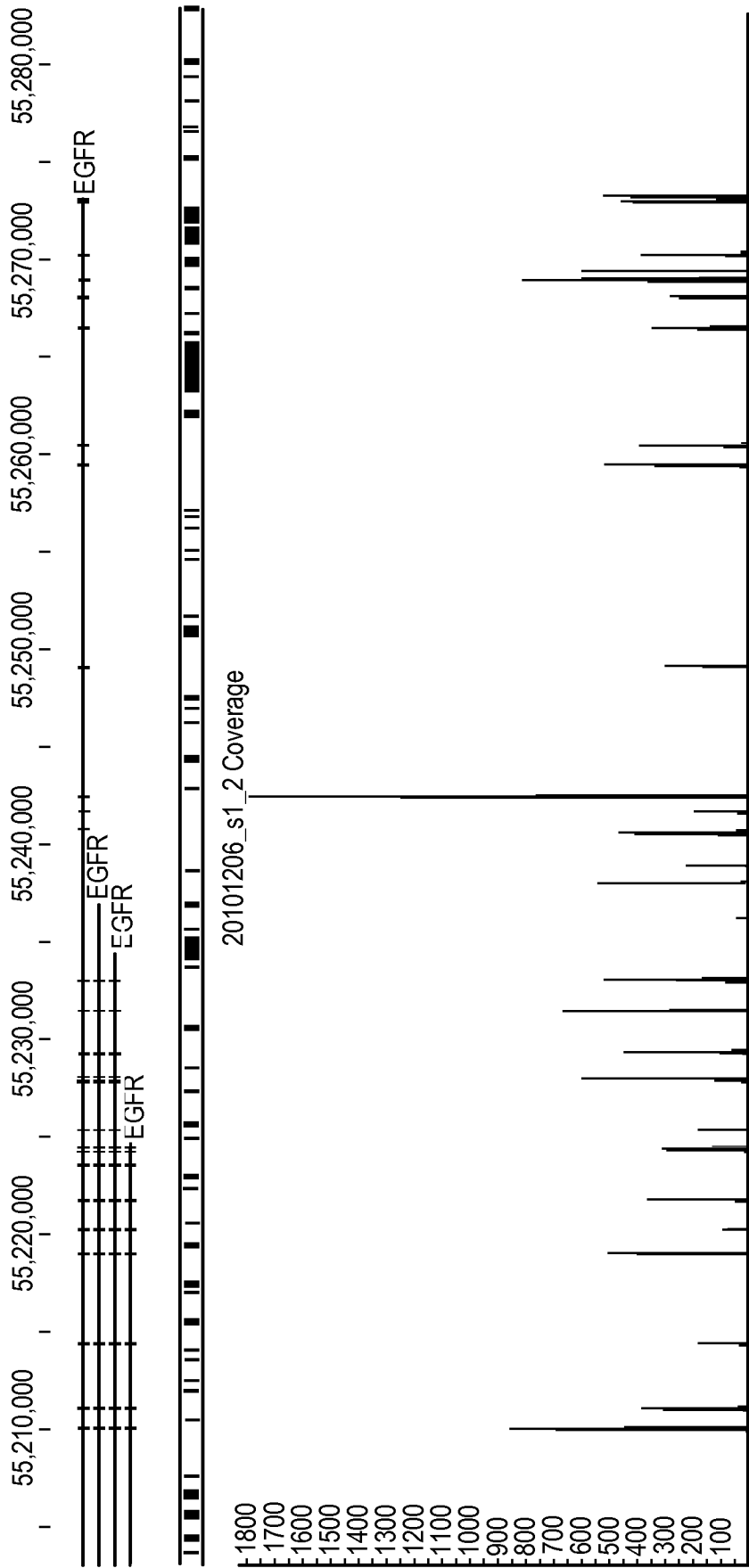
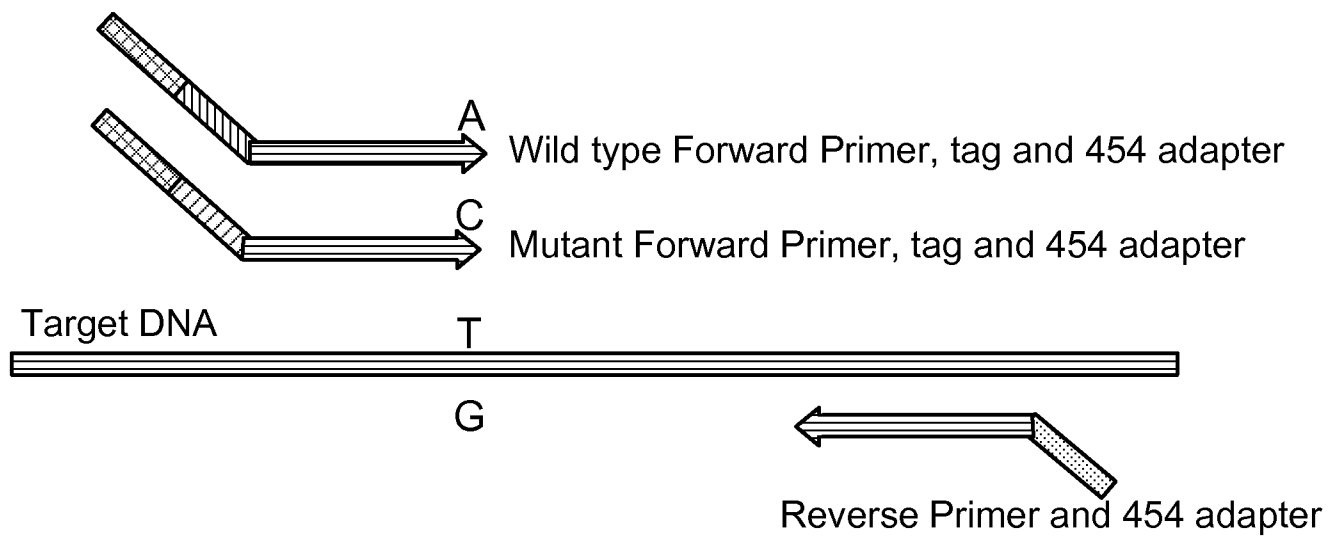


FIG. 30

41 / 43

**FIG. 31A**

Cycle 1 Wild type detection by priming on WT tag and extending all bases



Cycle 2 Mutant detection by priming on Mutant tag and extending all bases



Mutant – Few of these

FIG. 31B

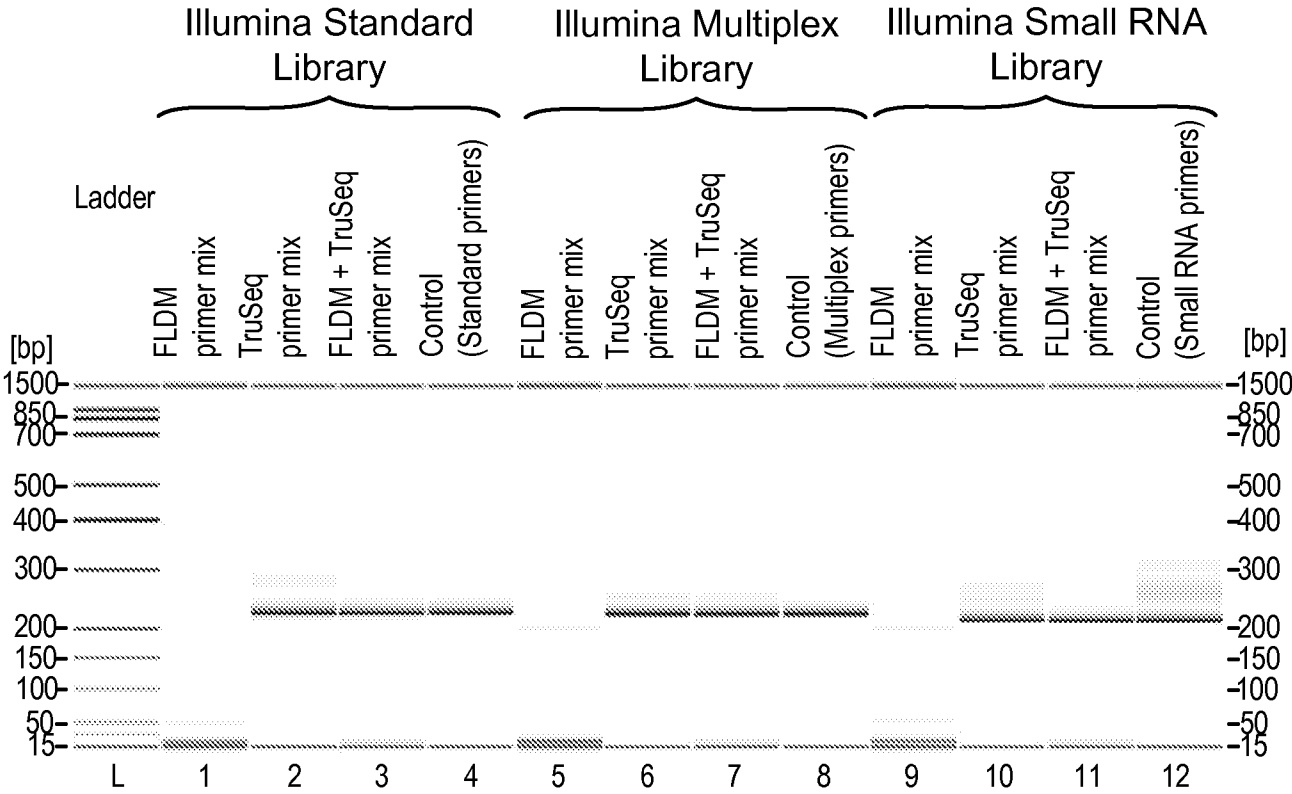


FIG. 32

Access Array Library

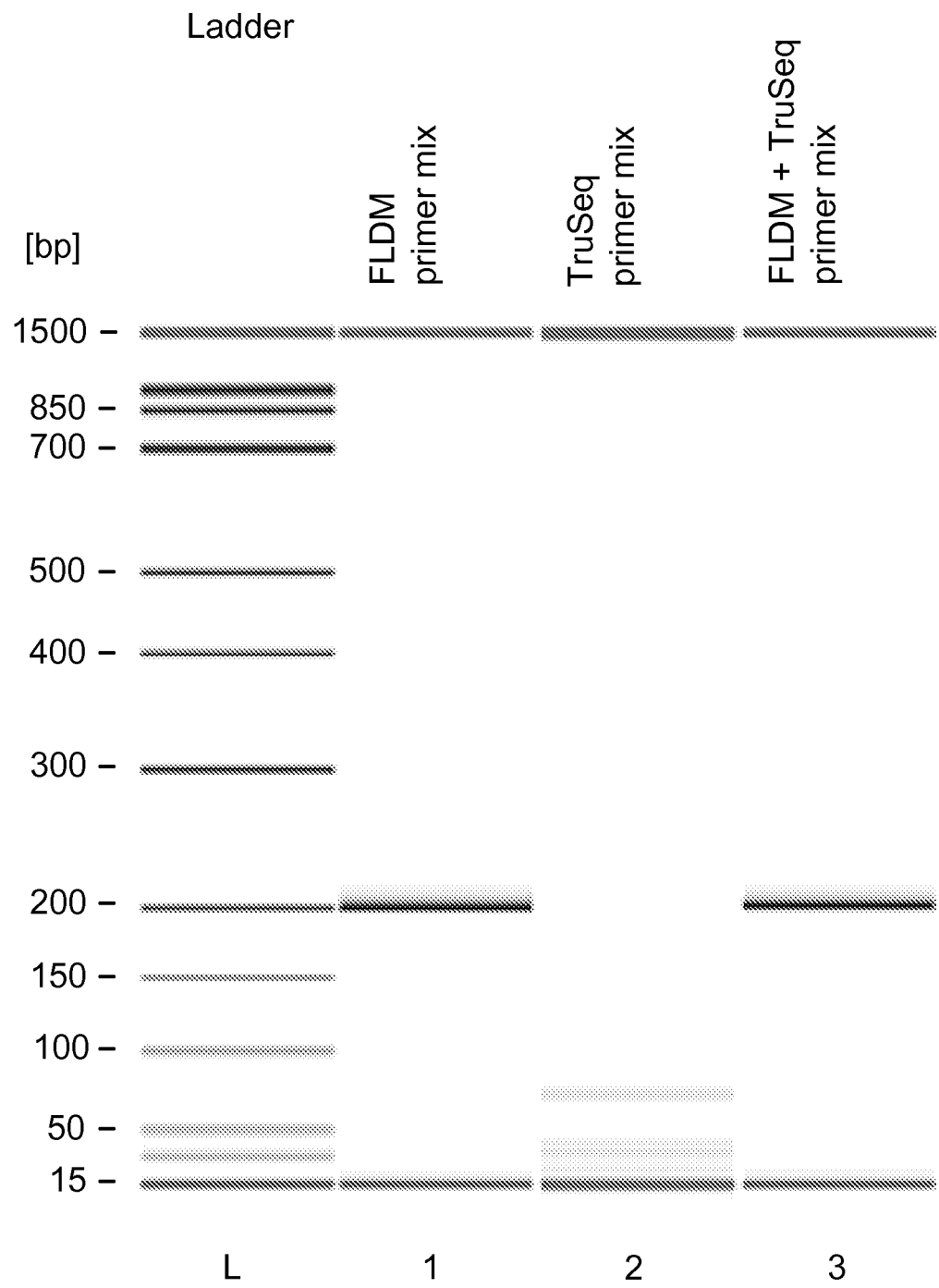


FIG. 33