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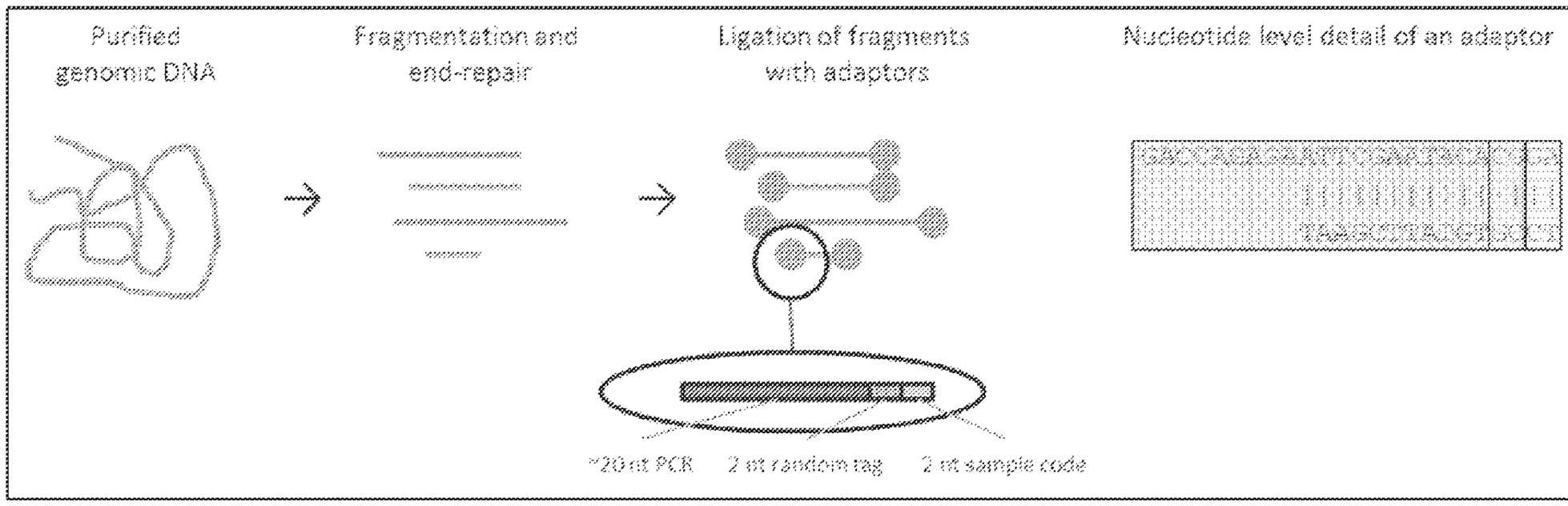
(71) **Demandeur/Applicant:**
RESOLUTION BIOSCIENCE, INC., US

(72) **Inventeurs/Inventors:**
RAYMOND, CHRISTOPHER K., US;
ARMOUR, CHRISTOPHER D., US;
LIM, LEE P., US

(74) **Agent:** BORDEN LADNER GERVAIS LLP

(54) **Titre : PROCEDES POUR ANALYSE GENOMIQUE CIBLEE**
(54) **Title: METHODS FOR TARGETED GENOMIC ANALYSIS**

FIG. 1



(57) **Abrégé/Abstract:**
The invention provides a method for genetic analysis in individuals that reveals both the genetic sequences and chromosomal copy number of targeted and specific genomic loci in a single assay. The present invention further provide methods for the sensitive and specific detection of target gene sequences and gene expression profiles.

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(71) **Applicant**: **CLEARFORK BIOSCIENCE, INC.** [US/US]; 2023 120th Avenue Ne, Bellevue, WA 98005 (US).

(72) **Inventors**: **RAYMOND, Christopher, K.**; 2626 Ne 86th Street, Seattle, WA 98115 (US). **ARMOUR, Christopher, D.**; 2023 120th Avenue Ne, Bellevue, WA 98005 (US). **LIM, Lee, P.**; 9520 Ne 120th Street, Apt. A304, Kirkland, WA 98034 (US).

(74) **Agents**: **MCDONALD, Michael, J.** et al.; Cooley LLP, 1299 Pennsylvania Avenue, Nw, Suite 700, Washington, DC 20004 (US).

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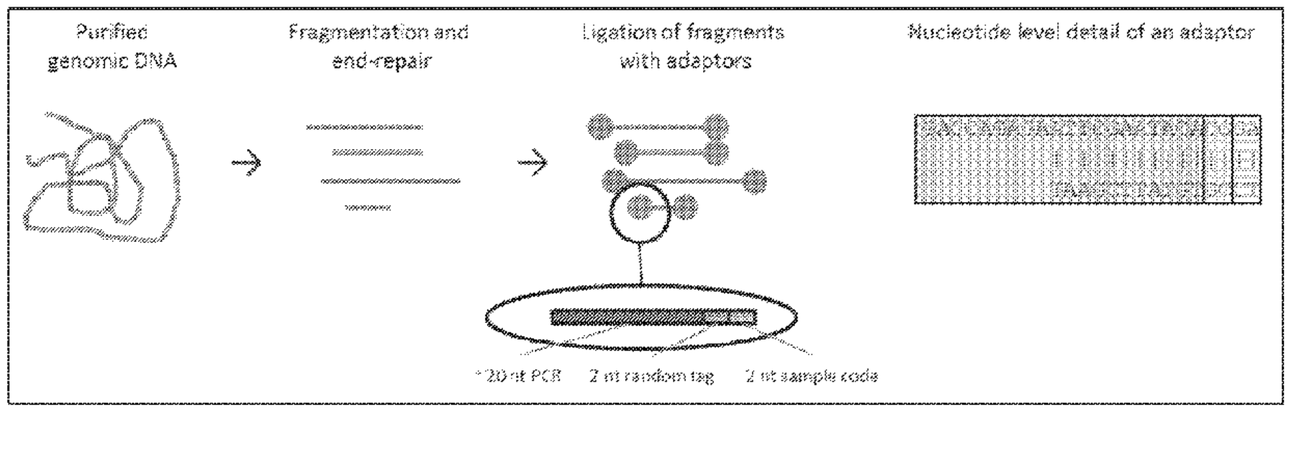
(54) Title: METHODS FOR TARGETED GENOMIC ANALYSIS

FIG. 1



(57) Abstract: The invention provides a method for genetic analysis in individuals that reveals both the genetic sequences and chromosomal copy number of targeted and specific genomic loci in a single assay. The present invention further provide methods for the sensitive and specific detection of target gene sequences and gene expression profiles.

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METHODS FOR TARGETED GENOMIC ANALYSIS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/794,049, filed March 15, 2013, and U.S. Provisional Application No. 61/735,417, filed December 10, 2012, which is incorporated by reference in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is CLFK_001_02WO_ST25.txt. The text file is 188 KB, was created on December 10, 2013, and is being submitted electronically via EFS-Web.

BACKGROUND

Technical Field

The invention relates generally to a method for genetic analysis in individuals that reveals both the genetic sequences and chromosomal copy number of targeted and specific genomic loci in a single assay. In particular, the present invention relates to methods that provide sensitive and specific detection of target gene sequences or gene transcripts and methods that reveal both variant sequences and overall gene copy number in a single assay.

Description of the Related Art

Both complete human genome sequences of individual human subjects and partial genome resequencing studies have revealed the basic theme that all humans appear to possess less than perfect genomes. In particular, normal healthy human subjects are found to harbor hundreds if not thousands of genetic lesions within their genome sequences. Many of these lesions are known or predicted to eliminate the

function of the gene in which they reside. The implication is that while normal diploid humans possess two functional copies of most genes, there are many instances in all humans where only one (or zero) functional gene copies are present. Similarly, instances where genes are overrepresented by gene duplication/amplification events are also encountered with significant frequency.

One of the key features in biological networks is functional redundancy. Normal, healthy individuals can tolerate the average load of genetic lesions because they possess, on average, two copies of every gene such that loss of one copy is inconsequential. Moreover, sets of genes often perform similar functions such that minor perturbations in specific gene functions are generally compensated for within a larger network of functional elements. While functional compensation in biological systems is a general theme, there are many instances in which specific gene loss can trigger acute disruptive events. By way of example, cancers appear to be the consequence of genetic disease in which the compound effect of multiple individual lesions is uncontrolled cell proliferation. Similarly, prescribed medicines are often specific chemical entities that are transported, metabolized and/or eliminated by very specific genes. Perturbations in these genes, while generally inconsequential under normal circumstances, can manifest as adverse events (*e.g.*, side effects) during chemical therapy.

The central aim of "personalized medicine", increasingly referred to as "precision medicine," is to merge genetic information that is specific to the patient with treatment options that are compatible with the individual's genetic profile. However, the vast potential of personalized medicine has yet to be realized. To realize this goal, there must be clinically acceptable, robust genetic diagnostic tests that can reliably determine the genetic status of relevant genes.

BRIEF SUMMARY

Particular embodiments contemplated herein provide a method for generating a tagged DNA library comprising treating fragmented DNA with end-repair enzymes to generate fragmented end-repaired DNA; and ligating a random nucleic acid tag

sequence, and optionally a sample code sequence and/or a PCR primer sequence to the fragmented end-repaired DNA to generate the tagged DNA library.

In particular embodiments, the random nucleic acid tag sequence is from about 2 to about 100 nucleotides. In some embodiments, the present invention provides that the random nucleic acid tag sequence is from about 2 to about 8 nucleotides.

In certain embodiments, the fragmented end-repaired DNA contains blunt ends. In some embodiments, the blunt ends are further modified to contain a single base pair overhang.

In certain embodiments, the ligating comprises ligating a multifunctional adaptor module to the fragmented end-repaired DNA to generate the tagged DNA library, wherein the multifunctional adaptor molecule comprises: i) a first region comprising a random nucleic acid tag sequence; ii) a second region comprising a sample code sequence; and iii) a third region comprising a PCR primer sequence.

In additional embodiments, the method further comprises hybridizing a tagged DNA library with at least one multifunctional capture probe module to form a complex, wherein the multifunctional capture probe module hybridizes to a specific target region in the DNA library.

In further embodiments, the method further comprises isolating the tagged DNA library-multifunctional capture probe module complex.

In some embodiments, the method further comprises 3′-5′ exonuclease enzymatic processing of the isolated tagged DNA library-multifunctional capture probe module complex to remove the single stranded 3′ ends. In some embodiments, the enzyme for use in the 3′-5′ exonuclease enzymatic processing is T4 polymerase.

In particular embodiments, the method further comprises 5’-3’ DNA polymerase extension of the isolated tagged DNA library-multifunctional capture probe module complex from the 3’ end of the multifunctional capture probe utilizing the isolated tagged DNA library fragments as template.

In certain embodiments, the method further comprises joining of the multifunctional capture probe and isolated tagged DNA library fragments through the concerted action of a 5’ FLAP endonuclease, DNA polymerization and nick closure by a DNA ligase.

In further embodiments, the method further comprises performing PCR on the 3′-5′ exonuclease enzymatically processed complex, wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence.

In various embodiments, a method for targeted genetic analysis is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged DNA library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; d) performing PCR on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

In various particular embodiments, a method for targeted genetic analysis is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged genomic library-multifunctional capture probe module complex from a); c) performing 5′-3′ DNA polymerase extension of multifunctional capture probe utilizing the isolated tagged DNA library fragments as template; d) performing PCR on the enzymatically processed complex from c) wherein the complement of the isolated target region is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the complement of the DNA target region, the target-specific region of the multifunctional capture probe and the multifunctional capture

probe module tail sequence; and e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

In various certain embodiments, a method for targeted genetic analysis is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing the creation of a hybrid multifunctional capture probe-isolated tagged DNA target molecule through the concerted action of a 5' FLAP endonuclease, DNA polymerization and nick closure by a DNA ligase; d) performing PCR on the enzymatically processed complex from c) wherein the of the multifunctional capture probe molecule is joined to the isolated tagged DNA target clone in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module; and e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

In particular embodiments, a method for determining copy number of a specific target region is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged DNA library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; e) performing PCR amplification of the hybrid nucleic acid in d); and e) quantitating the PCR reaction

in d), wherein the quantitation allows for a determination of copy number of the specific target region.

In certain embodiments, a method for determining copy number of a specific target region is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing 5′-3′ DNA polymerase extension of multifunctional capture probe utilizing the isolated tagged DNA library fragments as template; d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; e) performing PCR amplification of the hybrid nucleic acid in d); and e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific target region.

In further embodiments, a method for determining copy number of a specific target region is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing the creation of a hybrid multifunctional capture probe-isolated tagged DNA target molecule through the concerted action of a 5’ FLAP endonuclease, DNA polymerization and nick closure by a DNA ligase; d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; e) performing PCR amplification

of the hybrid nucleic acid in d); and e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific target region.

In additional embodiments, a method for targeted genetic analysis is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a); c) performing PCR on the complex from b) to replicate a region that is 3′ relative to the sequence of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged DNA library sequence that is located 3′ relative to the multifunctional capture probe; and d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

In particular embodiments, a method for targeted genetic analysis is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the genomic library; b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a); c) performing 5′-3′ DNA polymerase extension of multifunctional capture probe utilizing the isolated tagged DNA library fragments as template, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged DNA library sequence that is located 3′ relative to the multifunctional capture probe; and d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

In certain embodiments, a method for targeted genetic analysis is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a); c) performing the creation of a hybrid multifunctional capture probe-isolated tagged DNA target molecule through the concerted action of a 5’ FLAP endonuclease, DNA

polymerization and nick closure by a DNA ligase, wherein the hybrid nucleic acid molecule comprises the complement of the multifunctional capture probe hybrid module and a region of the tagged DNA library sequence that is located 5′ relative to the multifunctional capture probe; and d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

In particular embodiments, a method for determining copy number of a specific target region is provided comprising: a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a); c) performing PCR on the complex from b) to replicate a region that is 3' relative to sequence of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged DNA library sequence that is located 3' relative to the multifunctional capture probe; d) performing PCR amplification of the hybrid nucleic acid in c); and e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific target region.

In various embodiments, the targeted genetic analysis is sequence analysis.

In particular embodiments, the tagged DNA library is amplified by PCR to generate an amplified tagged DNA library.

In certain embodiments, the DNA is from a biological sample selected from the group consisting of blood, skin, hair, hair follicles, saliva, oral mucous, vaginal mucous, sweat, tears, epithelial tissues, urine, semen, seminal fluid, seminal plasma, prostatic fluid, pre-ejaculatory fluid (Cowper's fluid), excreta, biopsy, ascites, cerebrospinal fluid, lymph, and tissue extract sample or biopsy sample.

In further embodiments, a tagged DNA library comprises tagged DNA sequences, wherein each tagged DNA sequence comprises: i) fragmented end-repaired DNA; ii) a random nucleotide tag sequence; iii) a sample code sequence; and iv) a PCR primer sequence.

In additional embodiments, a hybrid tagged DNA library comprises hybrid tagged DNA sequences for use in targeted genetic analysis, wherein each hybrid tagged DNA sequence comprises: i) fragmented end-repaired DNA; ii) a random nucleotide tag sequence; iii) a sample code sequence; iv) a PCR primer sequence; and v) a multifunctional capture probe module tail sequence.

In further embodiments, a multifunctional adaptor module comprises: i) a first region comprising a random nucleotide tag sequence; ii) a second region comprising a sample code sequence; and iii) a third region comprising a PCR primer sequence.

In particular embodiments, a multifunctional capture probe module comprises: i) a first region capable of hybridizing to a partner oligonucleotide; ii) a second region capable of hybridizing to a specific target region; and iii) a third region comprising a tail sequence. In some embodiments, the first region of the capture probe module is bound to a partner oligonucleotide. In some embodiments, the partner oligonucleotide is chemically modified.

In one embodiment, compositions comprise a tagged DNA library, a multifunctional adaptor module and a multifunctional capture probe module.

In particular embodiments, a composition comprises a hybrid tagged genomic library according to the methods of the present invention.

In certain embodiments, a composition comprises a reaction mixture for performing the methods contemplated herein.

In particular embodiments, a reaction mixture capable of generating a tagged DNA library comprises: a) fragmented DNA and b) DNA end-repair enzymes to generate fragmented end-repaired DNA.

In certain embodiments, a reaction mixture further comprises a multifunctional adaptor module.

In additional embodiments, a reaction mixture further comprises a multifunctional capture probe module.

In some embodiments, a reaction mixture further comprises an enzyme with 3′-5′ exonuclease activity and PCR amplification activity.

In one embodiment, the reaction mixture comprises a FLAP endonuclease, a DNA polymerase, and DNA ligase.

In any of the foregoing embodiments, the DNA can be isolated genomic DNA or cDNA.

In various embodiments, a method for generating a tagged genomic library is provided comprising: treating fragmented genomic DNA with end-repair enzymes to generate fragmented end-repaired genomic DNA; and ligating a random nucleic acid tag sequence, and optionally a sample code sequence and/or a PCR primer sequence to the fragmented end-repaired genomic DNA to generate the tagged genomic library.

In particular embodiments, the random nucleic acid tag sequence is from about 2 to about 100 nucleotides.

In certain embodiments, the random nucleic acid tag sequence is from about 2 to about 8 nucleotides.

In additional embodiments, the fragmented end-repaired genomic DNA contains blunt ends.

In further embodiments, the blunt ends are further modified to contain a single base pair overhang.

In some embodiments, the ligating comprises ligating a multifunctional adaptor module to the fragmented end-repaired genomic DNA to generate the tagged genomic library, wherein the multifunctional adaptor molecule comprises: a first region comprising a random nucleic acid tag sequence; a second region comprising a sample code sequence; and a third region comprising a PCR primer sequence.

In particular embodiments, the methods contemplated herein comprise hybridizing a tagged genomic library with a multifunctional capture probe module to form a complex, wherein the multifunctional capture probe module hybridizes to a specific genomic target region in the genomic library.

In certain particular embodiments, the methods contemplated herein comprise isolating the tagged genomic library-multifunctional capture probe module complex.

In additional particular embodiments, the methods contemplated herein comprise 3′-5′ exonuclease enzymatic processing of the isolated tagged genomic library-multifunctional capture probe module complex to remove the single stranded 3′ ends.

In further particular embodiments, the enzyme for use in the 3′-5′ exonuclease enzymatic processing is T4 DNA polymerase.

In some particular embodiments, the methods contemplated herein comprise performing PCR on the 3′-5′ exonuclease enzymatically processed complex from the preceding claims, wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence.

In various embodiments, a method for targeted genetic analysis is provided comprising: (a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific genomic target region in the genomic library; (b) isolating the tagged genomic library-multifunctional capture probe module complex from a); (c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; (d) performing PCR on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and (e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

In particular embodiments, steps a) through d) are repeated at least about twice and the targeted genetic analysis of e) comprises a sequence alignment of the hybrid nucleic acid molecule sequences obtained from the at least two d) steps.

In further embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In some embodiments, at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

In various embodiments, a method for determining copy number of a specific genomic target region is provided comprising: (a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module complex selectively hybridizes to a specific genomic target region in the genomic library; (b) isolating the tagged genomic library-multifunctional capture probe module complex from a); (c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; (d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; (e) performing PCR amplification of the hybrid nucleic acid molecule in d); and (f) quantitating the PCR reaction in e), wherein the quantitation allows for a determination of copy number of the specific genomic target region.

In some embodiments, the methods contemplated herein comprise obtaining the sequences of the hybrid nucleic acid molecules from step e).

In further embodiments, steps a) through e) are repeated at least about twice and a sequence alignment is performed using the hybrid nucleic acid molecule sequences obtained from the at least two e) steps.

In additional embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In certain embodiments, at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

In various embodiments, a method for determining copy number of a specific genomic target region is provided comprising: (a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module complex selectively hybridizes to a specific genomic target region in the genomic library; (b) isolating the tagged genomic library-multifunctional capture probe module complex from a); (c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; (d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and (e) performing PCR amplification of the hybrid nucleic acid molecule in d).

In certain embodiments, the methods contemplated herein comprise obtaining the sequences of the hybrid nucleic acid molecules from step e).

In particular embodiments, steps a) through e) are repeated at least about twice and a sequence alignment is performed using the hybrid nucleic acid molecule sequences obtained from the at least two e) steps.

In some embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In additional embodiments, at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

In various embodiments, a method for determining copy number of a specific genomic target region is provided comprising: (a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module comprises selectively hybridizes to a specific genomic target region in the genomic library; (b) isolating the tagged genomic library-multifunctional

capture probe module complex from a); (c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; (d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; (e) performing PCR amplification of the hybrid nucleic acid molecule in d); and (f) performing targeted genetic analysis on the hybrid nucleic acid molecule from e).

In particular embodiments, steps a) through e) are repeated at least about twice and the targeted genetic analysis of f) comprises performing a sequence alignment of the hybrid nucleic acid molecule sequences from the at least two e) steps.

In certain embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In additional embodiments, at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

In various embodiments, a method for targeted genetic analysis is provided comprising: (a) hybridizing a tagged genomic library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific genomic target region in the genomic library; (b) isolating the tagged genomic library-multifunctional capture probe hybrid module complex from a); (c) performing 5′ to 3′ DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured, tagged genomic target region that is 3′ of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged genomic target region that is located in the 3′

direction from the location where the multifunctional capture probe hybrid module hybridizes to the genomic target region; and (d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

In further embodiments, steps a) through c) are repeated at least about twice and the targeted genetic analysis of d) comprises a sequence alignment of the hybrid nucleic acid molecule sequences obtained from the at least two d) steps.

In some embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In particular embodiments, at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

In various embodiments, a method for determining copy number of a specific genomic target region is provided comprising: (a) hybridizing a tagged genomic library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific genomic target region in the genomic library; (b) isolating the tagged genomic library-multifunctional capture probe hybrid module complex from a); (c) performing 5' to 3' DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured tagged genomic target region that is 3' of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged genomic target region that is located in the 3'direction from the location where the multifunctional capture probe hybrid module hybridizes to the genomic target region; and (d) performing PCR amplification of the hybrid nucleic acid molecule in c); and (e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific genomic target region.

In particular embodiments, the methods contemplated herein comprise obtaining the sequences of the hybrid nucleic acid molecules from step d).

In certain embodiments, steps a) through d) are repeated at least about twice and a sequence alignment of the hybrid nucleic acid molecules from the at least two d) steps.

In additional embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In further embodiments, at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

In some embodiments, the targeted genetic analysis is sequence analysis.

In particular embodiments, the tagged genomic library is amplified by PCR to generate an amplified tagged genomic library.

In related particular embodiments, the genomic DNA is from a biological sample selected from the group consisting of blood, skin, hair, hair follicles, saliva, oral mucous, vaginal mucous, sweat, tears, epithelial tissues, urine, semen, seminal fluid, seminal plasma, prostatic fluid, pre-ejaculatory fluid (Cowper's fluid), excreta, biopsy, ascites, cerebrospinal fluid, lymph, and tissue extract sample or biopsy sample.

In various embodiments, a tagged genomic library is provided comprising tagged genomic sequences, wherein each tagged genomic sequence comprises: fragmented end-repaired genomic DNA; a random nucleotide tag sequence; a sample code sequence; and a PCR primer sequence.

In various related embodiments, a tagged cDNA library is provided comprising tagged cDNA sequences, wherein each tagged cDNA sequence comprises: fragmented end-repaired cDNA; a random nucleotide tag sequence; a sample code sequence; and a PCR primer sequence.

In various particular embodiments, a hybrid tagged genomic library is provided comprising hybrid tagged genomic sequences for use in targeted genetic analysis, wherein each hybrid tagged genomic sequence comprises: fragmented end-repaired genomic DNA; a random nucleotide tag sequence; a sample code sequence; a PCR primer sequence; a genomic target region; and a multifunctional capture probe module tail sequence.

In various certain embodiments, a hybrid tagged cDNA library is provided comprising hybrid tagged cDNA sequences for use in targeted genetic analysis, wherein each hybrid tagged cDNA sequence comprises: fragmented end-repaired cDNA; a random nucleotide tag sequence; a sample code sequence; a PCR primer sequence; a cDNA target region; and a multifunctional capture probe module tail sequence.

In various certain embodiments, a multifunctional adaptor module is provided comprising: a first region comprising a random nucleotide tag sequence; a second region comprising a sample code sequence; and a third region comprising a PCR primer sequence.

In various additional embodiments, a multifunctional capture probe module is provided comprising: a first region capable of hybridizing to a partner oligonucleotide; a second region capable of hybridizing to a specific genomic target region; and a third region comprising a tail sequence.

In particular embodiments, the first region is bound to a partner oligonucleotide.

In particular embodiments, a multifunctional adaptor probe hybrid module is provided comprising: a first region capable of hybridizing to a partner oligonucleotide and capable of functioning as a PCR primer and a second region capable of hybridizing to a specific genomic target region.

In certain embodiments, the first region is bound to a partner oligonucleotide.

In some embodiments, the partner oligonucleotide is chemically modified.

In further embodiments, a composition comprising a tagged genomic library, a multifunctional adaptor module and a multifunctional capture probe module is provided.

In additional embodiments, a composition comprising a hybrid tagged genomic or c DNA library according to any of the preceding embodiments is provided.

In various embodiments, a reaction mixture for performing a method of any one of the preceding embodiments is provided.

In particular embodiments, a reaction mixture capable of generating a tagged genomic library is provided comprising: fragmented genomic DNA; and DNA end-repair enzymes to generate fragmented end-repaired genomic DNA.

In particular embodiments, a reaction mixture capable of generating a tagged genomic library is provided comprising: fragmented cDNA; and DNA end-repair enzymes to generate fragmented end-repaired cDNA.

In particular embodiments, a reaction mixture comprises a multifunctional adaptor module.

In some embodiments, a reaction mixture comprises a multifunctional capture probe module.

In certain embodiments, a reaction mixture comprises an enzyme with 3′-5′ exonuclease activity and PCR amplification activity.

In various embodiments, a method for DNA sequence analysis is provided comprising: obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence; performing a paired end sequencing reaction on the one or more clones and obtaining one or more sequencing reads; and ordering or clustering the sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

In particular embodiments, a method for DNA sequence analysis is provided comprising: obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence; performing a sequencing reaction on the one or more clones in which a single long sequencing read of greater than about 100 nucleotides is obtained, wherein the read is sufficient to identify both the first DNA sequence and the second DNA sequence; and ordering or clustering sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

In certain embodiments, the sequences of the one or more clones are compared to one or more human reference DNA sequences.

In additional embodiments, sequences that do not match the one or more human reference DNA sequences are identified.

In further embodiments, non-matching sequences are used to create a de novo assembly from the non-matching sequence data.

In some embodiments, the de novo assemblies are used to identify novel sequence rearrangements associated with the capture probe.

In various embodiments, a method for genomic copy number determination analysis is provided comprising: obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises a random nucleotide tag sequence and a targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence; performing a paired end sequencing reaction on the one or more clones and obtaining one or more sequencing reads; and ordering or clustering the sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

In some embodiments, a method for genomic copy number determination analysis is provided comprising: obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises a random nucleotide tag sequence and a targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence; performing a sequencing reaction on the one or more clones in which a single long sequencing read of greater than about 100 nucleotides is obtained, wherein the read is sufficient to identify both the first DNA sequence and the second DNA sequence; and ordering or clustering sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

In certain embodiments, the random nucleotide tag sequence is about 2 to about 50 nucleotides in length.

In further embodiments, the methods contemplated herein comprise analyzing all sequencing reads associated with a second read sequence by: determining the distributions of unique and redundant sequencing reads; counting the number of times a unique read is encountered; fitting a frequency distribution of the unique reads to a statistical distribution; inferring a total number of unique reads; and normalizing the total number of inferred unique read to an assumption that most human genetic loci are generally diploid.

In additional embodiments, an inferred copy number of one or more targeted loci are determined.

In some embodiments, the one or more target loci that deviate from an expected copy number value are determined.

In further embodiments, the one or more targeted loci of a gene are grouped together in a collection of loci and the copy number measurements from the collection of targeted loci are averaged and normalized.

In additional embodiments, the inferred copy number of a gene is represented by the normalized average of all the target loci representing that gene.

In certain embodiments, a method for generating a tagged RNA expression library is provided comprising: fragmenting a cDNA library; treating the fragmented cDNA library with end-repair enzymes to generate fragmented end-repaired cDNA; and ligating a multifunctional adapter molecule to the fragmented end-repaired c DNA to generate a tagged RNA expression library.

In particular embodiments, a method for generating a tagged RNA expression library is provided comprising: preparing a cDNA library from the total RNA of one or more cells; fragmenting the cDNA library; treating the fragmented cDNA with end-repair enzymes to generate fragmented end-repaired cDNA; and ligating a multifunctional adapter molecule to the fragmented end-repaired c DNA to generate a tagged RNA expression library.

In various embodiments, the cDNA library is an oligo-dT primed cDNA library.

In particular embodiments, the cDNA library is primed by random oligonucleotides comprising about 6 to about 20 random nucleotides.

In certain embodiments, the cDNA library is primed by random hexamers or random octamers.

In additional embodiments, the cDNA library is fragmented to a size of about 250 bp to about 750 bp.

In further embodiments, the cDNA library is fragmented to a size of about 500 bp.

In some embodiments, the multifunctional adaptor module comprises: a first region comprising a random nucleic acid tag sequence, and optionally; a second region

comprising a sample code sequence, and optionally a third region comprising a PCR primer sequence.

In related embodiments, the multifunctional adaptor module comprises a first region comprising a random nucleic acid tag sequence, a second region comprising a sample code sequence, and a third region comprising a PCR primer sequence.

In various embodiments, the methods contemplated herein comprise hybridizing a tagged cDNA library with a multifunctional capture probe module to form a complex, wherein the multifunctional capture probe module hybridizes to a specific target region in the cDNA library.

In some embodiments, the methods contemplated herein comprise isolating the tagged cDNA library-multifunctional capture probe module complex.

In particular embodiments, the methods contemplated herein comprise 3′-5′ exonuclease enzymatic processing of the isolated tagged cDNA library-multifunctional capture probe module complex to remove the single stranded 3′ ends.

In some embodiments, the enzyme for use in the 3′-5′ exonuclease enzymatic processing is T4 DNA polymerase.

In certain embodiments, the methods contemplated herein comprise performing PCR on the 3′-5′ exonuclease enzymatically processed complex, wherein a tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the cDNA target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence.

In further embodiments, a method for targeted gene expression analysis is provided comprising: (a) hybridizing a tagged RNA expression library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the tagged RNA expression library; (b) isolating the tagged RNA expression library-multifunctional capture probe module complex from a); (c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged RNA expression library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; (d) performing PCR on the enzymatically processed complex

from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and (e) performing targeted gene expression analysis on the hybrid nucleic acid molecule from d).

In additional embodiments, a method for targeted gene expression analysis is provided comprising: (a) hybridizing a tagged RNA expression library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the RNA expression library; (b) isolating the tagged RNA expression library-multifunctional capture probe hybrid module complex from a); (c) performing 5' to 3' DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured, tagged target region that is 3' of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of the tagged target region that is located in the 3'direction from the location where the multifunctional capture probe hybrid module hybridizes to the target region; and (d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

In various embodiments, a method for targeted gene expression analysis is provided comprising: (a) hybridizing a tagged cDNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the cDNA library; (b) isolating the tagged cDNA library-multifunctional capture probe hybrid module complex from a); (c) performing 5' to 3' DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured, tagged target region in the cDNA library that is 3' of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of the tagged target region in the cDNA library that is located in the

3'direction from the location where the multifunctional capture probe hybrid module hybridizes to the target region; and (d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

In particular embodiments, at least two different multifunctional capture probe modules are used in the at least two (a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In certain embodiments, at least one multifunctional capture probe module hybridizes downstream of the target region and at least one multifunctional capture probe module hybridizes upstream of the target region.

In additional embodiments, a method for cDNA sequence analysis is provided comprising: (a) obtaining one or more clones, each clone comprising a first cDNA sequence and a second cDNA sequence, wherein the first cDNA sequence comprises targeted genomic cDNA sequence and the second cDNA sequence comprises a capture probe sequence; (b) performing a paired end sequencing reaction on the one or more clones and obtaining one or more sequencing reads; and (c) ordering or clustering the sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

In various embodiments, a method for cDNA sequence analysis is provided comprising: (a) obtaining one or more clones, each clone comprising a first cDNA sequence and a second cDNA sequence, wherein the first cDNA sequence comprises targeted genomic DNA sequence and the second cDNA sequence comprises a capture probe sequence; (b) performing a sequencing reaction on the one or more clones in which a single long sequencing read of greater than about 100 nucleotides is obtained, wherein the read is sufficient to identify both the first cDNA sequence and the second cDNA sequence; and (c) ordering or clustering sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

In particular embodiments, the methods contemplated herein comprise analyzing all sequencing reads associated with a second read sequence by: determining the distributions of unique and redundant sequencing reads; counting the number of times a unique read is encountered; fitting a frequency distribution of the unique reads to a statistical distribution; inferring a total number of unique reads; and converting

unique read counts into transcript abundance using normalization to the total reads collected within each cDNA library sample.

In certain embodiments, a method for targeted genetic analysis is provided comprising: (a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the DNA library; (b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a); (c) performing a concerted enzymatic processing of the tagged DNA library-multifunctional capture probe hybrid module complex from b) that includes 5′ FLAP endonuclease activity, 5′ to 3′ DNA polymerase extension, and nick closure by a DNA ligase to join the complement of the multifunctional capture probe to the target region that is 5′ of the multifunctional capture probe binding site in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the complement of the multifunctional capture probe hybrid module and a region of the tagged target region that is located 5′ of the location where the multifunctional capture probe hybrid module hybridizes to the genomic target region; and (d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

In various embodiments, steps a) through c) are repeated at least about twice and the targeted genetic analysis of d) comprises a sequence alignment of the hybrid nucleic acid molecule sequences obtained from the at least two d) steps.

In certain embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In particular embodiments, at least one multifunctional capture probe module hybridizes downstream of the target region and at least one multifunctional capture probe module hybridizes upstream of the target region.

In additional embodiments, a method for determining copy number of a specific target region is provided comprising: (a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the genomic library; (b) isolating the tagged DNA library-multifunctional capture probe

hybrid module complex from a); (c) performing a concerted enzymatic processing of the tagged DNA library-multifunctional capture probe hybrid module complex from b) that includes 5′ FLAP endonuclease activity, 5′ to 3′ DNA polymerase extension, and nick closure by a DNA ligase to join the complement of the multifunctional capture probe to the target region that is 5′ of the multifunctional capture probe binding site in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the complement of the multifunctional capture probe hybrid module and a region of the tagged target region that is located 5′ of the location where the multifunctional capture probe hybrid module hybridizes to the target region; and (d) performing PCR amplification of the hybrid nucleic acid molecule in c); and (e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific target region.

In various embodiments, the methods contemplated herein comprise obtaining the sequences of the hybrid nucleic acid molecules from step d).

In particular embodiments, steps a) through d) are repeated at least about twice and a sequence alignment of the hybrid nucleic acid molecules from the at least two d) steps.

In particular embodiments, at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

In certain embodiments, at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

In additional embodiments, the targeted genetic analysis is sequence analysis.

In further embodiments, the target region is a genomic target region and the DNA library is a genomic DNA library.

In some embodiments, the target region is a cDNA target region and the DNA library is a cDNA library.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1: Construction of an amplifiable, sample coded, tagged genomic DNA library. Purified genomic DNA was isolated from a source such as whole blood or a buccal cheek swab. The DNA was fragmented (e.g. by mechanical, enzymatic or chemical means) and the ends of the DNA were repaired, in this example, to blunt ends. The repaired DNA was ligated to a multifunctional adaptor module that contained a universal amplification sequence, a random nucleotide tag sequence and a sample code sequence. A specific example of a typical adaptor duplex molecule is shown by way of example.

Figure 2: Genomic capture probe design. (A) Structure of a prototypical 114 nt probe. Region 1 is a 34 nt sequence that shares perfect complementarity to a highly modified, 34 nt partner oligonucleotide. The partner oligonucleotide was modified on its 5′ end with a biotin – TEG chemical entity that enabled capture on streptavidin-coated magnetic beads. B stands for the "Bio-TEG" modification. Region 2 is the 60 nt probe region designed to interact with genomic DNA targets. Region 3 is a 20 nt tail that introduced PCR amplification sequences into the captured genomic fragments. (B) An example of the highly modified partner strand that is complementary to region 1 of each individual probe.

Figure 3: Sequence "spreading" in conventional, hybridization-based capture experiments. (A) Flanking fragments can "hitch-hike" into capture libraries by way of legitimate hybridization triplexes involving probe-fragment-flanking fragment interactions. (B) The net result of cross-fragment hybridization is sequence "spreading", meaning sequences that are within proximity to the target region (dashed lines) but fall outside the boundaries of the desired target.

Figure 4: Enzymatic processing of fragment: probe hybridized complexes. (A) The purified complex of fragment (light gray) and probe (black) prior to processing. B – biotin affinity modification. (B) DNA polymerase (e.g. T4 DNA polymerase) encoded 3′ → 5′ exonuclease activity removes the 3′ segment of the captured fragment. (C) Upon encountering the probe: fragment duplex region, the polymerase copies the probe tail segment onto the hybridized genomic fragment. (D) The final modified

fragment in which the tail segment of the multifunctional capture probe hybrid module has been copied onto the target genomic fragment.

Figure 5: Enzymatic processing of capture complexes "focuses" sequencing reads on the target region. (A) Enzymatically processed fragment shown in relation to the capture probe. The orientation of the sequencing read is specified by the probe and is denoted by the thin black arrow. (B) Hypothetical trace of focused reads where "spreading" is minimized.

Figure 6: The "focusing effect" of directional probes. (A) Typical exons average 100 – 150 bp. Directional capture probes are positioned in intronic region flanking the target segment. (B) The sequence read distribution for each individual probe is shown by the dashed line. The additive coverage is shown by the solid line. The directional nature of coverage can be used, as shown in this example, to obtain sharp focus on query regions.

Figure 7: Demonstration of adaptor-dimer-free fragment libraries with "tunable, on/off" amplification properties. The exact same gel image is shown in four different color and contrast schemes. The samples were: (1) no insert, adaptor-only ligation amplified with ACA2 20; (2) no insert, adaptor-only ligation amplified with ACA2 (normal 25 nt PCR primer); (3) no insert, adaptor-only ligation amplified with ACA2 FLFP (full length forward primer); (4) 20 ng of ~200 bp hgDNA insert + adaptor ligation amplified with ACA2 20; (5) 20 ng of ~200 bp hgDNA insert + adaptor ligation amplified with ACA2 (normal 25 nt PCR primer); and (6) 20 ng of ~200 bp hgDNA insert + adaptor ligation amplified with ACA2 FLFP (full length forward primer). No amplified material was visible in the adaptor alone ligation → PCR products (lanes 1-3). The shorter, 20 nt ACA2 primer showed inefficient amplification (lane 4) relative to the "normal", 25 nt ACA2 primer (lane 5). Only the faintest trace of material was visible with the 58 nt ACA2 FLFP primer (lane 6).

Figure 8: Even distribution of average fragment size following Covaris fragmentation of gDNA. Male (M) and female (F) human gDNA (received from Promega Corporation, Madison, WI, USA) was sheared via Covaris conditions, and 2µl (~120ng) or 5µl (~300ng) of the pre-fragmented (U) or the post-fragmented (C)

samples were loaded on a 2% agarose gel. The average fragment size was an even distribution centered around 200bp.

Figure 9: Amplification traces for proteolipid protein 1 (PLP1) qPCR assay 6 demonstrating expected copy number differences between male and female samples. Triplicate male or female genomic DNA templates were amplified by real-time PCR on the Illumina Eco instrument using PLP1 qPCR assay 6. Amplification traces clearly demonstrated copy number differences between the female and male samples.

Figure 10: Demonstration of expected amplicon size and uniqueness following conventional PCR with PLP1 qPCR assay primers. Male or female genomic DNA templates were amplified by conventional PCR using qPCR assay primer sets 1-8 (Example 3) and the unpurified PCR reactions were loaded directly on 2% agarose gels. The upper bands of each doublet were consistent with the projected mobility of the assay PCR products. The lower "fuzzy" material was most likely unused PCR primers.

Figure 11: Analysis of PLP1 qPCR assay performance using ABI 2x SYBR mix and conditions. DNA fragments from genomic library I (constructed in Example 4) were used as template to measure the performance of the PLP1 qPCR assays when set up at room temperature using ABI 2x SYBR master mix in a 2 step PCR reaction. No template control traces (A) and +gDNA traces (B) are shown to provide a qualitative picture of assay performance.

Figure 12: Reduced insert size following post-capture processing with T4-DNA polymerase. Four samples of adapter ligated gDNA fragments from genomic library I (constructed in Example 4) were captured as described (Example 6 - PLP1 EXON 2). Two of these samples utilized universal binding oligo C1, while the other two samples were bound with oligo C10. Samples were then either treated with T4-DNA polymerase (T4 processed) or processed similarly in a reaction solution lacking the T4 polymerase (untreated). Post-capture processing with T4-polymerase induced an overall reduction in the size distribution of the sample, suggesting a reduction in the average size of insert. Additionally, T4 processing resulted in the appearance of two faint bands (~250 bp and ~175bp).

Figure 13: Direct measurement of post-capture processing sensitivity. First, PLP1 exon 2 specific genomic DNA fragments were isolated by pull down/pull out

from the female gDNA library (Example 1) using single PLP1 capture probes in independent reactions. Captured material was quantified using an adjacent PLP1 qPCR assay primer pair, as illustrated in (A). Following enzymatic processing the amount of processed complex was measured again by qPCR using one PLP1 specific primer and one probe-specific primer, as show in (B). The ratio of the measurements in [B/A x 100%] will yield an estimate of processing efficiency. The PCR products from real-time reactions were extracted and subjected to gel analysis to verify that amplicons of the expected length were produced (C). This was possible since both PCR reactions had discrete start and stop points. Processing efficiency was inferred from pull-outs that yielded interpretable data from A + B + C.

Figure 14: Gel analysis of qPCR products from real-time quantification of pre- and post-processed PLP1 exon 2 captured DNA fragments. Six independent capture reactions (two with probe #1, two with probe #4, one with probe #2, and one with probe #3) were processed as described in Figure 16. The probes were from the B10 universal oligo set (Example 4) and comprised universal oligo and probe ultramer. Under these conditions, assay sets 3 (probe 4), 5 (probe 2) and 6 (probe 3) yielded PCR products consistent with the assay amplicon (top gel) or the post-processed PLP1 to adaptor amplicons (bottom gel), while no detectable products were observed in the other assay sets.

Figure 15: Alternative enzymatic processing of fragment: probe hybridized complexes. In contrast to the method outlined in Figure 4, this alternative approach shifted from having the clone copy the probe to having the probe copy the clone. This reversal in polarity means that the 5′ end of the probe was used as both the pull-down sequence and the reverse PCR sequence. The 3′ end of the probe was left unmodified thus it was able to copy the clone using DNA polymerase; 5′-3′ DNA polymerase extended the multifunctional capture probe utilizing the isolated tagged DNA library fragments as template.

Figure 16: Experimental design employed to test the alternative enzymatic processing concept. Four well behaved qPCR assays, (10, 14, 15 and 16) were matched with probes that "pointed" at those assays. Importantly, although the target sequences of the probe and qPCR assays were directed to regions within proximity to one another,

they did not overlap. Thus the effect of the processing could be directly tested using these well-established assay sets.

Figure 17: Decrease in the average insert size of the library induced by the alternative processing method. DNA fragments were hybridized to capture probes/ultramers, and captured on streptavidin beads as previously described (Figure 12). Post-capture processing was performed using the alternative method described in Figure 17, and input samples as well as pre- and post-processed samples were analyzed by 2% agarose gel electrophoresis. As was expected, the average insert size of the library was decreased in the processed samples, thus supporting the conclusion that the processing worked. Collapsing of the library into an apparent band at the bottom of the processed sample was also observed, indicating some priming off of probe may have occurred

Figure 18: Enhanced focusing of target sequences by the alternative processing method. The sequence reads obtained in Example 13 were displayed in the UCSC Genome Browser to assess the coverage and distribution of captured fragments within specific target sites. The density of sequence reads corresponding to 'capture only' and 'processed' libraries are shown (black) for two target regions on the X chromosome, one corresponding to exons of the PLP1 gene (A and B) and the other an intronic segment of the ZNF630 gene (C and D). Reads from libraries generated by the alternative processing method (B and D) are more highly concentrated in target sites than libraries constructed by capture alone (A and C). Capture probe binding sites are shown in red. Each track is scaled to the maximal read density values (y-axis) observed for a given stretch of genomic coordinates (x-axis).

Figure 19: Diagram of vertical alignment. All next generation sequence (NGS) analysis starts with alignment to a reference genome. (A) Initial read alignment is based on configurable word string searches that can accommodate single nucleotide variants (SNVs) and, to a limited extent, insertions/deletions. (B) The collective set of aligned reads is analyzed for SNVs. In the example shown, the candidate SNV was observed twice, but the read coordinates for these two reads was identical. The vertical alignment paradigm generates a large number of SNVs and/or insertions/deletion hypotheses that require orthogonal validation.

Figure 20: Diagram of data analysis scheme. Step one is to match reads to probes. Step two will be to analyze the sequence information connected "horizontally" to each probe.

Figure 21: Diagram of horizontal alignment "forces" reads associated with probe 1 and probe 2. Assembly will generate two contigs, one with a wild-type exon structure and one with an insertion structure. Two important principles emerge: 1) Overlapping reads from adjacent probes will support or refute the hypothesis of an indel-containing allele of the captured exon and 2) micro-CNV alleles outside of capture probes are readily detectable by horizontal methodologies.

Figure 22: Diagram of "low confidence" SNV calls. Candidate nucleotide variants can be actual events harbored in the individual from whom the sample was collected and processed, but they can also be artifacts introduced during processing and sequencing of samples. The methods described here were designed to differentiate between actual, "high confidence" variant calls and artifactual "low confidence" variant calls. Sequencing reads covering a target region are collected from multiple different clones in both possible sequencing orientations, and each read is annotated with tag information. The tags allow reads derived from the same cloning event to be identified and grouped. SNVs and indels that arise within only one set of clones that are all derived from the same cloning event are low confidence calls that are discarded from further analysis.

Figure 23: Diagram of "high confidence" SNV calls. Candidate nucleotide variants can be actual events harbored in the individual from whom the sample was collected and processed, but they can also be artifacts introduced during processing and sequencing of samples. The methods described here were designed to differentiate between actual, "high confidence" variant calls and artifactual "low confidence" variant calls. Sequencing reads covering a target region are collected from multiple different clones in both possible sequencing orientations, and each read is annotated with tag information. The tags allow reads derived from distinct cloning events to be identified. Examples shown are (A) reads with the same start point but different sequence labels, (B) reads in the same orientation that have different start points and different labels and (C) reads in opposite orientations. In all these instances, the occurrence and detect of a

variant in independent cloning events marks that variant with high confidence, and such variants are followed up with further, orthogonal validation methods.

Figure 24: Molecularly annotated sequencing read. (A) Forward flow cell (Illumina chemistry) graft sequences and sequencing primer binding site. (B) Reverse flow cell graft sequence and reverse sequencing primer annealing site. (1) Sequence label. (2) Sample label. (3) Forward read start site. (4) Sequence of genomic fragment. (5) Genomic index (probe sequence). The combination of (1) + (3) constitutes the unique read tag that is critical for both variant calling and copy number determination.

Figure 25: The most significant classes of DNA sequence variants (insertions, deletions, runs of point mutations and/or translocations) are also the most difficult to detect by alignment based methods.

Figure 26: Dual probe interrogation of target regions (e. g. exons). (A) Typical exons average 100 – 150 bp. Capture probes are positioned in the intronic region flanking the target segment. These probes have opposite sequence polarity (one queries the "+" stand, the other the "-" strand. (B) The sequence read distribution for each individual probe is indicated by the shaded areas and the read orientations are specified by arrows. Key aspects are that target regions are sequenced by multiple reads in both orientations. Moreover, each probe captures reads that sequence the adjacent probe binding sites. This arrangement is one element that increases the confidence of variant calls.

Figure 27: The role of sequence tags in variant calling. Sequence "tags" are comprised of a nucleotide code (ovals; in the case of Clearfork a collection of 16 possible three nucleotide sequences) and a ragged, arbitrary clone fragment end sequence. (A) A false-positive variant call is one in which a variant is identified among a collection of sibling sequences that all bear identical sequence tags. (B) A high confidence variant call is found among a collection of sequences that have different sequence tags.

Figure 28: Copy determination using read observation statistics.

Figure 29: Molecularly annotated sequencing read. (A) Forward flow cell (Illumina SBS chemistry) graft sequences and sequencing primer binding site. (B)

Reverse flow cell graft sequence and reverse sequencing primer annealing site. (1) Sequence label. (2) Sample label. (3) Forward read start site. (4) Sequence of genomic fragment. (5) Genomic index (probe sequence). (6) Capture label. The combination of (1) + (3) constitutes the unique read tag that is critical for copy number determination. The combination of (5) + (6) constitutes a genomic index tag that can be used to monitor and quantify capture events. The forward sequencing read 1 that determines the sequences of annotation elements 1, 2, 3, and 4, and paired-end reverse read 2 that determines the sequences of annotation elements 5 and 6 are indicated.

Figure 30: Probes (*e.g.*, multifunctional capture probes) are generally directional, meaning they capture sequences on one side (generally the 3′3′ side) of their position. Tail sequences that add additional functionality (*e.g.*, PCR primer binding site, binding site for partner oligo that enables biotin pull-out, etc) are added in addition to the core targeting 60-mer. Sixty nucleotide targeting sequences are chosen with the following constraints and criterion: (1) The probe is positioned from -100 to +50 nt relative to the start of the target sequence. In the illustration at right, the "start" of the target sequence is the intron:exon junction; (2) Probes are designed with redundancy, as illustrated, such that the resulting sequences from a pair of probes are overlapping in opposite orientations; (3) Probes are selected (where possible) to possess GC content not less than 33% (>20 G's or C's per 60 mer) and not more than 67% (<40 G's or C's per 60 mer); (4) Probes are selected to avoid repeats wherever possible. This is done with the help of REPEATMASKER and/or unique alignability criterion, both of which can be viewed on the UCSC genome browser; (5) In cases the position requirement, GC requirement, and the uniqueness requirement cannot be met, selection rules are relaxed in the following order (GC >position>uniqueness). In other words, GC and positioning are flexible; the uniqueness criterion is not.

Figure 31. Processing to create targeted genomic sequencing libraries. (A) the initial capture complex comprises a "standard" tagged genomic library fragment, a tailed capture probe that targets a genomic "target region" that is 5′ to the probe, and a biotinylated partner Oligonucleotide that is common to all probes. (B) Processing of the complex into a sequence-ready clone comprises 3 steps: (1) the 5′ FLAP endonuclease of DNA polymerase holoenzyme (*e.g.,* full length *Bst* polymerase) clips

the 5′ tail of the genomic clone; (2) the polymerase extends the partner oligo sequence by polymerization (can occur concurrently with step 1); and (3) *Taq* ligase repairs the nick between the partner oligo and the genomic fragment. These concerted steps create a sequence-ready clone.

Figure 32 shows post-capture/processing of PCR products. Lane 1 is ACA2 single primer amplified, unprocessed capture complex. Lanes 2-4 were amplified with AF+CR dual PCR primers.

Figures 33-35 show a library free method for generating tagged genomic DNAs and related capture, processing, and analysis methods.

Figure 36 shows a suppressive PCR strategy for avoiding primer-dimer artifacts in library free methods for generating tagged genomic DNAs.

Figure 37 shows gel electrophoresis results of raw and sonicated gDNAs used in library free methods for generating tagged genomic DNAs.

Figure 38 shows a qPCR amplification plot of four gDNA samples prepared by the library free method.

Figure 39 shows the gel electrophoresis results from raw PCR products amplified from samples prepared by the library free methods.

Figure 40 shows the gel electrophoresis results from bead-cleaned PCR products amplified from samples prepared by the library free methods.

Figure 41 shows a qPCR amplification plot of samples prepared by the library free methods using different combinations of enzymes: T4 DNA polymerase (P), T4 DNA ligase (L), and T4 gene 32 protein (32), or no enzyme control.

Figure 42 shows the gel electrophoresis results of PCR amplified (10 cycles or 16 cycles) samples prepared by the library free methods using different combinations of enzymes: T4 DNA polymerase (P), T4 DNA ligase (L), and T4 gene 32 protein (32), or no enzyme control.

Figure 43 shows the gel electrophoresis results of individual samples prepared by the library free methods prior to pooling.

Figure 44 shows CNV for PLP1 in relation to the normalizing autosomal loci KRAS and MYC across samples with variable dosages of the X chromosome. Samples were prepared using library free methods.

Figure 45 shows the DNA sequence start points for chrX region 15 in the XXXX (4X dosage) sample relative to the capture probe sequence. Reads go from left to right and samples were prepared using library free methods.

Figure 46 shows the gel electrophoresis results of cDNA prepared from RNA samples using the RNA –seq methods contemplated herein, captured cDNAs, and cDNA preps sized with the Pippin automated DNA size selector.

Figure 47 shows the correlations of the gene expression of various transcripts in heart versus liver in libraries prepared using a total RNA versus targeted expression strategy for the library preparation.

Figure 48 shows the correlation of absolute expression levels of various transcript measured in total RNA-seq compared to targeted RNA-seq.

DETAILED DESCRIPTION

A. OVERVIEW

The present invention is based at least in part, on the discovery that the coordinated utilization of several key molecular modules can be employed in performing targeted genetic analyses.

The practice of the invention will employ, unless indicated specifically to the contrary, conventional methods of chemistry, biochemistry, organic chemistry, molecular biology, microbiology, recombinant DNA techniques, genetics, immunology, and cell biology that are within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982); Ausubel *et al.*, *Current Protocols in Molecular Biology* (John Wiley and Sons, updated July 2008); *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience; Glover, *DNA Cloning: A Practical Approach*, vol. I & II (IRL Press, Oxford, 1985); Anand, T*echniques for the Analysis of Complex Genomes*, (Academic Press, New York,

1992); T*ranscription and Translation* (B. Hames & S. Higgins, Eds., 1984); Perbal, *A Practical Guide to Molecular Cloning* (1984); and Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998).

All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety.

B. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred embodiments of compositions, methods and materials are described herein. For the purposes of the present invention, the following terms are defined below.

The articles "a," "an," and "the" are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The use of the alternative (*e.g.*, "or") should be understood to mean either one, both, or any combination thereof of the alternatives.

The term "and/or" should be understood to mean either one, or both of the alternatives.

As used herein, the term "about" or "approximately" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, the term "about" or "approximately" refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length ± 15%, ± 10%, ± 9%, ± 8%, ± 7%, ± 6%, ± 5%, ± 4%, ± 3%, ± 2%, or ± 1% about a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of

a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. In particular embodiments, the terms "include," "has," "contains," and "comprise" are used synonymously.

By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Reference throughout this specification to "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

As used herein, the term "isolated" means material that is substantially or essentially free from components that normally accompany it in its native state. In particular embodiments, the term "obtained" or "derived" is used synonymously with isolated.

As used herein, the term "DNA" refers to deoxyribonucleic acid. In various embodiments, the term DNA refers to genomic DNA, recombinant DNA, synthetic DNA, or cDNA. In one embodiment, DNA refers to genomic DNA or cDNA. In particular embodiments, the DNA comprises a "target region." DNA libraries contemplated herein include genomic DNA libraries and cDNA libraries constructed

from RNA, *e.g.,* an RNA expression library. In various embodiments, the DNA libraries comprise one or more additional DNA sequences and/or tags.

A "target region" refers to a region of interest within a DNA sequence. In various embodiments, targeted genetic analyses are performed on the target region. In particular embodiments, the target region is sequenced or the copy number of the target region is determined.

C. EXEMPLARY EMBODIMENTS

The present invention contemplates, in part, a method for generating a tagged genomic library. In particular embodiments, the method comprises treating fragmented DNA, *e.g.,* genomic DNA or cDNA, with end-repair enzymes to generate fragmented end-repaired DNA followed by ligating a random nucleic acid tag sequence to generate the tagged genomic library. In some embodiments, a sample code sequence and/or a PCR primer sequence are optionally ligated to the fragmented end-repaired DNA.

The present invention contemplates, in part, a method for generating a tagged DNA library. In particular embodiments, the method comprises treating fragmented DNA with end-repair enzymes to generate fragmented end-repaired DNA followed by ligating a random nucleic acid tag sequence to generate the tagged DNA library. In some embodiments, a sample code sequence and/or a PCR primer sequence are optionally ligated to the fragmented end-repaired DNA.

Illustrative methods for fragmenting DNA include, but are not limited to: shearing, sonication, enzymatic digestion; including restriction digests, as well as other methods. In particular embodiments, any method known in the art for fragmenting DNA can be employed with the present invention.

In some embodiments, the fragmented DNA is processed by end-repair enzymes to generate end-repaired DNA. In some embodiments, the end-repair enzymes can yield for example blunt ends, 5′-overhangs, and 3′-overhangs. In some embodiments, the end-repaired DNA contains blunt ends. In some embodiments, the end-repaired DNA is processed to contain blunt ends. In some embodiments, the blunt ends of the end-repaired DNA are further modified to contain a single base pair overhang. In some embodiments, end-repaired DNA containing blunt ends can be further processed to

contain adenine (A)/thymine (T) overhang. In some embodiments, end-repaired DNA containing blunt ends can be further processed to contain adenine (A)/thymine (T) overhang as the single base pair overhang. In some embodiments, the end-repaired DNA has non-templated 3′ overhangs. In some embodiments, the end-repaired DNA is processed to contain 3′-overhangs. In some embodiments, the end-repaired DNA is processed with terminal transferase (TdT) to contain 3′-overhangs. In some embodiments, a G-tail can be added by TdT. In some embodiments, the end-repaired DNA is processed to contain overhang ends using partial digestion with any known restriction enzymes (*e.g.*, with the enzyme Sau3A, and the like.

In particular embodiments, DNA fragments are tagged using one or more "random nucleotide tags" or "random nucleic acid tags." As used herein, the terms "random nucleotide tag" or "random nucleic acid tag" refer to a polynucleotide of discrete length wherein the nucleotide sequence has been randomly generated or selected. In particular illustrative embodiments, the length of the random nucleic acid tag is from about 2 to about 100 nucleotides, from about 2 to about 75 nucleotides, about 2 to about 50 nucleotides, about 2 to about 25 nucleotides, about 2 to about 20 nucleotides, about 2 to about 15 nucleotides, about 2 to about 10 nucleotides, about 2 to about 8 nucleotides, or about 2 to about 6 nucleotides. In certain embodiments, the length of the random nucleotide tag is from about 2 to about 6 nucleotides (*see*, *e.g.*, Figure 1). In one embodiment, the random nucleotide tag sequence is about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 nucleotides.

In particular embodiments, methods known in the art can be employed to add the random nucleotide tags of the present invention to the fragmented DNA. In some embodiments, "tagmentation" can be employed. Tagmentation is a Nextera Technology that is commercially available (from Illumina and Epicenter, USA) and which can be used to load transposon protein complexes with the random nucleotide tags and/or the multifunctional adaptor modules of the invention. The loaded transposon complexes can then be used in creation of the tagged genomic library according to described methods.

DNA for use in the present methods can come from any source known to those of skill in the art. DNA can be collected from any source, synthesized from RNA as

copy DNA (cDNA), and processed into pure or substantially pure DNA for use in the present methods. In some embodiments, the size of the fragmented DNA is in the range of about 2 to about 500 base pairs, about 2 to about 400 base pairs, about 2 to about 300 base pairs, about 2 to about 250 base pairs, about 2 to about 200 base pairs, about 2 to about 100 base pairs or about 2 to about 50 base pairs.

The combination of the introduced "random nucleic acid tag" or "random nucleic acid tags" with the DNA fragment end sequence constitutes a combination of two elements that will hereafter be referred to as "the genomic tag" or the "the cDNA tag." In some embodiments the uniqueness of "the genomic tag" or the "the cDNA tag" can be determined by the combinatorial product of the diversity within the attached random nucleotide tag pool multiplied by the diversity of the DNA fragment end sequence pool.

The present invention also contemplates, in part, a multifunctional adaptor module. As used herein, the term "multifunctional adaptor module" refers to a polynucleotide comprising: (i) a first region comprising a random nucleotide tag sequence; optionally (ii) a second region comprising a sample code sequence; and optionally (iii) a third region comprising a PCR primer sequence. In particular embodiments, a multifunctional adaptor module comprises a PCR primer sequence, a random nucleotide tag, and a sample code sequence. In certain embodiments, a multifunctional adaptor module comprises a PCR primer sequence and a random nucleotide tag or a sample code sequence. In some embodiments, the second region comprising the sample code is optional. In some embodiments, the multifunctional adaptor module does not include a second region, but instead only a first and third region. The multifunctional adaptor module of the present invention can include blunt or complementary ends appropriate for the ligation method employed, including those disclosed elsewhere herein, as well as any others known to those of skill in the art for ligating the multifunctional adaptor module to the fragmented DNA.

In various embodiments, a first region comprises a random nucleotide tag sequence. In particular embodiments, the first region comprises a random nucleotide tag sequence that is from about 2 to about 100 nucleotides, from about 2 to about 75 nucleotides, about 2 to about 50 nucleotides, about 2 to about 25 nucleotides, about 2 to

about 20 nucleotides, about 2 to about 15 nucleotides, about 2 to about 10 nucleotides, about 2 to about 8 nucleotides, or about 2 to about 6 nucleotides, or any intervening number of nucleotides.

In particular embodiments, a second region, when optionally present, comprises a sample code sequence. As used herein, the term "sample code sequence" refers to a polynucleotide that is used to identify the sample. In particular embodiments, the second region comprises a sample code sequence that is from about 1 to about 100 nucleotides, from about 2 to about 75 nucleotides, about 2 to about 50 nucleotides, about 2 to about 25 nucleotides, about 2 to about 20 nucleotides, about 2 to about 15 nucleotides, about 2 to about 10 nucleotides, about 2 to about 8 nucleotides, or about 2 to about 6 nucleotides, or any intervening number of nucleotides.

In certain embodiments, a third region, when optionally present, comprises a PCR primer sequence. In particular embodiments, the third region comprises a PCR primer sequence that is from about 5 to about 200 nucleotides, from about 5 to about 150 nucleotides, from about 10 to about 100 nucleotides from about 10 to about 75 nucleotides, about 10 to about 50 nucleotides, about 10 to about 40 nucleotides, about 20 to about 40 nucleotides, or about 20 to about 30 nucleotides, or any intervening number of nucleotides.

In particular embodiments, a ligation step comprises ligating a multifunctional adaptor module to the fragmented end-repaired DNA. This ligation reaction can be used to generate the tagged DNA library, which comprises end-repaired DNA ligated to a multifunctional adaptor molecule and/or random nucleotide tag. In some embodiments, a single multifunctional adaptor module is employed. In some embodiments, more than one multifunctional adaptor module is employed. In some embodiments, a single multifunctional adaptor module of identical sequence is ligated to each end of the fragmented end-repaired DNA.

The present invention also provides a multifunctional capture probe module. As used herein, the term "multifunctional capture probe module " refers to a polynucleotide comprising: (i) a first region capable of hybridizing to a partner oligonucleotide; (ii) a second region capable of hybridizing to a specific target region; and optionally (iii) a third region comprising a tail sequence.

In one embodiment, a multifunctional capture probe module comprises a region capable of hybridizing to a partner oligonucleotide, a region capable of hybridizing to a DNA target sequence, and a tail sequence.

In one embodiment, a multifunctional capture probe module comprises a region capable of hybridizing to a partner oligonucleotide and a region capable of hybridizing to a genomic target sequence.

In particular embodiments, the multifunctional capture probe module optionally comprises a random nucleotide tag sequence.

In various embodiments, a first region comprises a region capable of hybridizing to a partner oligonucleotide. As used herein, the term "partner oligonucleotide" refers to an oligonucleotide that is complementary to a nucleotide sequence of the multifunctional capture probe module. In particular embodiments, the first region capable of hybridizing to a partner oligonucleotide is a sequence that is from about 20 to about 200 nucleotides, from about 20 to about 150 nucleotides, about 30 to about 100 nucleotides, about 30 to about 75 nucleotides, about 20 to about 50 nucleotides, about 30 to about 45 nucleotides, or about 35 to about 45 nucleotides. In certain embodiments, the region is about 30 to about 50 nucleotides, about 30 to about 40 nucleotides, about 30 to about 35 nucleotides or about 34 nucleotides, or any intervening number of nucleotides.

In particular embodiments, a second region, when optionally present, comprises a region capable of hybridizing to a specific DNA target region. As used herein, the term "DNA target region" refers to a region of the genome or cDNA selected for analyses using the compositions and methods contemplated herein. In particular embodiments, the second region comprises a region capable of hybridizing to a specific target region is a sequence from about 20 to about 200 nucleotides, from about 30 to about 150 nucleotides, about 50 to about 150 nucleotides, about 30 to about 100 nucleotides, about 50 to about 100 nucleotides, about 50 to about 90 nucleotides, about 50 to about 80 nucleotides, about 50 to about 70 nucleotides or about 50 to about 60 nucleotides. In certain embodiments, the second region is about 60 nucleotides, or any intervening number of nucleotides.

In certain embodiments, a third region, when optionally present, comprises a tail sequence. As used herein, the term "tail sequence" refers to a polynucleotide at the 5′ end of the multifunctional capture probe module, which in particular embodiments can serve as a PCR primer binding site. In particular embodiments, the third region comprises a tail sequence that is from about 5 to about 100 nucleotides, about 10 to about 100 nucleotides, about 5 to about 75 nucleotides, about 5 to about 50 nucleotides, about 5 to about 25 nucleotides, or about 5 to about 20 nucleotides. In certain embodiments, the third region is from about 10 to about 50 nucleotides, about 15 to about 40 nucleotides, about 20 to about 30 nucleotides or about 20 nucleotides, or any intervening number of nucleotides.

In one embodiment, a multifunctional capture probe module comprises a region capable of hybridizing to a partner oligonucleotide and a region capable of hybridizing to a genomic target sequence. In particular embodiments, wherein the multifunctional capture probe module comprises a region capable of hybridizing to a partner oligonucleotide and a region capable of hybridizing to a genomic target sequence, the partner oligo may also function as a tail sequence or primer binding site.

In one embodiment, a multifunctional capture probe module comprises a tail region and a region capable of hybridizing to a genomic target sequence.

In various embodiments, the multifunctional capture probe comprises a specific member of a binding pair to enable isolation and/or purification of one or more captured fragments of a tagged DNA library that hybridizes to the multifunctional capture probe. In particular embodiments, the multifunctional capture probe is conjugate to biotin or another suitable hapten, *e.g.,* dinitrophenol, digoxigenin.

The present invention further contemplates, in part, hybridizing a tagged DNA library with a multifunctional capture probe module to form a complex. In some embodiments, the multifunctional capture probe module substantially hybridizes to a specific genomic target region in the DNA library.

Hybridization or hybridizing conditions can include any reaction conditions where two nucleotide sequences form a stable complex; for example, the tagged DNA library and multifunctional capture probe module forming a stable tagged DNA library—multifunctional capture probe module complex. Such reaction conditions are

well known in the art and those of skill in the art will appreciated that such conditions can be modified as appropriate and within the scope of the present invention. Substantial hybridization can occur when the second region of the multifunctional capture probe complex exhibits 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92% 91%, 90%, 89%, 88%, 85%, 80%, 75%, or 70% sequence identity, homology or complementarity to a region of the tagged DNA library.

In particular embodiments, the first region of the multifunctional capture probe module does not substantially hybridize to the region of the tagged DNA library to which the second region does substantially hybridize. In some embodiments, the third region of the multifunctional capture probe module does not substantially hybridize to the region of the tagged DNA library to which the second region of the multifunctional capture probe module does substantially hybridize. In some embodiments, the first and third regions of the multifunctional capture probe module do not substantially hybridize to the region of the tagged DNA library to which the second region of the multifunctional capture probe module does substantially hybridize.

In certain embodiments, the methods contemplated herein comprise isolating a tagged DNA library—multifunctional capture probe module complex. In particular embodiments, methods for isolating DNA complexes are well known to those skilled in the art and any methods deemed appropriate by one of skill in the art can be employed with the methods of the present invention (Ausubel *et al.*, *Current Protocols in Molecular Biology*, 2007-2012). In particular embodiments, the complexes are isolated using biotin—streptavidin isolation techniques. In some embodiments, the partner oligonucleotide capable of hybridizing to the first region of the multifunctional capture probe module is modified to contain a biotin at the 5′- end or 3′-end which is capable of interacting with streptavidin linked to a column, bead or other substrate for use in DNA complex isolation methods.

In particular embodiments, a first region of a multifunctional capture probe module is bound to a partner oligonucleotide. In some embodiments, the multifunctional capture probe module is bound to the partner oligonucleotide prior to formation of a tagged DNA library—multifunctional capture probe module complex. In some embodiments, the multifunctional capture probe module is bound to the partner

oligonucleotide after the formation of a tagged DNA library—multifunctional capture probe module complex. In some embodiments, the multifunctional capture probe module is bound to the partner oligonucleotide simultaneously with the formation of a tagged DNA library—multifunctional capture probe module complex. In some embodiments, the partner oligonucleotide is chemically modified.

In particular embodiments, removal of the single stranded 3′-ends from the isolated tagged DNA library-multifunctional capture probe module complex is contemplated. In certain embodiments, the methods comprise 3′-5′ exonuclease enzymatic processing of the isolated tagged DNA library-multifunctional capture probe module complex to remove the single stranded 3′ ends.

In certain other embodiments, the methods comprise performing 5′-3′ DNA polymerase extension of multifunctional capture probe utilizing the isolated tagged DNA library fragments as template.

In certain other embodiments, the methods comprise creating a hybrid multifunctional capture probe-isolated tagged DNA target molecule through the concerted action of a 5’ FLAP endonuclease, DNA polymerization and nick closure by a DNA ligase.

A variety of enzymes can be employed for the 3′-5′ exonuclease enzymatic processing of the isolated tagged DNA library-multifunctional capture probe module complex. Illustrative examples of suitable enzymes, which exhibit 3′-5′ exonuclease enzymatic activity, that can be employed in particular embodiments include, but are not limited to: T4 or Exonucleases I, III, V (*see also*, Shevelev IV, Hübscher U., “The 3′ 5′ exonucleases,” *Nat Rev Mol Cell Biol*. 3(5):364-76 (2002)). In particular embodiments, the enzyme comprising 3′-5′ exonuclease activity is T4 polymerase. In particular embodiments, an enzyme which exhibits 3′-5′ exonuclease enzymatic activity and is capable of primer template extension can be employed, including for example T4 or Exonucleases I, III, V. *Id.* 3′5′

In some embodiments, the methods contemplated herein comprise performing PCR on the 3′-5′ exonuclease enzymatically processed complex discussed *supra* and elsewhere herein. In particular embodiments, a tail portion of a multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule. In one

embodiment, the hybrid nucleic acid molecule generated comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence.

In various embodiments, a method for targeted genetic analysis is also contemplated. In certain embodiments a method for targeted genetic analysis comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the genomic library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged DNA library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; d) performing PCR on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

In various embodiments, methods for determining copy number of a specific target region are contemplated. In particular embodiments, a method for determining copy number of a specific target region comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module comprises selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged DNA library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends; d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe

module and the complement of the multifunctional capture probe module tail sequence; e) performing PCR amplification of the hybrid nucleic acid in d); and f) quantitating the PCR reaction in e), wherein the quantitation allows for a determination of copy number of the specific target region.

In various embodiments, a method for targeted genetic analysis is also contemplated. In certain embodiments a method for targeted genetic analysis comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the genomic library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing 5′-3′ DNA polymerase extension of multifunctional capture probe utilizing the isolated tagged DNA library fragments as template; d) performing PCR on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

In various embodiments, methods for determining copy number of a specific target region are contemplated. In particular embodiments, a method for determining copy number of a specific target region comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module comprises selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) performing 5′-3′ DNA polymerase extension of multifunctional capture probe utilizing the isolated tagged DNA library fragments as template; d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence;

e) performing PCR amplification of the hybrid nucleic acid in d); and f) quantitating the PCR reaction in e), wherein the quantitation allows for a determination of copy number of the specific target region.

In various embodiments, a method for targeted genetic analysis is also contemplated. In certain embodiments a method for targeted genetic analysis comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the genomic library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) creating a hybrid multifunctional capture probe-isolated tagged DNA target molecule through the concerted action of a 5' FLAP endonuclease, DNA polymerization and nick closure by a DNA ligase; d) performing PCR on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

In various embodiments, methods for determining copy number of a specific target region are contemplated. In particular embodiments, a method for determining copy number of a specific target region comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module comprises selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe module complex from a); c) creating a hybrid multifunctional capture probe-isolated tagged DNA target molecule through the concerted action of a 5' FLAP endonuclease, DNA polymerization and nick closure by a DNA ligase; d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the

multifunctional capture probe module tail sequence; e) performing PCR amplification of the hybrid nucleic acid in d); and f) quantitating the PCR reaction in e), wherein the quantitation allows for a determination of copy number of the specific target region.

In particular embodiments, PCR can be performed using any standard PCR reaction conditions well known to those of skill in the art. In certain embodiments, the PCR reaction in e) employs two PCR primers. In one embodiment, the PCR reaction in e) employs a first PCR primer that hybridizes to the target region. In a particular embodiment, the PCR reaction in e) employs a second PCR primer hybridizes to the hybrid molecule at the target region/tail junction. In certain embodiments, the PCR reaction in e) employs a first PCR primer that hybridizes to the target region and a second PCR primer hybridizes to the hybrid molecule at the target genomic region/tail junction. In particular embodiments, the second primer hybridizes to the target region/tail junction such that at least one or more nucleotides of the primer hybridize to the target region and at least one or more nucleotides of the primer hybridize to the tail sequence. In certain embodiments, the hybrid nucleic acid molecules obtained from step e) are sequenced and the sequences aligned horizontally, *i.e.*, aligned to one another but not aligned to a reference sequence. In particular embodiments, steps a) through e) are repeated one or more times with one or more multifunctional capture probe module complexes. The multifunctional capture probe complexes can be the same or different and designed to target either DNA strand of the target sequence. In some embodiments, when the multifunctional capture probe complexes are different, they hybridize near the same target region within the tagged DNA library. In one embodiment, one or more multifunctional capture probe hybridize within about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, or more bp of the target region in a tagged DNA library, included all intervening distances from the target region

In some embodiments, the method can be performed using two multifunctional capture probe modules per target region, wherein one hybridizes to the "Watson" strand (non-coding or template strand) upstream of the target region and one hybridizes to the "Crick" strand (coding or non-template strand) downstream of the target region.

In particular embodiments, the methods contemplated herein can further be performed multiple times with any number of multifunctional probe modules, for example 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more multifunctional capture probe modules per target region any number of which hybridize to the Watson or Crick strand in any combination. In some embodiments, the sequences obtained can be aligned to one another in order to identify any of a number of differences.

In certain embodiments, a plurality of target regions are interrogated, *e.g.,* 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 10000, 50000, 100000, 500000 or more in a single reaction, using one or more multifunctional probe modules.

Copy number can provide useful information regarding unique reads and duplicative reads, as well as assisting with searching for variants of known reads. As used herein, the terms "read," "read sequence," or "sequencing read" are used synonymously and refer to a polynucleotide sequence obtained by sequencing a polynucleotide. In particular embodiments, DNA tags, *e.g.,* a random nucleotide tag, can be used to determine copy number of a nucleic acid sequence being analyzed.

In one embodiment, a multifunctional capture probe hybrid module comprises: (i) a first region capable of hybridizing to a partner oligonucleotide and capable of functioning as a PCR primer and (ii) a second region capable of hybridizing to a specific genomic target region.

In various embodiments, a first region of a multifunctional capture probe hybrid module comprises a PCR primer sequence. In particular embodiments, this first region comprises a PCR primer sequence that is from about 5 to about 200 nucleotides, from about 5 to about 150 nucleotides, from about 10 to about 100 nucleotides from about 10 to about 75 nucleotides, about 10 to about 50 nucleotides, about 10 to about 40 nucleotides, about 20 to about 40 nucleotides, or about 20 to about 30 nucleotides, including any intervening number of nucleotides.

In particular embodiments, a first region of a multifunctional capture probe hybrid module is bound to a partner oligonucleotide. In certain embodiments, the multifunctional capture hybrid probe module is bound to the partner oligonucleotide prior to formation of a tagged DNA library—multifunctional capture probe hybrid

module complex. In particular embodiments, the multifunctional capture probe hybrid module is bound to the partner oligonucleotide after the formation of a tagged DNA library—multifunctional capture probe hybrid module complex. In some embodiments, the multifunctional capture probe hybrid module is bound to the partner oligonucleotide simultaneously with the formation of a tagged DNA library—multifunctional capture hybrid probe module complex. In some embodiments, the partner oligonucleotide is chemically modified.

In various embodiments, the methods contemplated herein comprise performing PCR on the tagged DNA library—multifunctional capture probe hybrid module complex so as to copy the captured tagged DNA library sequence to generate a hybrid nucleic acid molecule comprising the multifunctional capture probe hybrid module complex and a sequence complementary to a region of the captured tagged DNA library sequence located 3′ or 5′ of the multifunctional capture probe sequence relative to where the hybrid module hybridizes to the genomic target. In particular embodiments, the copied target region is anywhere from 1 to 5000 nt from the, 3′ or 5′-end of sequence where the multifunctional capture probe hybrid module hybridizes to the genomic target. In certain embodiments, the complementary sequence of the region that is 3′ to the location where the multifunctional capture probe hybrid module hybridizes is copied in order to generate a hybrid nucleic acid molecule. The hybrid nucleic acid molecule generated comprises the multifunctional capture probe hybrid module and the complement of a region of captured tagged DNA library sequence that is located 3′ or 5′ from the location where the multifunctional capture probe hybrid module hybridizes to the target region.

In various embodiments, the methods contemplated herein comprise processing a tagged DNA library-multifunctional capture probe module complex to generate a hybrid nucleic acid molecule (*i.e.,* a hybrid multifunctional capture probe-isolated tagged DNA target molecule). In particular embodiments, a hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged DNA library sequence that is located 3′ relative to the location where the multifunctional capture probe hybrid module hybridizes to the target region. In one non-limiting embodiment, the hybrid nucleic acid molecule is

generated by 3′-5′ exonuclease enzymatic processing that removes single stranded 3′-ends from an isolated tagged DNA library-multifunctional capture probe module complex and/or 5′-3′ DNA polymerase extension of the multifunctional capture probe.

In other particular embodiments, a hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged DNA library sequence that is located 5′ relative to the location where the multifunctional capture probe hybrid module hybridizes to the target region. In one non-limiting embodiment, the hybrid nucleic acid molecule is generated by the concerted action of a 5' FLAP endonuclease, DNA polymerization and nick closure by a DNA ligase.

In various embodiments, a method for targeted genetic analysis is provided. In one embodiment, a method for targeted genetic analysis comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a); c) performing PCR on the complex from b) to form a hybrid nucleic acid molecule and d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c). In particular embodiments, the hybrid nucleic acid molecules obtained from step c) are sequenced and the sequences aligned horizontally, *i.e.*, aligned to one another but not aligned to a reference sequence. In certain embodiments, steps a) through c) are repeated one or more times with one or more multifunctional capture probe module.

The multifunctional capture probe modules can be the same or different and designed to hybridize against either strand of the genome. In some embodiments, when the multifunctional capture probe modules are different, they hybridize anywhere from 1 to 5000 nt of the same target region in the tagged DNA library.

In particular embodiments, the method can be performed twice, using two multifunctional capture probe modules, wherein one hybridizes upstream of the genomic target region (*i.e.*, at the 5′-end; *i.e.,* a forward multifunctional capture probe module or complex) and one hybridizes downstream of the genomic target region on

the opposite genomic strand (*i.e.*, at the 3′-end; *i.e.*, a reverse multifunctional capture probe module or complex).

In one embodiment, one or more multifunctional capture probe hybridize within about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, or more bp of the target region in a tagged DNA library, included all intervening distances from the target region.

In some embodiments, the method can further be performed multiple times with any number of multifunctional probe modules, for example 2, 3, 4, 5, 6, 7, 8, 9, 10 or more multifunctional capture probe modules per target region, any number of which hybridize to the Watson or Crick strand in any combination.

In certain embodiments, a plurality of target regions are interrogated, *e.g.,* 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 10000, 50000, 100000, 500000 or more in a single reaction, using one or more multifunctional probe modules.

In particular embodiments, the sequences obtained by the method can be aligned to one another in order to identify mutations and without being aligned to a reference sequence. In certain embodiments, the sequences obtained in can optionally be aligned to a reference sequence.

In various embodiments, methods for determining copy number of a specific target region are contemplated. In particular embodiments, a method for determining copy number of a specific target region comprises: a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module comprises selectively hybridizes to a specific target region in the DNA library; b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a); c) performing PCR on the complex from b) to form a hybrid nucleic acid molecule; d) performing PCR amplification of the hybrid nucleic acid in c); and e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific target region. In particular embodiments, PCR can be performed using any standard PCR reaction conditions well known to those of skill in the art. In certain embodiments, the PCR reaction in d) employs two PCR primers. In particular

embodiments, the PCR reaction in d) employs two PCR primers each of which hybridize to a region downstream to the location where the multifunctional capture probe hybrid module hybridizes to the tagged DNA library. In further embodiments, the region where the PCR primers hybridize is located in the region amplified in step c). In various embodiments, the hybrid nucleic acid molecules obtained from step c) are sequenced and the sequences aligned horizontally, *i.e.*, aligned to one another but not aligned to a reference sequence. In particular embodiments, steps a) through c) are repeated one or more times with one or more multifunctional capture probe module. The multifunctional capture probe modules can be the same or different and designed to hybridize to either strand of the genome.

In one embodiment, one or more multifunctional capture probe hybridize within about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, or more bp of the target region in a tagged DNA library, included all intervening distances from the target region.

In some embodiments, the method can further be performed multiple times with any number of multifunctional probe modules, for example 2, 3, 4, 5, 6, 7, 8, 9, 10 or more multifunctional capture probe modules per target region, any number of which hybridize to the Watson or Crick strand in any combination.

In certain embodiments, a plurality of target regions are interrogated, *e.g.,* 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 10000, 50000, 100000, 500000 or more in a single reaction, using one or more multifunctional probe modules.

In particular illustrative embodiments, a tagged DNA library is amplified, *e.g.,* by PCR, to generate an amplified tagged DNA library.

All genomic target regions will have a 5′-end and a 3′-end. In particular embodiments, the methods described herein can be performed with two multifunctional capture probe complexes which provide for amplification of a targeted genomic region from both the 5′ and 3′ directions, respectively. In one embodiment, one or more multifunctional capture probe hybridize within about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500,

4000, 4500, 5000, or more bp of the target region in a tagged DNA library, included all intervening distances from the target region.

In some embodiments, the method can further be performed multiple times with any number of multifunctional probe modules, for example 2, 3, 4, 5, 6, 7, 8, 9, 10 or more multifunctional capture probe modules per target region, any number of which hybridize to the Watson or Crick strand in any combination.

In certain embodiments, a plurality of target regions are interrogated, *e.g.,* 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 10000, 50000, 100000, or more in a single reaction, using one or more multifunctional probe modules.

In particular embodiments, the targeted genetic analysis is a sequence analysis. In particular embodiments, sequence analysis comprises any analysis wherein one sequence is distinguished from a second sequence. In various embodiments, sequence analysis excludes any purely mental sequence analysis performed in the absence of a composition or method for sequencing. In certain embodiments, sequence analysis includes, but is not limited to: sequencing, single nucleotide polymorphism (SNP) analysis, gene copy number analysis, haplotype analysis, mutation analysis, methylation status analysis (as determined by example, but not limited to, bisulfite conversion of unmethylated cytosine residues), targeted resequencing of DNA sequences obtained in a chromatin-immunoprecipitation experiment (CHIP-seq), paternity testing in sequence captured fetal DNA collected from pregnant maternal plasma DNA, microbial presence and population assessment in samples captured with microbe-specific capture probes, and fetal genetic sequence analysis (for example, using fetal cells or extracellular fetal DNA in maternal samples).

Copy number analyses include, but are not limited to analyses that examine the number of copies of a particular gene or mutation that occurs in a given genomic DNA sample and can further include quantitative determination of the number of copies of a given gene or sequence differences in a given sample.

Also contemplated herein, are methods for sequence alignment analysis that can be performed without the need for alignment to a reference sequence, referred to herein as horizontal sequence analysis (exemplified in, for example, Figure 20). Such analysis

can be performed on any sequences generated by the methods contemplated herein or any other methods. In particular embodiments, the sequence analysis comprises performing sequence alignments on the hybrid nucleic acid molecules obtained by the methods contemplated herein. In one embodiment, one or more multifunctional capture probe hybridize within about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, or more bp of the target region in a tagged DNA library, included all intervening distances from the target region.

In some embodiments, the method can further be performed multiple times with any number of multifunctional probe modules, for example 2, 3, 4, 5, 6, 7, 8, 9, 10 or more multifunctional capture probe modules per target region, any number of which hybridize to the Watson or Crick strand in any combination.

In certain embodiments, a plurality of target regions are interrogated, *e.g.,* 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 10000, 50000, 100000, or more in a single reaction, using one or more multifunctional probe modules.

In particular embodiments DNA can be isolated from any biological source. Illustrative sources for DNA include, but are not limited to: blood, skin, hair, hair follicles, saliva, oral mucous, vaginal mucous, sweat, tears, epithelial tissues, urine, semen, seminal fluid, seminal plasma, prostatic fluid, pre-ejaculatory fluid (Cowper's fluid), excreta, biopsy, ascites, cerebrospinal fluid, lymph, or tissue extract sample or biopsy sample.

In one embodiment, tagged DNA libraries for use with the methods contemplated herein are provided. In some embodiments, a tagged DNA library comprises tagged genomic sequences. In particular embodiments, each tagged DNA sequence includes: i) fragmented end-repaired DNA; ii) one or more random nucleotide tag sequences; iii) one or more sample code sequences; and iv) one or more PCR primer sequences.

In one embodiment, a hybrid tagged DNA library is contemplated. In particular embodiments, a hybrid tagged DNA library comprises hybrid tagged DNA sequences. In certain embodiments, each hybrid tagged DNA sequence includes: i) fragmented

end-repaired DNA comprising a target region; ii) one or more random nucleotide tag sequences; iii) one or more sample code sequences; iv) one or more PCR primer sequences; and v) a multifunctional capture probe module tail sequence.

In various embodiments, kits and compositions of reagents used in the methods contemplated herein. In some embodiments, the composition includes a tagged DNA library, a multifunctional adaptor module and a multifunctional capture probe module. In particular embodiments, the composition includes a tagged genomic library. In certain embodiments, the composition includes a hybrid tagged genomic library.

In various embodiments, reaction mixtures for carrying out the methods contemplated herein are provided. In particular embodiments, the reaction mixture is a reaction mixture for performing any of the methods contemplated herein. In certain embodiments, the reaction mixture is capable generating a tagged DNA library. In some embodiments, the reaction mixture capable of generating a tagged DNA library includes: a) fragmented DNA and b) DNA end-repair enzymes to generate fragmented end-repaired DNA. In particular embodiments, the reaction mixture further comprises a multifunctional adaptor module. In various embodiments, the reaction mixture further comprises a multifunctional capture probe module. In certain embodiments, the reaction mixture further comprises an enzyme with 3′-5′ exonuclease activity and PCR amplification activity.

In various embodiments, methods for DNA sequence analysis are provided for the sequence of one or more clones contemplated herein. In one embodiment, the method comprises obtaining one or more or a plurality of tagged DNA library clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises targeted DNA sequence and the second DNA sequence comprises a capture probe sequence; performing a paired end sequencing reaction on the one or more clones and obtaining one or more sequencing reads or performing a sequencing reaction on the one or more clones in which a single long sequencing read of greater than about 100, 200, 300, 400, 500 or more nucleotides is obtained, wherein the read is sufficient to identify both the first DNA sequence and the second DNA sequence; and ordering or clustering the sequencing reads of the one or more clones according to the probe sequences of the sequencing reads.

The sequence reads can be compared to one or more human reference DNA sequences. Sequence reads that do not match the reference sequence may be identified and used to create a *de novo* assembly from the non-matching sequence data. In particular embodiments, the *de novo* assemblies are used to identify novel sequence rearrangements associated with the capture probe.

In various embodiments, a method for copy number determination analysis is provided comprising obtaining one or more or a plurality of clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises a random nucleotide tag sequence and a targeted DNA sequence and the second DNA sequence comprises a capture probe sequence. In related embodiments, a paired end sequencing reaction on the one or more clones is performed and one or more sequencing reads are obtained. In another embodiment, a sequencing reaction on the one or more clones is performed in which a single long sequencing read of greater than about 100 nucleotides is obtained, wherein the read is sufficient to identify both the first DNA sequence and the second DNA sequence. The sequencing reads of the one or more clones can be ordered or clustered according to the probe sequence of the sequencing reads.

In particular embodiments, a method for determining the copy number is provided. In particular embodiments, the method comprises obtaining one or more or a plurality of clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises a random nucleotide tag sequence and a targeted DNA sequence and the second DNA sequence comprises a capture probe sequence; ordering or clustering sequencing reads of the one or more clones according to the probe sequence of the sequencing reads. In particular embodiments, the random nucleotide tag is about 2 to about 50 nucleotides in length.

The methods may further comprise analyzing all sequencing reads associated with a second read sequence by determining the distributions of unique and redundant sequencing reads; counting the number of times a unique read is encountered; fitting a frequency distribution of the unique reads to a statistical distribution; inferring a total number of unique reads; and normalizing the total number of inferred unique read to an assumption that humans are generally diploid.

In particular embodiments, the methods contemplated herein can be used to calculate an inferred copy number of one or more targeted loci and the deviation of that calculation, if any, from an expected copy number value. In certain embodiments, one or more targeted loci of a gene are grouped together in a collection of loci and the copy number measurements from the collection of targeted loci are averaged and normalized. In one embodiment, the inferred copy number of a gene can be represented by the normalized average of all the target loci representing that gene.

In various embodiments, the compositions and methods contemplated herein are also applicable to generating and analyzing RNA expression. Without wishing to be bound to any particular theory, it is contemplated that any of the methods and compositions that are used to make tagged gDNA libraries may also be used to create tagged cDNA libraries, and capture and process target regions corresponding to RNA sequences embodied in the cDNA for subsequent RNA expression analysis, including, without limitation, sequence analysis.

In various embodiments, a method for generating a tagged RNA expression library comprises first obtaining or preparing a cDNA library. Methods of cDNA library synthesis are known in the art and may be applicable to various embodiments. The cDNA library may be prepared from one or a plurality of the same or different cell types depending on the application. In one embodiment, the method comprises fragmenting a cDNA library; treating the fragmented cDNA library with end-repair enzymes to generate fragmented end-repaired cDNA; and ligating a multifunctional adapter molecule to the fragmented end-repaired cDNA to generate a tagged RNA expression library.

In a particular embodiment a tagged RNA expression library (cDNA library) is prepared by obtaining or preparing a cDNA library from the total RNA of one or more cells; fragmenting the cDNA library; treating the fragmented cDNA with end-repair enzymes to generate fragmented end-repaired cDNA; and ligating a multifunctional adapter molecule to the fragmented end-repaired cDNA to generate a tagged RNA expression library.

In certain embodiments, the cDNA library is an oligo-dT primed cDNA library.

In certain embodiments, the cDNA library is primed by random oligonucleotides comprising about 6 to about 20 random nucleotides. In particular preferred embodiments, the cDNA library is primed by random hexamers or random octamers.

The cDNA libraries may be sheared or fragmented using known methods in order to achieve a desired average library fragment size. In one embodiment, the cDNA library is fragmented to an average size of about 250 bp to about 750 bp. In a certain embodiment, the cDNA library is fragmented to an average size of about 500 bp.

In various embodiments, RNA expression libraries contemplated herein may be captured, processed, amplified, and sequenced, *etc*., using any of the methods contemplated herein for capturing, processing, and sequencing tagged genomic DNA libraries, without or without minor variations.

In one embodiment, a method for targeted gene expression analysis is provided comprising: hybridizing a tagged RNA expression library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the tagged RNA expression library; isolating the tagged RNA expression library-multifunctional capture probe module complex; performing 3′-5′ exonuclease enzymatic processing and/or 5’-3’ DNA polymerase extension on the isolated tagged RNA expression library-multifunctional capture probe module complex; performing PCR on the enzymatically processed complex, wherein a tail portion (*e.g.,* a PCR primer binding site) of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the complement of the target region, the specific multifunctional capture probe sequence and the capture module tail sequence; and performing targeted gene expression analysis on the hybrid nucleic acid molecule.

In one embodiment, a method for targeted gene expression analysis comprises: hybridizing a tagged RNA expression library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the RNA expression library; isolating the tagged RNA expression library-multifunctional capture probe hybrid

module complex; performing PCR on the complex to form a hybrid nucleic acid molecule.

In particular embodiments, at least two different multifunctional capture probe modules are used in the at least two hybridization steps, wherein the at least two hybridization steps employ one multifunctional capture probe module each. In certain embodiments, at least one multifunctional capture probe module hybridizes 5′ of the target region and at least one multifunctional capture probe module hybridizes 3′ of the target region.

In one embodiment, one or more multifunctional capture probe hybridize within about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, or more bp of the target region in a tagged RNA expression or cDNA library, included all intervening distances from the target region.

In some embodiments, the method can further be performed multiple times with any number of multifunctional probe modules, for example 2, 3, 4, 5, 6, 7, 8, 9, 10 or more multifunctional capture probe modules per target region, any number of which hybridize to the Watson or Crick strand in any combination.

In certain embodiments, a plurality of target regions are interrogated, *e.g.,* 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 10000, 50000, 100000, or more in a single reaction, using one or more multifunctional probe modules.

In a further embodiment, methods for cDNA sequence analysis are provided, which allow the skilled artisan to conduct gene expression analysis from the cDNA library. In particular embodiments, any of the sequencing methods contemplated herein may be adapted to sequence the cDNA libraries with little or no deviation from their application to sequencing tagged genomic clones. As described above, the statistical distribution of tagged cDNA sequencing reads of a target region of a cDNA in the RNA expression analyses contemplated herein, correlates to the level of gene expression of the target region in a cell from which the cDNA library was prepared or obtained.

All publications, patent applications, and issued patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or issued patent were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

EXAMPLES

EXAMPLE 1

PREPARATION OF TARGET GENOMIC REGION FOR GENETIC ANALYSES

OVERVIEW

In particular embodiments, the methods contemplated herein comprise the coordinated utilization of several key molecular modules. In the following section, each module is described separately. At the end of this section, the interconnection of modules is described.

SECTION 1: TAGGING OF GENOMIC DNA FRAGMENTS

Genomic DNA from an individual can be collected, processed into pure DNA, fragmented and random nucleotide sequences of one nucleotide or more, in some embodiments in the range of 2 – 100 nucleotides, or in the range of 2 – 6 nucleotides are attached to the random ends of genomic DNA fragments (Figure 1). The combination of the introduced random nucleotide tag sequence together with the genomic fragment end sequence constitutes a unique combination of two elements that will hereafter be referred to as a the first region of the multifunctional adaptor module.

The uniqueness of first region of the multifunctional adaptor module is determined by the combinatorial product of the diversity within the attached first region of the multifunctional adaptor module pool times the diversity of the genomic fragment end sequences.

SECTION 2: ADDITION OF SAMPLE-SPECIFIC CODES AND UNIVERSAL AMPLIFICATION SEQUENCES

Multifunctional adapter molecules can further comprise sample-specific codes (referred to herein as the second region of the multifunctional adaptor module) and universal amplification sequences (referred to herein as the PCR primer sequence or the third region of the multifunctional adaptor module). In addition to the introduced random nucleotides from the first region of the multifunctional adaptor module, each segment that is attached to fragmented genomic DNA may include an additional set of nucleotides that is common to each sample but different between samples such that the DNA sequence of this region can be used to uniquely identify a given samples sequences in a set of sequences where multiple samples have been combined together (in other words, sample barcoding). In addition, the attached nucleotide sequence may contain a universal sequence that can be used to amplify (*e.g.*, by PCR) the polynucleotides. The combined elements of the random nucleotide tag sequence, the sample code, and the universal amplification sequence constitute an "adaptor" (also refer to as a multifunctional adaptor module) that is most commonly attached to the fragmented genomic DNA by means of nucleotide ligation.

An illustrative example of a multifunctional adaptor module ligated to fragmented gDNA are illustrated in Figure 1 and (while not wishing to be limited by example) an exemplary set of such sequences is shown in Table 1. Within Table 1, the sets of adaptor sequences are clustered into four sets of adaptor sequences. Within each column, all adaptors share the same two base code and all 16 possible random tags are represented. The 16 possible adaptors are mixed prior to ligation to fragment. Only the top, "ligation strand" of each adaptor is shown; this is the strand that becomes covalently attached to end-repaired DNA fragments. The bottom, partner strand that is eventually lost, while shown in Figure 1, is not included in Table 1.

Table 1: 5′ to 3′ DNA sequence of a set of 64 tagging and sample ID adapters

Sample code AC	SEQ ID NO:	Sample code GA	SEQ ID NO:
GACCAGAGAATTCGAATACAAAAC	1	GACCAGAGAATTCGAATACAAAGA	17
GACCAGAGAATTCGAATACAACAC	2	GACCAGAGAATTCGAATACAACGA	18
GACCAGAGAATTCGAATACAAGAC	3	GACCAGAGAATTCGAATACAAGGA	19
GACCAGAGAATTCGAATACAATAC	4	GACCAGAGAATTCGAATACAATGA	20
GACCAGAGAATTCGAATACACAAC	5	GACCAGAGAATTCGAATACACAGA	21
GACCAGAGAATTCGAATACACCAC	6	GACCAGAGAATTCGAATACACCGA	22
GACCAGAGAATTCGAATACACGAC	7	GACCAGAGAATTCGAATACACGGA	23
GACCAGAGAATTCGAATACACTAC	8	GACCAGAGAATTCGAATACACTGA	24
GACCAGAGAATTCGAATACAGAAC	9	GACCAGAGAATTCGAATACAGAGA	25
GACCAGAGAATTCGAATACAGCAC	10	GACCAGAGAATTCGAATACAGCGA	26
GACCAGAGAATTCGAATACAGGAC	11	GACCAGAGAATTCGAATACAGGGA	27
GACCAGAGAATTCGAATACAGTAC	12	GACCAGAGAATTCGAATACAGTGA	28
GACCAGAGAATTCGAATACATAAC	13	GACCAGAGAATTCGAATACATAGA	29
GACCAGAGAATTCGAATACATCAC	14	GACCAGAGAATTCGAATACATCGA	30
GACCAGAGAATTCGAATACATGAC	15	GACCAGAGAATTCGAATACATGGA	31
GACCAGAGAATTCGAATACATTAC	16	GACCAGAGAATTCGAATACATTGA	32
Sample code CT	**SEQ ID NO:**	**Sample code TG**	**SEQ ID NO:**
GACCAGAGAATTCGAATACAAACT	33	GACCAGAGAATTCGAATACAAATG	49
GACCAGAGAATTCGAATACAACCT	34	GACCAGAGAATTCGAATACAACTG	50
GACCAGAGAATTCGAATACAAGCT	35	GACCAGAGAATTCGAATACAAGTG	51
GACCAGAGAATTCGAATACAATCT	36	GACCAGAGAATTCGAATACAATTG	52
GACCAGAGAATTCGAATACACACT	37	GACCAGAGAATTCGAATACACATG	53
GACCAGAGAATTCGAATACACCCT	38	GACCAGAGAATTCGAATACACCTG	54
GACCAGAGAATTCGAATACACGCT	39	GACCAGAGAATTCGAATACACGTG	55
GACCAGAGAATTCGAATACACTCT	40	GACCAGAGAATTCGAATACACTTG	56
GACCAGAGAATTCGAATACAGACT	41	GACCAGAGAATTCGAATACAGATG	57
GACCAGAGAATTCGAATACAGCCT	42	GACCAGAGAATTCGAATACAGCTG	58

GACCAGAGAATTCGAATACAGGCT	43	GACCAGAGAATTCGAATACAGGTG	59
GACCAGAGAATTCGAATACAGTCT	44	GACCAGAGAATTCGAATACAGTTG	60
GACCAGAGAATTCGAATACATACT	45	GACCAGAGAATTCGAATACATATG	61
GACCAGAGAATTCGAATACATCCT	46	GACCAGAGAATTCGAATACATCTG	62
GACCAGAGAATTCGAATACATGCT	47	GACCAGAGAATTCGAATACATGTG	63
GACCAGAGAATTCGAATACATTCT	48	GACCAGAGAATTCGAATACATTTG	64

The application of a single group of adaptors (*i.e.*, a universal amplification sequence, a sample-specific code, and a set of random tags; also referred to as a multifunctional adaptor module) with a single amplification sequence on both ends of the genomic fragment has several significant advantages, including the fact that the same genomic fragment is tagged independently on its two ends. As described in the next few sections, the two strands of any given fragment are eventually separated from one another and will behave in the present invention as independent molecules. Therefore the presence of two different tags at the two ends of the same fragment becomes an advantage rather than a liability of the present invention. Additionally, there is the fact that adaptor-to-adaptor ligation events are a huge problem in next-generation library construction where the initial goal is to create amplicons with dissimilar ends. Using the methods of the present invention, the methods introduce this asymmetry later in the process and therefore identical ends are acceptable with the present invention. An unforeseen and surprising benefit of the present methods is that adaptor dimers are not observed in the library construction methods of the present invention. While not being bound by theory, present inventors contemplate that this may be because the rare adaptor dimer species that are formed rapidly form tight hairpin structures during the steps of denaturation and annealing that are necessary for PCR amplification and it is further contemplated that these hairpin structures are completely resistant to further primer-directed amplification. The ability to make adaptor-dimer free libraries is a significant technical feature in extremely low input applications like single-to-few cell genomic analysis, circulating DNA analysis (as in fetal diagnostics, tissue transplantation rejection surveillance, or cancer screening applications) or single cell transcriptome analysis. As such, the present methods

provide significant utility in such applications. An additional significant feature of single-primer amplicons is that it is possible to "turn on" amplification with a PCR primer of 25 nt and to "turn off" amplification with a longer 58 nt primer. This is described in more detail, and the significance to the present invention is highlighted, in section 6-5 below.

Overall

The adaptor strategy in which a single universal amplification sequence is used on both ends of target fragments eliminates issues with adaptor dimer. This is clearly demonstrated by way of example in Example 3: Construction of single-adaptor genomic library.

SECTION 3: LIBRARY QUANTIFICATION

An additional aspect of the present methods for genomic analysis strategy is that the "coverage depth" is known, that is, the average number of genomic copies present in a library are known or can be determined. The cover depth is measured using purified ligation reactions prior to the bulk amplification of the library that is necessary for subsequent steps. By way of illustration, if 50 genomes worth of DNA is put into the library scheme of an embodiment of the present invention and there is100% efficient ligation of adaptors to both ends of the fragments, then the coverage depth is 100 because each adaptor end acts independently of the other, then 2 ends times 50 genomes = 100 in coverage. The simple fact that adaptor-dimers do not amplify with the universal PCR primer contemplated herein but fragments adapted on both ends do means that library quantitation will simply be a matter of measuring library complexity with quantitative PCR (qPCR) using universal primer and calibrating the results against standards with known coverage depth. Here the phrase "genome copies" and "coverage depth" mean the same thing and may be used interchangeably. The present methods will carry forward anywhere from 4 – 1000, preferably 20 – 100 fold coverage depth into the next phase of sample processing according to the present invention.

SECTION 4: LIBRARY AMPLIFICATION

In particular embodiments, a portion of the adaptor ligated genomic fragment library equivalent to 20 – 100 fold coverage depth will be amplified using standard PCR techniques with a single, universal primer sequence driving amplification. At this stage it is advantageous, in particular embodiments, to convert the picograms of material in the initial library into micrograms of amplified material, implying a 10,000-fold amplification.

SECTION 5: HYBRIDIZATION OF TARGET LIBRARY FRAGMENTS TO CAPTURE PROBES

Advances in oligonucleotide synthesis chemistries have created new opportunities for sophisticated genome capture strategies. In particular, the availability of long oligonucleotides (100 – 200 nt in length) that have reasonable per base synthesis costs, relatively high yields, and exquisite base accuracy are now commercially available from a variety of vendors. This capability has the present inventor to create multifunctional capture probes (Figure 2). The elements of an illustrative example of a multifunctional capture probe comprises:

Region 1 comprises a 34 nt region common to all probes that hybridizes to a modified complementary oligonucleotide (also referred to as a partner oligonucleotide). This modified oligonucleotide further comprises on the 5′ end a biotin – TEG modification that is a biotin capable of binding tightly to streptavidin protein, a long hydrophilic spacer arm that alleviates steric hindrance of biotin binding. On the 3′ end, the oligonucleotide terminates with a dideoxy-cytosine residue that renders this partner oligonucleotide inert to primer extension. This element of probe design allows for adapting an unlimited number of probes with biotin capture functionality without having to directly modify said probes.

Region 2 comprises custom 60 nt regions that are target-specific and that interact with gDNA fragment molecules. This region is designed by computational methods that account for uniqueness of sequence within the genome, the presence of common SNPs that may compromise binding efficiency, and secondary structure considerations.

Region 3 comprises a 20 nt segment that will serve as a PCR primer binding site in subsequent fragment amplification. This feature is described in further detail in the next paragraph.

A multiplicity of probes can be used to capture genomic regions of interest (multiplexing of probes). At least two probes may be employed to thoroughly query a typical coding exon of 100 – 150 bp in length. By way of example, this indicates that 20 probes will be used to capture a typical 10 exon gene and a total of 2000 probes will be used to interrogate a 100 gene panel. The hybridization of genomic library fragments to probes can be performed by thermal denaturation followed by reannealing. In one embodiment, the steps comprise:

1. Combining genomic library fragments with pooled probe sequences (in this case "probe sequences" refers to the combination of individual probes together with an equal molar quantity of the highly modified partner oligonucleotide) at a specific target to probe ratio ranging anywhere from 1 part target to 1 part probe to 1 part target to 1,000,000 parts probe. In one embodiment, the optimal ratio is about 1 part fragment to 10,000 parts probe.
2. Heating the combined fragment + probe in a solution containing 1M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA and 0.1% Tween 20 (non-ionic detergent) to 95°C for >30 sec to denature all double-strand DNA structure.
3. Cooling the combined probe and fragment in controlled steps, for example 1°C decrease in temperature every 2 minutes, down to <60°C. This slow cooling will result in duplexes between target genomic fragments and probe sequences.
4. Binding of the probe: fragment complexes to carboxyl coated, streptavidin modified paramagnetic beads and "pull out" of these beads using a strong magnet.
5. Washing of the bound complexes with a solution containing 25% (v/v) formamide, 10 mM Tris pH 8.0, 0.1 mM EDTA and 0.05% Tween 20. In particular embodiments, the wash step is performed at least twice.
6. Resuspension of the washed beads in a solution that is amenable to the subsequent enzyme processing step(s).

Capture Reactions

Embodiments of the capture reaction are demonstrated in Example 3 (Construction of single-adaptor genomic library) and employ qPCR assays developed and further described in Example 5 (Validation of PLP1 qPCR assays).

SECTION 6: ENZYMATIC PROCESSING OF HYBRIDIZED PROBE:TARGET COMPLEXES

As currently practiced in the art, hybridization-based sequence capture methods generally result in a suboptimal enrichment of target sequences. From literature and commercial publications it can be estimated that, at best, about 5% - 10% of reads map to their intended target sequences. The remaining reads often map ***near*** the intended target and commercial vendors have resorted to redefining "on-target" as reads that land anywhere within ~1000 bases of the intended locus. The reasons for this "spreading" effect are incompletely understood, but they are likely a result of legitimate sequence hybridization events (*see*, *e.g.*, Figure 3).

The enzymatic processing of complexes as contemplated herein more sharply focuses the captured sequences on the exact regions of interest. In this step, a DNA polymerase that also possesses a 3′ to 5′ exonuclease activity is employed. An illustrative example of such an enzyme is T4 DNA polymerase. This enzyme will "chew off" dangling tail sequences down to the duplex region formed between the probe and target sequence. It will then copy the tail segment on the probe. *See, e.g.,* Figure 4. Benefits provided by this step include, but are not limited to:

1. By employing this type of enzymatic processing, only those fragments that were in direct, duplexed hybridization with probe will be carried forward. The final sequencing library is a chimeric (hybrid) set of molecules derived from both the fragment and the probe.
2. The probe is strand-specific and therefore the captured target has an inherent directionality to it (illustrated in Figure 5). This means that only one of two strands generated from a single fragment will interact with probe, and processing will "focus" reads to a region 5′ of the probe sequence. At this point, the complementary strand of the fragment becomes a completely independent species. By placing directional probes on either side of a target region (*e.g.* an

exon), the technology enables highly specific focusing of sequencing reads on target regions (Figure 6).

3. Target molecules that are legitimately cross-hybridized with target fragments (but not with probe; Figure 3) do not acquire essential probe sequence and are therefore lost in the subsequent amplification step.
4. The actual "tail" sequence of the probe becomes copied into target fragments as part of the amplification sequence. All commercially practicable sequencing platforms (*e.g.* Illumina's reversible terminator chemistry sequencing platforms) require sequencing libraries in which the target fragments have asymmetric ends; this is often referred to as a "forward" and a "reverse" adaptor sequence, or in the shorthand of sequencing labs, "P1" and "P2". In particular embodiments, up to this point, fragment libraries contemplated herein have a single species on the end; called "P1". The enzymatic processing step achieves two things. First, it "erases" (by 3′ to 5′ exonuclease activity) one of these P1 ends. Second, it "adds" (by DNA polymerase copying of the probe tail sequence) the basis of a P2 end that is dissimilar from P1.
5. Enzymatically-modified target molecules with legitimate P1-P2 ends can be selectively enriched in the PCR amplification step that follows processing. This is achieved by the use of long PCR primers. In particular long primers are necessary to add the full functionality required for next-generation sequencing, and they also confer selectivity to amplification. Residual P1-P1 library fragments that are "contaminants" from the first round of amplification fail to amplify with longer P1 primers. This is a significant advantage of the present method. The initial P1-P1 library amplifies effectively with a single, 25 nt PCR primer. When this primer length is extended to 57 nt – to add sequencing functionality – these same P1-P1 molecules are not amplified to any appreciable extent. Therefore amplification of the initial library can be "turned on" with a 25 nt primer and "turned off" with a 57 nt primer.

Overall

The inability of P1-insert-P1 libraries to amplify is demonstrated Example 3 (Construction of single-adaptor genomic library). The preferential amplification of P1-

insert-P2 processed DNA fragments is shown in Example 3 (Construction of single-adaptor genomic library). Example 3 further demonstrates the substantial improvement in target specificity that accompanies processing. Finally, the "sensitivity" of processing, meaning the percent of initial complexes that are processed, is demonstrated to be on the order of 10% of all captured complexes in Example 9 (Direct measurement of post-capture processing).

SECTION 7: AMPLIFICATION AND SEQUENCING

The core adaptor and primer sequences applied to initial proof-of-concept experiments are shown in Table 2. Enzymatically processed complex from step 6 is added directly to a PCR amplification reaction that contains full-length forward and reverse PCR primers. Following amplification, the library can be purified, quantified, loaded on a high-throughput, next-generation sequencer (in this embodiment, the libraries are configured for the Illumina reversible terminator-based platforms), and the sequence of ~millions of fragments is determined. At this stage, single reads of >36 nt, preferably 72 or 100+ nt in length can be observed.

Table 2: Genesis of primer sequences

Description	Sequence (5′ to 3′)	SEQ ID NO:
Forward amplification primer for full-length library.	AATGATACGGCGACCACCGA	65
Forward primer sequence present on Illumina's paired-end (PE) flow cell.	AATGATACGGCGACCACCGAGATCTACAC	66
Non-palindromic 4mer of all four bases. This was added to make the read 1 sequencing primer the same length and position as the Illumina standard.	GTCA	
ACA2. PCR primers ACA2 and CAC3 predate NuGEN	TGCAGGACCAGAGAATTCGAATACA	67
ACA2 forward sequencing primer (FSP) - read 1	ACACGTCATGCAGGACCAGAGAATTCGAA TACA	68
ACA2 full length forward amplification	AATGATACGGCGACCACCGAGATCTACAC	69

primer (FLFP). Final length = 58 nt.	GTCATGCAGGACCAGAGAATTCGAATACA	
Reverse amplification primer for full-length library.	CAAGCAGAAGACGGCATACG	70
PE flow cell reverse primer	CAAGCAGAAGACGGCATACGAGAT	71
These are the nine 5′ bases from the TruSeq Reverse adaptor that were inserted to create a read 2 primer site	GTGACTGG	
CAC3 reverse primer	CACGGGAGTTGATCCTGGTTTTCAC	72
CAC3 reverse sequencing primer (RSP) - read 2	GTGACTGGCACGGGAGTTGATCCTGGTTTTCAC	73
CAC3 full length reverse primer (FLRP). Final length = 57 nt.	CAAGCAGAAGACGGCATACGAGATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC	74

SECTION 8: DATA ANALYSIS.

There are at least two major aspects to post-sequencing data analysis. The first is the identification of sequence variants – single nucleotide variants, micro-insertions and/or micro-deletions relative to an established set of reference sequences. While complex, these methods are well documented in the field and one of skill in the art would understand such methods. The second is the determination of copy number variation from the targeted sequencing data.

EXAMPLE 2

COPY NUMBER DETERMINATIONS

Copy number determination finds a variety of uses in the field of DNA sequencing. By way of non-limiting example, massive parallel DNA sequencing technologies provide at least two opportunities to interrogate and analyze biological samples. One well-established aspect is determination of DNA sequences, meaning the *de novo* sequences present in the sample (*e.g.*, the sequencing of a newly isolated microbe) or the resequencing of known regions for variants (*e.g.*, the search for variants within known genes). A second aspect of massive parallel sequencing is quantitative

biology and the ability to count the number of times a particular sequence is encountered. This is a fundamental aspect of technologies like "RNA-seq" and "CHIP-seq", where counting is used to infer gene expression or the association of a particular protein with genomic DNA, respectively. This example relates to the quantitative, counting-based aspects of DNA sequencing.

DNA fragments are most often counted as constellations of sequences that share high degrees of similarity (*i.e.*, they align to specific regions of a known genomic sequence). Often the sequences within these clusters are identical. Note that DNA sequences with either a) different starting and ending DNA sequence reads or b) with high quality sequence differences from other reads within the set are most often considered "unique reads". Thus, different starting sequence location and sequence variation are one form of "tagging" that is used to differentiate unique events from clones. In the present example, random nucleotide tags (*e.g.*, random six nucleotide sequences) are also introduced onto genomic fragments during the course of library construction. The combination of 1) the random nucleotide tag sequence with 2) the start point of the DNA sequencing read and 3) the actual sequence of the read collectively make up a tag. This tag enables one to differentiate between convergent events where the same fragment was cloned twice (such fragments will have different random nucleotide tag sequences that were introduced during library construction) and fragments of the same origin that were replicated during library amplification (these "clones" will have the same random nucleotide segment and the same clone start points). This type of tagging further enables quantitative analysis of genomic DNA specifically, and populations of DNA molecules (*e.g.*, RNA-seq libraries) more generally.

The introduction of random nucleotide tags (random Nmer combined with DNA clone ends) into DNA sequencing libraries allows, in theory, each unique clone within the library to be identified by its unique tag sequence. The specification of "in theory" acknowledges confounding features of ordinary experimental data sets that may occur such as errors in sequencing, errors introduced during library amplification, introduction of contaminating clones from other libraries, *etc.* All of these sources can and will confound the theoretical consideration posed here. In the context of sequence

capture and targeted resequencing, the tagging of libraries can enable quantitative analysis of locus copy number within a captured library.

Consider, by way of non-limiting example, a library constructed from the equivalent of 100 diploid genomes of input that was created from a male subject. The prediction is that approximately 200 library clones will be present at each autosomal locus, and 100 clones will be present at each X-chromosome locus. If an autosomal region is captured and sequenced 2000 times, then all 200 tags will be encountered with a confidence interval exceeding 99% certainty. For the X-chromosomal region, 2000 reads would, in theory, reveal a total of 100 tags. By way of illustration, this example supports the general concept that the creation of DNA tags within a DNA sequencing library can preserve copy number differences. This general framework can be applied to the methods described herein. Empirical evidence suggest that adjustments may need to be made for differences in cloning efficiencies on a locus-by-locus basis, for sporadic introduction of artifactual tags from experimental error, etc. The implementation of this concept into practice may differ in different contexts and may involve case-by-case sequence analysis methods, but the general principle outlined here will underlie all such applications.

To this point, the creation of tagged DNA libraries has been considered in the context of genomic DNA analysis, yet it must be emphasized that this concept applies to all counting-based DNA sequencing applications. In particular embodiments, tagging may be applied to RNA-seq, where cDNA molecules produced from mRNA of a sample are cloned by methods that create tags. Such approaches may substantially increase the fidelity of sequence-based gene expression analysis. In certain embodiments, it is contemplated that tagging can increase the resolution of chromatin immunoprecipitation (CHIP-seq) experiments. In various embodiments, tagging will enhance the quantitative aspects of sequence counting used to determine the presence and abundance of microbes in microbiome compartments and environmental samples.

EXAMPLE 3

CONSTRUCTION OF SINGLE ADAPTOR GENOMIC LIBRARY

PURPOSE

The goal of this example was to create a genomic DNA library from acoustically fragmented ProMega female hgDNA (~200 bp).

SUMMARY

The results clearly demonstrated the significant features of the present methods for adaptor design. In particular, the adaptor-alone ligation reaction had no detectable adaptor dimer species present. This was very significant in the context of extremely low input sequencing library preparation technology, as with current methods, the limits of input are invariably determined by the background levels of adaptor dimer. Highly specialized technologies have been applied in attempts to keep adaptor dimer contamination in check. These include size exclusion methods such as columns or gel purification, expensive custom oligonucleotide modifications designed to minimize adaptor self-ligation events, and adaptor sequence modifications that allow for restriction digestion destruction of adaptor dimers following library construction.

The simple, single adaptor, single primer concept contemplated herein addresses the adaptor dimer issue with a simple solution that evokes the basic principles of DNA structural principles. This extremely low input technology will be useful for the construction of genomic libraries for genomic analysis, for transcriptome analysis of cloned double-stranded cDNA as, for example, in RNA-seq applications of one or a few specific cells, and in rescuing the few intact fragments that may be present in a highly modified, poorly preserved formalin-fixed, paraffin-embedded (FFPE) nucleic acid sample.

Another essential feature of the adaptor design of the present invention is the ability to "turn on" and "turn off" PCR amplification of the target amplicon library by using different PCR primer lengths. As has been clearly demonstrated, the optimal primer length for library amplification was 25 nt primer species with a projected Tm (under standard ionic strength conditions) $\geq 55°C$. A shorter, lower Tm primer displayed less efficient amplification of and appeared to favor amplicons with smaller

average insert sizes. There is ample precedent that primers in this size class work well when paired with opposite primers of dissimilar sequence.

Taken together, these data demonstrated that the adaptor and PCR amplification methods of the present invention produce adaptor-dimer-free fragment libraries with "tunable, on/off" amplification properties.

METHODS

Hydrated primers that were received from IDT to 100 μM in TEzero (10 mM Tris pH 8.0, 0.1 mM EDTA).

Fragment repair. Thawed gDNA and 500 ng gDNA was end repaired by combining:

- 14 μl water
- 5 μl hgDNA
- 2.5 μl 10X end repair buffer
- 2.5 μl 1 mM dNTPs
- Mix and add 1 μl end repair enzyme and 0.5 μl PreCR enzyme repair mix.

The mixture was incubated at 20°C for 30 min and 70°C for 10 min; and held at 10°C.

Adaptor annealing. Combined 68 μl of TEzero, 2 μl of 5M NaCl, 20 μl of oligo 11 and 10 μl of oligo 12. Heated to 95°C for 10 sec, 65°C for 5 min and cooled to RT.

Table 3: Adapter sequences Adap_P23 and Adapt_L23

SEQ ID NO:	Oligo ID	Name	Sequence	Description
75	11	Adap_P23	TCCGTGTATTCGAAT	Number 23 of the initial 64 adapter set, partner strand
23	12	Adap_L23	GACCAGAGAATTCGA ATACACGGA	Number 23 of the initial 64 adapter set, ligation strand

Ligations. Combined in a total volume of 20 μl; 1 = no insert, 2 = 100 ng end-repaired hgDNA.

- 13 μl or 8 μl water
- 0 or 5 μl end-repaired fragment = 100 ng.
- 2 μl 10X T4 ligase buffer
- 3 μl 50% PEG8000
- 1 μl 10 μM ACA2 adaptor #23 duplex
- Mix and add 1 μl of T4 DNA ligase

Incubated at 23°C for 30 min and 65°C for 10 min. Added 80 µl/rxn of TEz and 120 µl of beads. Mixed and incubated at RT for 10 min. Washed twice with 200 µl aliquots of 70% EtOH:water v/v and resuspended in 50 µl of TEz.

PCR amplification. Each 10 µl aliquot of ligation mix = 20 ng of library. Planned to amplify for 18 cycles.

Table 4: Reaction mixtures

	100 µl	600 µl
Water	50	300
10X STD Taq buffer	10	60
25 mM $MgCl_2$	10	60
10 µM PCR primer	10	60
Template	10	60
DMSO	5	30
10 mM dNTPs	5	30
Taq polymerase	1	6

Made 600 µl mix containing all components except primer and template. Made 6 of 80 µl aliquots. Added 10 µl no insert ligation to set 1 and 10 µl of hgDNA insert to set 2. Added 10 µM primer of the primers shown below in pairs to the no insert and hgDNA insert ligation mixes. Mixed. Thermal cycled for 18 cycles of 94°C – 30 sec, 60°C – 30 sec and 72°C – 60 sec; finish for 2 min at 72°C and held at 10°C.

Table 5: Primer sequences

SEQ ID NO:	Oligo ID	Name	Sequence
76	3	ACA2_20	GACCAGAGAATTCGAATACA
77	5	ACA2	TGCAGGACCAGAGAATTCGAATACA
78	8	ACA2_FLFP	AATGATACGGCGACCACCGAGATCACACGTCATG CAGGACCAGAGAATTCGAATACA

Purified PCR product with 120 µl of beads. Washed twice with 200 µl of 70% EtOH. Dry beads and elute DNA with 50 µl of TEz. Analyzed 5 µl of each sample on a 2% agarose gel.

RESULTS

The exact same gel image is shown in four different color and contrast schemes in Figure 7. The samples loaded on the gel were:

1. No insert, adaptor-only ligation amplified with ACA2 20
2. No insert, adaptor-only ligation amplified with ACA2 (normal 25 nt PCR primer)
3. No insert, adaptor-only ligation amplified with ACA2 FLFP (full length forward primer)
4. 20 ng of ~200 bp hgDNA insert + adaptor ligation amplified with ACA2 20
5. 20 ng of ~200 bp hgDNA insert + adaptor ligation amplified with ACA2 (normal 25 nt PCR primer)
6. 20 ng of ~200 bp hgDNA insert + adaptor ligation amplified with ACA2 FLFP (full length forward primer)

It was clear that there was no amplified material in the adaptor alone ligation → PCR products (lanes 1-3). The shorter, 20 nt ACA2 primer showed inefficient amplification (lane 4) relative to the "normal", 25 nt ACA2 primer (lane 5). Only the faintest trace of material was visible with the 58 nt ACA2 FLFP primer (lane 6).

In further embodiments, it may be useful to titrate in the amount of the ACA2 primer and monitor yield. Normal high-yield PCR primers possess 1 μM of both the forward and reverse primers for a total of 2 μM in primer (per 100 μl PCR reaction). Therefore, addition of ACA2 to 2 μM (since it is both the forward and reverse primer) may increase yield. Similarly, in particular embodiments, it may be useful to monitor amplification characteristics of libraries at primer annealing temperatures lower than 60°C.

EXAMPLE 4

FRAGMENTATION OF GDNA

PURPOSE

For initial proof-of principle experiments sheared human gDNA from male and female was needed. The present example employs human female and male gDNA from Promega. Based on quantities shown on the tubes, these were diluted to 1000 μl of 100

ng/ μl DNA and subjected them to Covaris conditions that were intended to generate fragments in the range of 200 bp.

SUMMARY

There are at least two components to the laboratory research infrastructure. One is the ability to quantify DNA, and the other is the ability to visualize the size distribution of DNA on gels. In the present example, the Qubit 2.0 instrument from Life Technologies was employed to measure DNA concentration. It was found that the readings recorded were generally lower than our previous experience with Nanodrop. The Qubit reading was based on dsDNA-specific dye binding and fluorescence. One major advantage of the Qubit is that it can be used to quantify DNA amplification reactions (*e.g.* PCR) without prior clean-up. In these experiments it was found that the Promega gDNA thought to be 100 ng/μl was measured at ~60 ng/μl by Qubit. With respect to gels and qualitative assessment of size distribution, there was electrophoresis and documentation that the system that worked effectively. In the present example, fragmented gDNA was found to have a mean size distribution centered on the desired ~200 bp.

METHODS AND RESULTS

After Covaris treatment, DNA concentrations were measured using the Qubit instrument. The gDNA was diluted 10-fold, and 2 μl were added to 200 μl final volume of assay solution. The reading for both female and male samples registered at ~60 ng/mL, meaning the starting solutions are 60 ng/μl. While this was below what was initially anticipated, it was well within the appropriate range for particular embodiments. We then loaded 2 μl (120 ng) and 5 μl (300 ng) of both pre-fragmented and post-fragmented material on a 2% agarose gel (Figure 8). In the top row, the labels stand for M – male gDNA and F – female gDNA. In the bottom row, the labels are U – unfragmented and C- Covaris fragmented. One important observation was that the average fragment size was an even distribution centered around 200 bp.

EXAMPLE 5

VALIDATION OF PLP1 QPCR ASSAYS

PURPOSE

The proteolipid protein 1 (PLP1) gene on chromosome X was examined for initial proof-of-concept capture studies. This gene was chosen because it is relevant to cancer and resides on the X chromosome, meaning it has a natural copy variation between males and females. The 187 nt exon 2 region of Ref-Seq transcript NM000533.3 of PLP1 was used as the target region. For proof of principle studies, the ability to monitor regions in and around PLP1 exon2 by qPCR was needed. This example provides description of the design and validation of eight such assays.

SUMMARY

Eight qPCR assays were designed (in this case, meaning simple primer pairs) to monitor PLP1 exon 2 capture. Five were on-target, meaning they are within a region targeted by capture probes. Two are "near-target," meaning one assay is positioned at a genomic coordinate 200 bp of the target region and one assay is positioned 1000 bp from the target region on the opposite strand. These two assays were designed to quantify "spreading", the phenomenon where region near-by the target locus are pulled along as "hitch-hikers" in capture experiments. Finally, one assay was designed against a region of chromosome 9, and it is designed to monitor an arbitrary and unrelated segment of human gDNA. Here, the example shows that all eight assays produce a PCR fragment that is consistent with the predicted sizes of the amplicons. The example showed that the PLP1 assays, which were situated on chromosome X, appropriately had higher specific activities per ng of input gDNA in females than in males. These data validated the use of these assays in further experiments to monitor gDNA capture.

METHODS, RESULTS AND DISCUSSION

A 400 bp region centered around the PLP1 exon 2 was submitted to PRIMER3 for generation of amplicons that were 80-100 bp in length, for primers that were 24 nt in length, on average, and that had Tms of 60°-65°C. The search region was manipulated to obtain primer pairs (qPCR amplicons) that "walk" from the 5′ intron-

exon boundary of exon 2 through the CDS and into the 3′ exon-intron boundary. Nearby, proximity capture assays were also designed that were distal to exon 2, toward exon 3, and positioned ~200 nt and ~1000 nt away from exon 2. These would be used to monitor "hitchhiker" genomic fragments that are captured in secondary hybridization events. Finally, one assay was created on chr9 to monitor bulk genomic DNA levels during experiments. The primer sequences of these assays are shown below and the details are appended to the end of this example.

Table 6: qPCR primer sequences "walking" the 5′ introns-exon boundary of PLP1 exon 2 through the CDS

Assay	SEQ ID NO:	Name	Sequence	Description
Assay 1	79	F1	TTAGAGTGCTGTGCAAGATGTCTG	Forward qPCR primer that sits within PLP1 exon2
	80	R1	ACCCCAAAGAAACACAATCCAGT	Reverse qPCR primer that sits within PLP1 exon2
Assay 2	81	F2	GCCACTGGATTGTGTTTCTTTG	Forward qPCR primer that sits within PLP1 exon2
	82	R2	TCAATTAGCTTTTCTGTGCCAGTG	Reverse qPCR primer that sits within PLP1 exon2
Assay 3	83	F3	ACTGTTCTGTGGCTGTGGACAT	Forward qPCR primer that sits within PLP1 exon2
	84	R3	TCTTGGTAGTTTTTGGAGAAATAGGTC	Reverse qPCR primer that sits within PLP1 exon2
Assay 4	85	F4	ttcttcttccccagGCTTGTTAGA	Forward qPCR primer that sits within and flanks upstream region of PLP1 exon2 (lower case).
	86	R4	CACCCCAAAGAAACACAATCCAGT	Reverse qPCR primer that sits within and flanks upstream region of PLP1 exon2 (lower case).

Assay 5	87	F5	CCCTCACTGGCACAGAAAAGCTAA	Forward qPCR primer that sits within and flanks downstream region of PLP1 exon2 (lower case).
	88	R5	tgggagggcaggtacttacACATT	Reverse qPCR primer that sits within and flanks downstream region of PLP1 exon2 (lower case).
Assay 6	89	F6	CCCCTTGTTTTCTTACACGTGTTCT	Forward qPCR primer that is 200 bp downstream of PLP1 exon2
	90	R6	CCTCCCTTGGCTTCTCCATACCTA	Reverse qPCR primer that is 200 bp downstream of PLP1 exon2
Assay 7	91	F7	GTGTGTCATTGTTTGGGAAAATGG	Forward qPCR primer that is 1000 bp downstream of PLP1 exon2 (actually in exon3)
	92	R7	CACCCCTTGTTATTGCCACAAAAT	Reverse qPCR primer that is 1000 bp downstream of PLP1 exon2 (actually in exon3)
Assay 8	93	F8	TACCAGCCAAGCCCATACTAGAGG	Forward qPCR primer unlinked to chrX PLP1 locus that sits in chr9
	94	R8	GGGATCAACAGTGGCATAATTGAA	Reverse qPCR primer unlinked to chrX PLP1 locus that sits in chr9

To validate primer pair performance, PCR reactions were set-up that contained either male or female genomic DNA as template. These were then amplified by real-

time PCR on the Illumina Eco instrument or by conventional PCR. The reasoning was that by qPCR, female should have slightly more PLP1 (chrX) signal than male. By conventional PCR, we were able to check amplicon size and uniqueness. Both tests yielded data consistent with the interpretation that all eight assays performed well.

PCR reaction set up: For each female or male PCR reaction, a 250 µl master mix was made on ice containing:

- 100 µl of water
- 25 µl of 10X STD Taq buffer
- 25 µl of 25 mM MgCl2
- 25 µl of 60 ng/µl sheared gDNA (female and male were the same concentration by Qubit)
- 12.5 µl DMSO
- 12.5 µl 10 mM dNTPs
- 6.25 µl EvaGreen dye (Biotum)
- 5 µl ROX dye (InVitrogen)
- Mix well and add 2.5 µl Taq DNA polymerase.

Table 7: Cq values from PLP1 qPCR assay validation

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
	chrX	chrX	chrX	chrX	chrX	chrX	chrX	chr9
F	23.27371	24.01229		24.39554	22.3413	22.10594	22.65759	22.31407
	23.33794	22.49233	23.87286	22.53665	22.49127	22.19686	22.73586	22.0446
	22.33889	22.40355	23.68538	22.50178	22.42961	22.23099	22.83415	22.32891
M	24.00953	23.56869		23.2648	23.51487	23.11367	23.65077	22.15477
	23.63462	23.48547	23.95081	23.18794	23.25046	22.93675	23.66965	21.23208
	23.77216	23.16852	24.16562	23.21774	22.93373	22.95635	23.61168	22.16391
	22.98351	22.96939	23.77912	22.51921	22.42073	22.17793	22.74253	22.2292
	23.80544	23.40756	24.05821	23.22349	23.23302	23.00226	23.64403	21.85026
	0.8	0.4	0.3	0.7	0.8	0.8	0.9	-0.4

For the experiment, 24 µl of mix was aliquoted into two sets of 8 strip tubes (female or male) and added 6 µl of primer mix that contained 10 µM of the forward and reverse primers from each assay. After mixing, three identical 5 µl amounts were aliquoted into columns of a 48-well Eco PCR plate (triplicate female on top in columns, triplicate male samples in columns on the bottom). The instrument was set to monitor SYBR and ROX and to cycle to 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec for 40 cycles. A JPG image of the amplification traces for assay 6 is shown in Figure 9. The copy difference between the female and male samples was clear. All of the "Cq"

values were gathered (value where the fluorescence curve passes some auto-defined baseline) for the female and male samples, then differences between averages of triplicate measurements were calculated. This is shown in Table 7 above (bottom line = M-F), where all values are positive except for the chr9 assay. The overall data indicated that all eight assays perform similarly (Cq values from 22 – 24) and that the chrX assays generally had a higher signal in females.

The conventional PCR reaction was cycled for 30 cycles of 94°C – 30sec, 60°C – 30 sec and 72°C – 30 sec; 72°C rest for 2 min, 10°C hold. A total of 5 µl of product was loaded directly on a 2% agarose gel without purification and is shown in Figure 10. The upper bands of each doublet were consistent with the projected mobility of the assay PCR products. The lower "fuzzy" material was most likely unused PCR primers.

From the results of real – time PCR and of conventional PCR followed by gel analysis it can be concluded that these eight assays amplify their intended regions exclusively and that they are suitable for monitoring fragment enrichment.

Example 5 Appendix: Details on assay design

PLP1 gene: Transcript ID NM_000533.3; Exon 2 – 187 nt; CDS2 From UCSC browser CDS in BOLD UPPERCASE UNDERLINED; primer sequences are shaded.

Flanking sequence in lowercase

SEQ ID NO:	NAME	SEQUENCE
95	Forward strand	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct acctactggatgtgcctgactgtttccccttcttcttccccagGCTTGTTAGAGTGC TGTGCAAGATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG GTGGCCACTGGATTGTGTTTCTTTGGGGTGGCACTGTTC TGTGGCTGTGGACATGAAGCCCTCACTGGCACAGAAAAG CTAATTGAGACCTATTTCTCCAAAAACTACCAAGACTAT GAGTATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttcc ctctctccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
96	Reverse complement	tccaccccagtcccctgctagttactaaggtactgaagagttctctatctccaggatggagagaggg aaaaaaaagatgggtctgtgtgggagggcaggtacttacACATTGATGAGATACT CATAGTCTTGGTAGTTTTTGGAGAAATAGGTCTCAATTAGC

		TTTTCTGTGCCAGTGAGGGCTTCATGTCCACAGCCACAGAA CAGTGCCACCCCAAAGAAACACAATCCAGTGGCCACCAGG GAAGCAAAGGGGGCCCCTACCAGACATCTTGCACAGCACT CTAACAAGCctggggaagaagaaggggaaacagtcaggcacatccagtaggtagctca tgccactcaaacccctctgccaggccctgtgctcacaggctcggagatagtgggcac
97	qPCR assay 1, 77nt:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct **acctactggatgtgcctgactgtttcccttcttcttccccag**GCTTGTTAGAGTGC TGTGCAAG**ATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG GTGGCCACTGGATTGTGTTTCTTTGGGGT**GGCACTGTTC TGTGGCTGTGGAC**ATGAAGCCCTCACTGGCACAGAAAAG CTAATTGAGACCTATTTCTCCAAAAACTACCAAG**ACTAT GAGTATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttcc ctctctccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
98	qPCR assay 2, 83 nt:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct **acctactggatgtgcctgactgtttcccttcttcttccccag**GCTTGTTAGAGTGC TGTGCAAG**ATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG GTGGCCACTGGATTGTGTTTCTTTGGGGT**GGCACTGTTC TGTGGCTGTGGAC**ATGAAGCCCTCACTGGCACAGAAAAG CTAATTGAGACCTATTTCTCCAAAAACTACCAAG**ACTAT GAGTATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttcc ctctctccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
99	qPCR assay 3, 81 nt:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct **acctactggatgtgcctgactgtttcccttcttcttccccag**GCTTGTTAGAGTGC TGTGCAAG**ATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG GTGGCCACTGGATTGTGTTTCTTTGGGGT**GGCACTGTTC TGTGGCTGTGGAC**ATGAAGCCCTCACTGGCACAGAAAAG CTAATTGAGACCTATTTCTCCAAAAACTACCAAG**ACTAT GAGTATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttcc ctctctccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
100	qPCR assay 4, 97 nt:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct **acctactggatgtgcctgactgtttcccttcttcttccccag**GCTTGTTAGAGTGC

		TGTGCAAG**ATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG** **GTGGCCACTGGATTGTGTTTCTTTGGGGT**GGCACTGTTC TGTGGCTGTGGAC**ATGAAGCCCTCACTGGCACAGAAAAG** **CTAATTGAGACCTATTTCTCCAAAAACTACCAAG**ACTAT GAGTATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttcc ctctctccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
101	qPCR assay 5, 95 nt:	agtgcccactatctccgagcctgtgagcacagggcctggcagagggg**tttgagtggcatgagct** **acctactggatgtgcctgactgtttccccttcttcttccccag**GCTTGTTAGAGTGC TGTGCAAG**ATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG** **GTGGCCACTGGATTGTGTTTCTTTGGGGT**GGCACTGTTC TGTGGCTGTGGAC**ATGAAGCCCTCACTGGCACAGAAAAG** **CTAATTGAGACCTATTTCTCCAAAAACTACCAAG**ACTAT GAGTATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttcc ctctctccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
102	qPCR assay 6, 200 bp distal to exon 2, 100 bp product >chrX:103040918+103041017 100bp	CCCCTTGTTTTCTTACACGTGTTCTgacttctgctaggtgtggttcatat tgcccaagttggagcctccagcgtagTAGGTATGGAGAAGCCAAGGGAGG
103	qPCR assay 7, 1000 bp distal to exon 2 (in exon 3), 81 bp product >chrX:103041614+103041694 81bp	GTGTGTCATTGTTTGGGAAAATGGctaggacatcccgacaaggtgatcat cctcaggATTTTGTGGCAATAACAAGGGGTG
104	qPCR assay 8, unlinked assay on chr 9, 96 bp product >chr9:103041653+103041748 96bp	TACCAGCCAAGCCCATACTAGAGGctgtccccagatgctagcaaccatct gattgaataaccatctgtatcaTTCAATTATGCCACTGTTGATCCC

EXAMPLE 6

CAPTURE OF PLP1 EXON 2

PURPOSE

In one embodiment, the Clearfork Bioscience v1.0 DNA capture strategy entails the use of multifunctional probes targeted at specific genomic target regions. The goal was to validate the approach using ultramers™ (Integrated DNA Technologies (IDT), Coralville, IA; ultramers is the trade name given to specialty synthesis synthetic oligonucleotides ranging in length from 45 – 200 nt) targeted to PLP1 exon 2.

SUMMARY

In this example, the capture reaction was demonstrated. Ultramers from IDT-DNA worked well for capture; the basic protocol through the capture step in terms of reagent stochiometries was sound; and PEG molecular crowding agent interfered with effective capture. Post capture enzymatic processing was subsequently addressed.

BRIEF DESCRIPTION

The multifunctional probes are diagrammed in Figure 2. The goal of this experimental data set was to test all three features of these probes. Region 1 was the binding site for a 34 nt universal, 5′ biotin-TEG and 3′ dideoxycytosine-modified universal "pull-down" oligo. Two of these universal regions were designed to validate/verify equivalent (hopefully) performance.

The sequences of these two universal oligos are shown below in Table 8.

Table 8: Pull-down oligo sequences targeting PLP exon 2:

SEQ ID NO:	Name	Sequence	Description
105	Univ_seq_1	/5BioTEG/CACTGGACTATGTAGTACCTCACTCAGCAATAC/3ddC/	One of two 5' biotin-TEG and 3' dideoxycytosine modified universal capture oligos
106	Univ_seq_10	/5BioTEG/GGCAACGAACGGACTGGAATCTACGGTCACCAA/3ddC/	One of two 5' biotin-TEG and 3' dideoxycytosine modified universal capture oligos

Below is a brief description of how these sequences were selected. The functional role of these oligos was to hybridize to capture probes and to thereby provide a stably bound biotin extension that could be used for capture on streptavidin modified magnetic beads.

10 random sequences were generated by a random DNA sequence generator set to have a rough base composition of 50% GC. The web site used was www.faculty.ucr.edu/mmaduro/random.htm. The ten sequences were then screened by BLAT to the hg19 build of the human genome. Only sequence 3 showed a significant alignment. The two sequences ending in "C" were chosen since these could be blocked with ddC. Both sequences were analyzed by the IDT OligoAnalyzer. Sequence 1 is 47% GC and has a 76°C melting temp in 1M NaCl. Sequence 2 has a 57% GC content and an 86°C melting temp in high salt. The sequences selected, 1 and 10, are the actual "universal" 5' Biotin TEG--ddC complementary probe sequences. The reverse complements of these were used as the tails on capture probes. These sequences were subsequently altered by adding four bases, A,G,C and T, to increase the length to 34 bases. This length worked well with SBC and there was no compelling reason to change. Second, some of the CGCG type motifs were disrupted to lower self-dimer formation.

Region 2 encompassed the portion of the probe that was designed to contact genomic sequences in sample genomic libraries. In this experiment, the target region was exon 2 of PLP1. The DNA sequence of PLP1 exon 2 is shown below. The CDS exon 2 is highlighted in BOLD UPPERCASE UNDERLINED type. The evenly spaced-capture probe sequences are shaded.

SEQ ID NO:	NAME	SEQUENCE
107	Forward strand	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagctacctactggatgtgcc tgactgtttccccttcttcttccccagGCTTGTTAGAGTGCTGTGCAAGATGTCTGGTAGG GGCCCCCTTTGCTTCCCTGGTGGCCACTGGATTGTGTTTCTTTGGGGTG GCACTGTTCTGTGGCTGTGGACATGAAGCCCTCACTGGCACAGAAAAGCT AATTGAGACCTATTTCTCCAAAAACTACCAAGACTATGAGTATCTCATCA

		ATGTgtaagtacctgccctcccacacagacccatctttttttccctctctccatcctggagatagagaactcttcagtacctta gtaactagcaggggactggggtgga

Region 3 was complementary to a validated PCR primer called CAC3. The sequence of the CAC3 PCR primer is: CACGGGAGTTGATCCTGGTTTTCAC (SEQ ID NO: 72).

The sequences of the ultramers that include these probe regions are shown in Table 9.

Table 9: Sequences of ultramers used in the PLP1 exon 2 capture

SEQ ID NO:	Name	Sequence	Description
108	B1_PLP1ex2_p1	GAGTTGATCCTGGTTTTCACTTTGAGTGGCATGAGCTACCTACTGGATGTGCCTGACTGTTTCCCCTTCTTCTTCCCCAGGGTATTGCTGAGTGAGGTACTACATAGTCCAGTG	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 1
109	B1_PLP1ex2_p2	GAGTTGATCCTGGTTTTCACATGTCTGGTAGGGGCCCCCTTTGCTTCCCTGGTGGCCACTGGATTGTGTTTCTTTGGGGTGGTATTGCTGAGTGAGGTACTACATAGTCCAGTG	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 1
110	B1_PLP1ex2_p3	GAGTTGATCCTGGTTTTCACCTTGGTAGTTTTTGGAGAAATAGGTCTCAATTAGCTTTTCTGTGCCAGTGAGGGCTTCATGGTATTGCTGAGTGAGGTACTACATAGTCCAGTG	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 1
111	B1_PLP1ex2_p4	GAGTTGATCCTGGTTTTCACATCTCCAGGATGGAGAGAGGGAAAAAAAAGATGGGTCTGTGTGGGAGGGCAGGTACTTACGGTATTGCTGAGTGAGGTACTACATAGTCCAGTG	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 1
112	B10_PLP1ex2_p1	GAGTTGATCCTGGTTTTCACTTTGAGTGGCATGAGCTACCTACTGGATGTGCCTGACTGTTTCCCCTTCTTCTTCCCCAGGTTGGTGACCGTAGATTCCAGTCCGTTCGTTGCC	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 10
113	B10_PLP1ex2_p2	GAGTTGATCCTGGTTTTCACATGTCTGGTAGGGGCCCCCTTTGCTTCCCTGGTGGCCACTGGATTGTGTTTCTTTGGGGTGTTGGTGACCGTAGATTCCAGTCCGTTCGTTGCC	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 10

114	B10_PLP1ex2_p3	GAGTTGATCCTGGTTTTCACCTTGGTAGTTTTTGGAGAAATAGGTCTCAATTAGCTTTTCTGTGCCAGTGAGGGCTTCATGTTGGTGACCGTAGATTCCAGTCCGTTCGTTGCC	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 10
115	B10_PLP1ex2_p4	GAGTTGATCCTGGTTTTCACATCTCCAGGATGGAGAGAGGGAAAAAAAAGATGGGTCTGTGTGGGAGGGCAGGTACTTACGTTGGTGACCGTAGATTCCAGTCCGTTCGTTGCC	Ultramer targeted to PLP1 exon 2 and compatible with Universal seq 10

Considerations about moles, micrograms and molecules: The genomic library constructed in Example 3 (hgDNA library from Promega female) was used. A large scale (800 µl) amplification of this library was performed starting with 20 µl of ligation mix as input. The purified library (400 µl) had a final concentration of 22 ng/µl. One microgram per experiment described here was used. Furthermore, based on the total adaptor being 50 bp and the inserts being 150 – 200 bp, there was an assumption that 75% of the library mass is genomic DNA. Based on this assumption, and that one human genome has a mass 3 pg, then there were roughly ($750 \times 10^{-9}/3 \times 10^{-12} = 250{,}000$) 250,000 copies of any given genomic region present. Previous experience and literature suggested that a 10,000-fold molar excess of probes is a reasonable place to start. This implies 2,500,000,000 molecules of probe. 2.5×10^{9} molecules/6.02×10^{23} molecules/mole = 4.15×10^{-15} moles = 4 amol probe. Translating this to volume of a stock solution, 1 µl of 4 nM (in each probe) = 4 amol of probe. Finally, Invitrogen's MyOne strep-coated C1 beads bind about 1 pmol of biotinylated 500 bb dsDNA/1 µl of beads. In this experiment, a total of 4 amol x 4 probes = 16 amol in probe were added. 1 µl of beads binds 1000 amol, 1 µl is a practical amount of beads to work with, and 1 µl of beads is a 60-fold excess of binding capacity over added probe. Therefore, in this example the following parameters were calculated:

- the number of target molecules in a unit mass of library (250,000 copies of a unique diploid locus/1 µg of library);
- the molarity of probe necessary to address the target loci with a 10,000-fold molar excess of probe (4 amol of each probe, 16 amol total probe (4 probes), 1 µl of a 4 nM solution of probes; and

- the amount of beads necessary to quantitatively capture all of the added probe (1 µl binds 1000 amol of dsDNA and/or unbound probe).

BUFFERS AND WORKING SOLUTIONS

Solution 1 – binding probes: hydrated universal binding partners and PLP1 probes to 100 µM. In two separate tubes, combined 92 µl TEz + 0.05% Tween-20 buffer, 4 µl universal oligo and 1 µl each of the four cognate (with the universal oligo) probes. This generated two of 1 µM stock probe solution. Diluted each of these 4 µl into 1000 µl of TEz+Tween to provide a 4 nM working solution of probes.

4X Binding buffer = 4M NaCl, 40 mM Tris pH 8.0, 0.4 mM EDTA, and 0.4% Tween 20. Made 50 mls by combining 40 mls of 5M NaCl, 2 mls of 1M Tris pH 8.0, 2 mls of 10% Tween20, 40 µl of 0.5 M EDTA and 6 mls of water.

Wash buffer = 25% formamide, 10 mM Tris pH 8.0, 0.1 mM EDTA and 0.05% Tween 20. Made 50 mls by combining 37 mls of water, 12.5 mls of formamide, 500 µl of 1M Tris pH8.0, 10 µl of 0.5M EDTA, and 250 µl of 10% Tween 20.

Beads. Combined 250 µl of 4X bind and 750 µl of water to make 1X binding buffer. Added 10 µl of beads to 90 µl of 1X bind, pulled aside with magnet, washed the beads 2X with 100 µl of 1X bind, and resuspended washed beads in 100 µl 1X bind. Ten microliters of washed beads is equivalent to 1 µl of beads as they come out of the manufacturer's tube.

METHODS

The following three parameters were tested:

1. Universal biotin oligo 1 versus oligo 10;
2. Binding in 1X bind versus 1X bind plus 7.5% PEG8000 (a molecular crowding agent that may enhance the rate of annealing); and
3. Fold-enrichment of PLP1 region after straight binding and after binding plus enzymatic processing

To test these parameters eight samples (2 x 2 x 2) were generated. These samples contained 50 µl of 20 ng/ µl genomic DNA, 25 µl of 4X binding buffer, 1 µl of binding probes and either 24 µl of water or 20 µl of 50% PEG8000 + 4 µl of water (four sample with and 4 samples without PEG). From the IDT DNA website OligoAnalyzer,

it was described that the Tm of oligos shifts to dramatically higher temperatures in high salt (*e.g.,* 1M NaCl). Thus, the sample was melted at 95°C and then dropped the temperature in 1°C and 2min increments to 60°C (35 cycles of AutoX on our ABI2720 thermal cycler where each cycle drops by one degree and each cycle lasts 2 min). After the samples cooled to room temperature (RT), 10 µl of washed beads were added per sample and incubated for 20 min. The beads were pulled out with a strong magnet and the solution aspirated and discarded. The beads were washed four times with 200 µl washes of wash buffer; each time the beads were resuspended, they were incubated a RT for 5 min. After the final wash, the majority of remaining wash was carefully aspirated from the tubes.

A set of four tubes was treated with T4 DNA polymerase. A cocktail was made by combining 10 µl of New England Biolab 10X Quick blunting buffer, 10 µl of 1 mM dNTPs from the same kit, 10 µl of water and 1 µl of T4 DNA polymerase. 20 µl was added to a set of four tubes and the reaction was incubated at 20°C for 15 min.

For PCR amplification following capture, the non-T4 treated samples (captured only) were amplified with ACA2-25 (TGCAGGACCAGAGAATTCGAATACA; SEQ ID NO: 67) in single primer reactions. The T4-treated samples were amplified with ACA2FL and CAC3FL primers (AATGATACGGCGACCACCGAGATCTACACGTCATGCAGGACCAGAGAATTCGAATACA (SEQ ID NO: 69) and CAAGCAGAAGACGGCATACGAGATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC (SEQ ID NO: 74), respectively). The core reaction mixes contained, per 400 µl reaction, 120 µl of water, 40 µl of 10X STD Taq buffer (NEB), 40 µl of 25 mM MgCl2, 80 µl of 10 µM single primer or 40 µl + 40 µl of F and R primer, 20 µl of DMSO, 20 µl of 10 mM dNTPs and 4 µl of Taq polymerase. 80 µl aliquots were added to beads that had been resuspended in 20 µl of TEz (binding only) or 20 µl of T4 mix. The final volumes were 100 µl. These samples were amplified by PCR for 30 cycles of 94°C-30 sec, 60°C-30 sec and 72°C-60 sec. The gel analysis – 5 µl of post-PCR material was loaded per lane - is shown in the results section. Qubit readings indicated that each PCR reaction had a concentration of ~20-25 ng/µl.

For post-amplification analysis, a 500 µl (final volume) master mix of conventional PCR mix was made by combining 200 µl water, 50 µl 10X Taq buffer, 50 µl 25 mM MgCl2, 25 µl of DMSO, 25 µl of 10 mM dNTPs, 12.5 µl EvaGreen (Biotum) and 5 µl Taq polymerase (NEB). 42 µl aliquots were distributed into 8 tubes and 12 µl of F+R 10 µM PLP1 primer mixes (the assays are described in Example 5: Validation of PLP1 qPCR assays) were added. 9 µl of mix was distributed, each assay in 8 columns. A total of 6 samples, 1 µl of sample per well, was assayed. These samples were:

Row 1: gDNA library starting material

Row 2: Biotin oligo 1 capture material

Row 3: Biotin oligo 1 + PEG capture

Row 4: Biotin oligo 10 capture material

Row 5: Biotin oligo 10 + PEG capture material

Row 6: TEz NTC control

The T4 treated samples were not assayed because gel analysis showed that only aberrant material was treated by PCR amplification.

RESULTS

The capture only libraries produced a smear that looked like the input genomic library, as expected. The samples left to right were (1) oligo 1, (2) oligo 1 + PEG, (3) oligo 10, and (4) oligo 10 + PEG. The T4-treated samples were contaminated with residual T4 polymerase (5-8). In particular embodiments, T4 polymerase is heat inactivated.

The Qubit measured yields of the four capture only libraries is shown below in Table 10.

Table 10: Qubit measured yields of the four capture only libraries

C1 universal oligo	23.2	ng/ul
C1+PEG	27.2	ng/ul
C10 universal oligo	24.8	ng/ul
C10+PEG	25.6	ng/ul

For qPCR, all eight validated PLP1 assays (Example 5) were used in columns and samples in rows. The array of the samples was:

Row 1: 1 µl of 25 ng/µl gDNA library

Row 2: 1 µl of ~25 ng/µl C1 capture sample

Row 3: 1 µl of ~25 ng/µl C1+P capture sample

Row 4: 1 µl of ~25 ng/µl C10 capture sample

Row 5: 1 µl of ~25 ng/µl C10+P capture sample

Row 6: 1 µl TEz (NTC)

In this configuration, 1 sample per well, the data were meant to be more of a qualitative overview than a rigorous quantitative measurement. The data are shown in the tables below. The top table is raw Cq values. The next table is Cq values converted to absolute values based on the assumption that all samples and assays conform to the same two-fold standard curve. The bottom table shows the quotient of captured sample divided by gDNA library. This provides a sense of fold-enrichment following capture.

Cq values	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA lib	22	25	29	25	25	26	25	25
C1	14	11	11	11	9	15	23	22
C1+P	18	15	18	14	13	18	29	26
C10	12	12	13	12	10	15	22	25
C10+ P	16	13	15	13	12	18	27	25
NTC	29	25	34	28	30	28	30	31

Cq values	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA lib	33	3	0	4	3	2	3	2
C1	4782	56660	50773	43156	138885	2950	10	28
C1+P	293	3196	407	6187	16082	450	0	1
C10	29864	34343	11961	28507	123383	2894	31	3
C10+ P	1234	11632	3976	10060	25772	413	1	2
NTC	0	3	0	0	0	1	0	0

cap/gDNA	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
C1	147	19303	330609	11735	48601	1720	3	11
C1+P	9	1089	2649	1682	5628	262	0	1
C10	917	11700	77886	7752	43176	1688	10	1
C10+ P	38	3963	25890	2736	9019	241	0	1

Table 11: Results of the eight PLP1 qPCR assays demonstrating successful capture-induced enrichment of target sequences

Several conclusions were drawn from the data: (1) The capture worked. The average capture enrichment across on-target assays 1-5 for C1 was 82,000X. The

average for C10 was 28,000X. Anywhere from hundreds to tens of thousands fold enrichment at assay sites was observed. This implies that ultramers work and that the basic probe design is effective. This meant that the basic stoichiometry of gDNA to probe to beads was correct; (2) The two biotin designs worked approximately the same; (3) PEG inhibits rather than enhanced capture efficiency; and (4) Significant "by-catch" at assay 6, which was 200 bp from target was observed. Less stray activity was seen for a region 1000 bp away.

In particular embodiments, it may be important to determine if enzymatic processing of the captured complex contributes to sensitivity (fold-enrichment) and specificity (degree of "by-catch") in this scheme.

EXAMPLE 7

PLP1 qPCR Assays in SYBR Space

Purpose

In some cases it is useful for real-time conditions to exactly mimic non-real-time amplification conditions. In this example this meant to set up on ice and three stage, relatively slow PCR reactions. Alternatively, some assays do not require replication of a set amplification conditions; rather they are intended strictly to make quantitative measurements. For example, the PLP1 qPCR assays are preferably not used to produce fragments, but only to measure locus enrichment. In this type of situation, qPCR reaction set up at room temperature and fast cycling are advantages. In this experiment, the eight PLP1 assays in ABI 2X SYBR mix were tested. These are the same primer assays as described in the Example 5 (Validation of PLP1 qPCR assays).

Summary

These data suggested that at least six of eight PLP1 qPCR assays could be used with SYBR green qPCR mix and conditions.

Method

PLP1 assay performance was measured against the female gDNA library (Example 3: hgDNA library from Promega female). Per 10 µl well combined 5 µl ABI 2X SYBR master mix, 0.2 µl of 10 µM stock F+R primer, 1 µl of gDNA library (20 ng/

µl) and 3.8 µl water (larger volume master mixes were made and aliquoted). Made triplicate no-template-control and triplicate gDNA library measurements across each assay. Cycled for 40 cycles on the Illumina Eco real-time PCR using standard 2 step PCR (15 sec at 95°C, 45 sec at 60°C) with ROX passive reference dye normalization.

RESULTS

The called Cq values for each well are shown in Table 12 below. The NTC is very clean; the gDNA Cq's are variable, likely due to pipetting. The general theme is that Assay 1 and 7 were poor performers while the remaining assays worked reasonably well in SYBR space. In Figure 11, the NTC traces (A) and +gDNA traces (B) were copied to provide a qualitative picture of assay performance.

Table 12: Called Cq values from rapid SYBR-green-based PLP1 assays

Assay	1	2	3	4	5	6	7	8
NTC	38	40	40	40	40	40	40	40
	40	40	40	40	40	40	40	40
	35	40	40	40	40	40	40	40
gDNA	35	25	25	24	27	22	32	26
	35	25	25	24	26	23	32	27
	32	24	22	22	23	21	29	24

EXAMPLE 8

MEASUREMENT OF PLP1 EXON2 ENRICHMENT BEFORE AND AFTER ENZYMATIC PROCESSING OF COMPLEXES

PURPOSE

In this example the enzymatic processing of the complex was tested directly for yield by measuring the "specific activity" of PLP1 exon2 DNA in pre- and post-processed capture complex. ultramers support excellent capture efficiency and the core capture protocol performed well.

SUMMARY

This experiment demonstrated that post-capture processing with T4-DNA polymerase dramatically improved the specificity of the capture reaction.

BACKGROUND

In Example 6 (Capture of PLP1 exon 2) the successful capture was described, however the post-capture processing step where T4 polymerase was not removed prior to PCR yielded an artifactual library. Here, the same basic experiment is repeated except that T4 was heat inactivated at 95°C for 1 min prior to PCR.

METHODS, RESULTS, DISCUSSION

In this experiment, four samples were generated which included two universal biotin capture probes in order to assess capture efficiency of complexes before and after enzyme processing. Each sample contained 50 µl of 20 ng/µl genomic DNA, 20 µl of 4X binding buffer, 1 µl of binding probes and either 9 µl of water for a final volume of 80 µl. Samples were melted at 95°C for one minute and annealed by cooling the temperature in 1°C, 2min increments to 60°C (35 cycles of AutoX on our ABI2720 thermal cycler) followed by cooling to RT. A total of 10 µl of washed beads (equivalent to 1 µl of MyOne bead solution – streptavidin-coated C1 – Invitrogen) were then added per sample and incubated for 20 min. The beads were pulled out with a magnet and the solution aspirated and discarded. The beads were washed four times with 200 µl washes of wash buffer; each time the beads were resuspended, they were incubated at RT for 5 min. After the final wash, the majority of remaining wash was carefully aspirated from the tubes, leaving beads coated with capture complex.

For T4 processing of two of the samples, we prepared 50 µl of enzymatic processing mix that contained 40 µl of water, 5 µl of 10X quick blunt buffer (New England Biolabs), 5 µl of 1 mM dNTPs and 0.5 µl of T4 DNA polymerase. Two aliquots of complex were suspended in 20 µl (each) of T4 mix and incubated at 20°C for 15 min, 95°C for 1 min, and cooled to RT. The "untreated" controls were suspended in 20 µl of the same buffer (40 µl of water, 5 µl of 10X quick blunt buffer (New England Biolabs), 5 µl of 1 mM dNTPs) that lacked T4 polymerase.

To measure specific activity, both the capture alone and capture + processing samples were amplified by 30 cycles of PCR. The DNA was then quantified and PLP1 assay signals measured in a specific and known amount of amplified DNA. In this example two amplification reactions were set-up. For capture alone, amplification was performed with ACA2-25 (TGCAGGACCAGAGAATTCGAATACA; SEQ ID NO: 67) since these libraries are only amplifiable with this single primer. For enzyme-processed complexes, amplification was performed with ACA2FL and CAC3FL primers (AATGATACGGCGACCACCGAGATCTACACGTCATGCAGGACCAGAGAATTCGAATACA (SEQ ID NO: 69) and CAAGCAGAAGACGGCATACGAGATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC (SEQ ID NO: 74), respectively). 100 µl of PCR mix contained 10 µl of 10X STD Taq buffer (all reagents NEB unless otherwise specified), 10 µl of 25 mM MgCl2, 20 µl of 10 µM single primer or 10 µl + 10 µl of 10 µM dual primers, 20 µl of template (untreated control or T4 processed, beads and all), 5 µl of DMSO, 5 µl of 10 mM dNTPs and 1 µl of Taq DNA polymerase (all set up one ice prior to amplification). Samples were amplified for 30 cycles of PCR in a three step, 95°C-30sec, 60°C-30sec, 72°C-60sec, protocol followed by 72°C for 2 min and rested at 10°C.

Following amplification, DNA yield was measured and the PCR amplified material examined by DNA gel electrophoresis. Qubit (InVitrogen) measured (DNA HS kit) yields are shown in Table 13 below. These data highlight a basic feature that dual primer amplification supports overall yields than single primer amplification.

Table 13: Qubit quantification of amplified DNA

Sample	yield (ng/ul)
C1 biotin, unprocessed complex	23
C10 biotin, unprocessed complex	24
C1 biotin, T4 processed complex	38
C10 biotin, T4 processed complex	34

The gel image (2% agarose, 100 ng of material loaded) is shown in Figure 12. Processing had two noticeable effects. First, it produced two faint bands of ~250 bp (upper arrow) and ~175 bp (lower arrow) in addition to an expected smear. The lower

band was consistent with inadvertent cloning of probe (115 bp of adaptor + 60 bp probe = 175 bp). Second, processing reduced the overall size distribution of the sample. This was noteworthy since the 50 bp single adaptors were replaced by 115 bp full-length adaptors, which was expected to create an overall upward shift of 65 bp in processed material. The interpretation was that processing reduced the average insert size of the library significantly.

Two efforts were made to measure enrichment efficiency by qPCR. In the first, more qualitative effort, all eight PLP1 assays (described in detail in Example 5: Validation of PLP1 qPCR assays) were used to measure six samples:

1. 25 ng/assay of starting gDNA library
2. 0.25 ng/assay of untreated C1
3. 0.25 ng/assay of untreated C10
4. 0.25 ng/assay of T4 treated C1
5. 0.25 ng/assay of T4 treated C10
6. No template control

The Cq values from these single measurements are shown in Table 14 below. The gDNA and NTC controls did perform well (top and bottom, lightest shades) and were not evaluated further.

Table 14: Cq values of qualitative PLP1 assay analysis of enrichment efficiencies

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA	N/A	27	29	40	27	30	23	32
un-C1	12	8	8	9	5	11	24	29
un-C10	16	14	14	15	9	10	30	31
T4-C1	7	6	3	3	4	19	25	N/A
T4-C10	4	3	4	6	3	17	26	28
NTC	32	32	31	23	24	28	24	30

The T4-treated samples (darkest shading) had so much signal (Cq's less than 10) that quantitative analysis was not very informative. But at a qualitative level, two trends were clear in comparison to untreated capture complexes (middle shading). One was that on-target signal from assays 1-5 increases (lower Cq's) dramatically. The other was that off-target signal from Assay 6, which is 200 bp away from the target region, decreased significantly upon processing. While the data have some bumps and

warts the central message was that processing greatly enhanced specificity of PLP1 exon2 signal.

To capture more quantitative aspects of this experiment, the untreated C10 capture amplicons were diluted 1000-fold and the processed C10 amplicons 15,000 fold prior to qPCR; this was done to bring Cq values into a measurable range. The starting gDNA library was then examined and these diluted samples in quadruplicate wells of the qPCR plate across two on-target assays (Assays 2 and 5) and two off-target assays (Assays 6 and 7). The Cq values of the quadruplicate wells were averaged and these values are shown in Table 15 below. Once again the gDNA signal was poor; the impact of poor signal on data interpretation was not terribly significant because the goal of these experiments was to compare PLP1 exon2 signal in unprocessed versus T4 polymerase treated capture complexes. The Cq values were converted to absolute values using a "universal" standard curve that assumes 2-fold amplification with every PCR cycle. The third segment of the table shows the adjustment for dilutions. The fourth segment, ratios of unprocessed and T4-treated to gDNA is not as useful; however, at the bottom of the table is the quantitative ratios of unprocessed versus T4-treated complex. In Example 6, it was observed that untreated capture enrichment of 82,000X for C1 and 28,000X for C10 (as in all these experiments, the gDNA denominator was derived from very low signal, so the fold range had a qualitative aspect to it), so a reasonable estimate was that capture alone produced a 50,000X enrichment of the 300 bp PLP1 exon2 region. Processing increased this enrichment another 50-fold (average of 83X and 24X from the Table 15), pushing enrichment to 2.5 million-fold and 10 million fold (3 billion bases per genome over 300 bp target). At the level of qPCR measurements, capture + processing therefore appeared to approach best-case scenarios in terms of enrichment. It was noteworthy that the off-target signal that was 200 bp removed from the target monitored by Assay 6, while greatly enriched by capture alone (the hitch-hiker, cross-hyb effect), dropped significantly with processing.

Table 15: Cq values of quantitative PLP1 assay analysis of enrichment efficiencies

Raw Cq values				
Cq	Assay 2	Assay 5	Assay 6	Assay 7
gDNA	27	33	35	27
capture	15	12	17	32
cap+enz	13	11	24	31

Cq converted to absolute values				
abs val	Assay 2	Assay 5	Assay 6	Assay 7
gDNA	81	1	0	95
capture	253464	2538822	89450	3
cap+enz	1408479	4050620	506	4

Absolute values adjusted for dilutions of samples				
abs val	Assay 2	Assay 5	Assay 6	Assay 7
gDNA	81	1	0	95
capture	253463648	2538821701	89449912	2573
cap+enz	21127189405	60759299218	7584546	66931

Ratios of captured samples to gDNA				
/gDNA	Assay 2	Assay 5	Assay 6	Assay 7
capture	3126652	2394181716	239697693	
cap+enz	260618748	57297762664	20324204	

Ratios of unprocessed to processed sample				
enz/cap	Assay 2	Assay 5	Assay 6	Assay 7
cap+enz	83	24	0.08	

This experiment addressed the specificity of capture + processing – the non-target qPCR signal. The specific activity per ng of amplified DNA - from PLP1 exon 2 was greatly enhanced by post-capture processing. This experiment did not address sensitivity, that is, the percent of capture complexes that are converted by enzyme. A quantitative understanding of both specificity and sensitivity of the present method may also be important in particular embodiments.

EXAMPLE 9

DIRECT MEASUREMENT OF POST-CAPTURE PROCESSING

PURPOSE

In the Example 8, it was determined that post-capture processing achieved the desired aim of substantially increasing target capture specificity. The other critical parameter to be examined is sensitivity, *i.e.* the percentage of the initially captured complexes that are recovered in the final sequencing library. In this example, we

demonstrated by direct measurement of sensitivity that enzymatic processing is effective for >10% of the initially captured sequences.

SUMMARY

The data from this experiment indicated that 10% of the on-target capture complexes were processed by T4 polymerase into post-capture sequencing library fragments.

CONSIDERATIONS

By way of reference, a schematic illustration of post-capture processing is shown in Figure 4. Here, the sensitivity of processing was measured in a three-step procedure, which is illustrated on the lower right of Figure 13. First, single PLP1 capture probes were used in independent reactions to pull down/pull out PLP1 exon 2 specific genomic DNA fragments from the female gDNA library (Example 3: hgDNA library from Promega female). As there were four probes, four pull-downs were performed. The amount of captured material was measured using an adjacent PLP1 qPCR assay primer pair, as illustrated in Figure 13(A). Following enzymatic processing of the complex, again by qPCR, the amount of processed complex was measured by using one PLP1 specific primer and one probe-specific primer, as show in Figure 13(B). The ratio of the measurements in [B/A x 100%] yielded an estimate of processing efficiency. Critical to the correct interpretation of the experimental results, the PCR products were extracted from real-time reactions and verified that amplicons of the expected length were produced by gel analysis Figure 13(C). This was possible because both PCR reactions had discrete start and stop points. Pull-outs that yield interpretable data from A + B + C were used to determine processing efficiency.

ASSAYS

Individual probes needed to be matched to qPCR assays. Six combinations of probes matched to pre- and post-process qPCR assays were elected. These are shown below with probe sequences in italics, PLP1 exon2 specific primers shaded. The darker shaded primers are those that are paired with the CAC3 primer after processing. The expected product sizes of the PCR amplicons are also shown for each assay set:

SEQ ID NO:	NAME	SEQUENCE
116	Probe 1 and assay 1; qPCR assay 1 = 77 bp; CAC3↔R1 = 167 bp	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct acctactggatgtgcctgactgtttccccttcttcttccccagGCTTGTTAGAGTGCTG TGCAAGATGTCTGGTAGGGGCCCCCTTTGCTTCCCTGGT GGCCACTGGATTGTGTTTCTTTGGGGTGGCACTGTTCTGT GGCTGTGGACATGAAGCCCTCACTGGCACAGAAAAGCTA ATTGAGACCTATTTCTCCAAAAACTACCAAGACTATGAG TATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttccctctct ccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
117	Probe 1 and assay 2; qPCR assay 2 = 83 bp; CAC3↔R2 = 224 bp:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct acctactggatgtgcctgactgtttccccttcttcttccccagGCTTGTTAGAGTGCTG TGCAAGATGTCTGGTAGGGGCCCCCTTTGCTTCCCTGGT GGCCACTGGATTGTGTTTCTTTGGGGTGGCACTGTTCTGT GGCTGTGGACATGAAGCCCTCACTGGCACAGAAAAGCTA ATTGAGACCTATTTCTCCAAAAACTACCAAGACTATGAG TATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttccctctct ccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
118	Assay 3 and probe 4; qPCR assay 3 = 81 bp; F3↔CAC3 = 187 bp:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct acctactggatgtgcctgactgtttccccttcttcttccccagGCTTGTTAGAGTGC TGTGCAAGATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG GTGGCCACTGGATTGTGTTTCTTTGGGGTGGCACTGTTC TGTGGCTGTGGACATGAAGCCCTCACTGGCACAGAAAAG CTAATTGAGACCTATTTCTCCAAAAACTACCAAGACTAT GAGTATCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttc cctctctccatcctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
119	Assay 2 and probe 4; qPCR assay 2 = 83 bp; F2↔CAC3 = 216 bp:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct acctactggatgtgcctgactgtttccccttcttcttccccagGCTTGTTAGAGTGC TGTGCAAGATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG GTGGCCACTGGATTGTGTTTCTTTGGGGTGGCACTGTTC TGTGGCTGTGGACATGAAGCCCTCACTGGCACAGAAAAG

		CTAATTGAGACCTATTTCTCCAAAAACTACCAAGACTAT GAGTATCTCATCAATGT*gtaagtacctgccctcccacacagacccatctttttttc cctctctccatcctggagat*agagaactcttcagtaccttagtaactagcaggggactggggtgga
120	Probe 2 and assay 5; qPCR assay 5 = 95 bp; CAC3↔R5 = 209 bp:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct **acctactggatgtgcctgactgtttccccttcttcttccccag**GCTTGTTAGAGTGC TGTGCAAG***ATGTCTGGTAGGGGCCCCCTTTGCTTCCCTGGT GGCCACTGGATTGTGTTTCTTTGGGGT***GGCACTGTTCTGTGG CTGTGGAC**ATGAAGCCCTCACTGGCACAGAAAAGCTAAT TGAGACCTATTTCTCCAAAAACTACCAAG**ACTATGAGTA TCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttccctctctccat cctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga
121	Assay 4 and probe 3; qPCR assay 4 = 97 bp; F4↔CAC3 = 204 bp:	agtgcccactatctccgagcctgtgagcacagggcctggcagaggggtttgagtggcatgagct **acctactggatgtgcctgactgtttccccttcttcttccccag**GCTTGTTAGAGTGC TGTGCAAG**ATGTCTGGTAGGGGCCCCCTTTGCTTCCCTG GTGGCCACTGGATTGTGTTTCTTTGGGGT**GGCACTGTTC TGTGGCTGTGGAC***ATGAAGCCCTCACTGGCACAGAAAAGCT AATTGAGACCTATTTCTCCAAAAACTACCAAG***ACTATGAGTA TCTCATCAATGTgtaagtacctgccctcccacacagacccatctttttttccctctctccat cctggagatagagaactcttcagtaccttagtaactagcaggggactggggtgga

METHODS

Probes. In these assays the B10 universal oligo set of probes (24 August 2012 Experiment 4 - Capture of PLP1 exon 2) was chosen. To make individual capture probes, 1 µl of universal oligo 10 (100 µM) was combined with 1 µl of 100 µM probe ultramer and 98 µl of TEz+0.05% Tween20. This was further diluted 4 µl into 996 µl TEz + Tween to provide a 4 nM working solution.

Capture. For capture 50 µl of 22 ng/µl gDNA library, 20 µl of 4X binding buffer, 1 µl of probe and 9 µl of water were combined. There were six independent capture reactions (two with probe #1, two with probe #4, one with probe #2 and one with probe #3). These were heated to 95°C for 1 min then cooled in 35 "cycles" of -1°C and 2 min to 60°C as described earlier. Following annealing, 10 µl of washed

beads (= 1 µl of stock beads) was added and binding was incubated for 20 min at RT. Beads were then pulled aside and washed 4 times, 5 min each, with 200 µl aliquots of wash buffer. After the last wash, all remaining accessible fluid was aspirated from the beads.

Processing. Beads were resuspended in 10 µl of quick blunt solution (200 µl = 20 µl of 10X quick blunting buffer, 20 µl of 1 mM dNTP and 160 µl water). Each of the six aliquots of beads was split into two 5 µl aliquot. 5 µl of QB buffer without enzyme was added to one set of tubes (these are the capture-only aliquot). To the other 5 µl aliquots, 5 µl of QB buffer containing 0.025 µl of T4 polymerase (this was made by combining 100 µl QB buffer with 0.5 µl of T4 polymerase and distributing 5 µl aliquots) was added. Both the capture only and the capture + processing tubes were incubated at 20°C for 15 min, 98°C for 1 min, cooled to RT and placed immediately on magnets. ~10 µl of supernatant was pulled from the six pairs of capture-only and T4-processed complexes (now 12 tubes total). These supernatants were used directly in qPCR as described below.

qPCR. For these assays, standard Taq reaction mix and 3 step thermal cycling were chosen. Twelve of 40 µl qPCR mix were constructed, each containing:

- 14 µl of water
- 4 µl of 10X STD Taq buffer
- 4 µl of 25 mM MgCl2
- 4 µl of a blend of F and R primer at 10 µM each
- 8 µl of template (supernatant from above)
- 2 µl of DMSO
- 2 µl of 10 mM dNTPs
- 1 µl of EvaGreen
- 0.8 µl of ROX
- 0.4 µl of Taq polymerase

Reactions were distributed in quads and cycled for 40 cycles of 94°C-30sec, 55°C-30sec, 72°C-60sec. Following PCR, reaction mix was pooled from each of the four wells of the quad and 5 µl was analyzed on 2% agarose gels.

RESULTS

To interpret the experimental data, the agarose gels that are shown in Figure 14 were examined. Under the cycling conditions used with the primers used (etc.) it was observed that assay sets 3, 5 and 6 yielded PCR products consistent with the assay amplicon (top gel) or the post-processed PLP1 to adaptor amplicons (bottom gel). The more successful assay sets corresponded to:

- Probe 4 with assay 3
- Probe 2 with assay 5
- Probe 3 with assay 4

The qPCR Cq values are shown in Table 16 below. Assays 1 and 2 failed gel analysis. Successful assays are shown in assays 3, 5 and 4. To derive % processing values, Cq's were converted to absolute values (In "Excel speak", Abs value = power(10,log10(1/2)*Cq+10). The quotient of post-processed over capture only was then expressed as a percentage. This measurement assumed that the amplification efficiency of the all amplicons was the same and conforms to an idealized standard curve (probably reasonably accurate). Assuming this was correct, then approximately 10% of captured material appears to be processed.

Table 16: qPCR analysis of post-capture processing sensitivity

	probe 1	probe 1	probe 4	probe 4	probe 2	probe 3
	assay 1	assay 2	assay 3	assay 2	assay 5	assay 4
capture			25		25	26
post-process			29		28	29
% processed			7%		17%	10%

EXAMPLE 10

CONSTRUCTION OF EXPANDED CODE MALE AND FEMALE GDNA LIBRARIES

PURPOSE

Build set of 16 coded male and female gDNA libraries that will be used to test multiple capture parameters in a single MiSeq sequencing run.

METHODS

Step 1: gDNA. Repaired gDNA was prepared.

Step 2: All 16 possible adaptor codes were generated. These codes are four base structures in which the base positions at -4 and -3 (relative to the insert) are random bases and the base positions at -2 and -1 are sample codes. There are four "clusters" of sample codes. These are:

- Cluster 1: AC, GA, CT, TG
- Cluster 2: AA, GC, CG, TT
- Cluster 3: AG, GT, CA, TC
- Cluster 4: AT, GG, CC, TA

Clusters 2 – 4 were ordered as 100 μM oligos in plates. One set of plates had ligation strand and one set of plates had partner strands. The plate array was A1-H1, A2-H2, etc. To anneal adaptors in two sets of 96 well PCR plates, 70 μl/well of "annealing solution" containing 68 μl of TEz and 2 μl of 5M NaCl was added to 20 μl partner strand oligo and 10 μl ligation strand oligo, covered with tape, and annealed 10 sec at 95°C, 5 min 65°C, and cooled to RT. Pooled sets of 16 – random codes that have the same sample code – into sets of four. Red = set AA, GC, CG and TT. Purple = set AG, GT, CA and TC. Blue = set AT, GG, CC and TA (laid out in this order).

Step 3: It is easiest to create 16 ligation for female DNA and 16 ligations for male DNA in which both types received the same set of 16 unique adaptor types. This will allow us to decide later which combinations of samples we want to create with maximum flexibility. To do this, End-repaired gDNA from the experiment was used. I will perform the requisite 32 ligation in 20 μl/rxn as follows:

Two gDNA cocktails were made, one female and one male, that contain:

- 144 μl water
- 32 μl 10X lig buffer
- 48 μl 50% PEG8000
- 64 μl gDNA

The cocktails were mixed and aliquoted into 16 tubes with 18 μl each. 2 μl of adaptors and 0.5 μl HC T4 ligase were added and the resulting reactions were incubated at 22°C for 60 min, 65°C for 10 min, and cooled to RT. 80 μl TEz, then 120 μl Ampure beads were also added to the reactions, mixed and incubated 10 min at RT.

The reactions were wash twice with 200 µl of 70% EtOH/water (v/v), air dried, and resuspended in 100 µl TEz.

Step 4: qPCR. Make qPCR master mix containing:

- 175 µl of water
- 50 µl of 10X STD Taq buffer
- 50 µl of 25 mM MgCl2
- 100 µl of ACA2 primer (10 µM)
- (50 µl of template – added later)
- 25 µl of DMSO
- 25 µl of 10 mM dNTPs
- 12.5 µl of Eva green
- 10 µl of ROX
- 5 µl of Taq DNA polymerase

9 µl was distributed into the 48 wells of an Illumina Eco qPCR plate. Two serial dilutions of library calibration standard were made that are 10 pg/µl and 1 pg/µl. The remainder of the plate was loaded with libraries as shown in the tables below.

Table 17: Sample key for 48 well assay plate 1

	1	2	3	4	5	6	7	8
A	10 pg/ul STD	1 pg/ul STD	F1	F2	F3	F4	F5	F6
B	10 pg/ul STD	1 pg/ul STD	F1	F2	F3	F4	F5	F6
C	F7	F8	F9	F10	F11	F12	F13	F14
D	F7	F8	F9	F10	F11	F12	F13	F14
E	F15	F16	M1	M2	M3	M4	M5	M6
F	F15	F16	M1	M2	M3	M4	M5	M6

The second plate had the layout shown in Table 18 below.

Table 18: Sample key for 48 well assay plate 2

	1	2	3	4	5	6	7	8
A	100 pg/ul STD	10 pg/ul STD	1 pg/ul STD	NTC	F15	M1	M2	M3
B	100 pg/ul STD	10 pg/ul STD	1 pg/ul STD	NTC	F15	M1	M2	M3
C	M1	M2	M3	M4	M5	M6	M7	M8
D	M1	M2	M3	M4	M5	M6	M7	M8
E	M9	M10	M11	M12	M13	M14	M15	M16
F	M9	M10	M11	M12	M13	M14	M15	M16

Ligation efficiency was measured by the following cycling program:

- 72°C – 2min
- 94°C – 30 sec, 60°C – 30 sec, and 72°C – 60 sec; 40 cycles

RESULTS

Table 19 below shows the Cq values of STDs and samples (the average of duplicate measurements except (i) the experiment repeated on plate 2 and (ii) M1, M2 and M3 were measured in three sets of duplicates –average of the three measurements taken).

Table 19

	Raw Ct value averages							
plate 1	9	13	9	10	9	10	9	9
	10	9	9	9	9	9	9	9
	14	9	9	9	9	9	9	9
plate 2	#DIV/0!	9	13	#DIV/0!	9	9	9	9
	9	9	9	9	9	9	10	9
	9	9	10	9	9	9	9	9
	Converted to abs values							
plate 1	1583	164	1984	1338	1739	970	2225	1756
	869	1729	1981	2142	1555	1751	2002	1688
		1729	2346	1947	1817	1545	2337	1697
plate 2	#DIV/0!	1469	151	#DIV/0!	1843	2218	1495	1489
	2308	1622	1885	1439	1834	1500	852	1733
	1822	2061	1340	1812	2100	1689	1857	2168
	Normalized to pg/ul							
plate 1	10	1	13	8	11	6	14	11
	5	11	13	14	10	11	13	11
		11	15	12	11	10	15	11
plate 2	#DIV/0!	10	1	#DIV/0!	13	15	10	10
	16	11	13	10	12	10	6	12
	12	14	9	12	14	11	13	15
	Genomes per ul of ligation mix							
plate 1			4	2	3	2	4	3
	2	3	4	4	3	3	4	3
		3	4	4	3	3	4	3
plate 2					4	4	3	3
	5	3	4	3	4	3	2	3
	4	4	3	4	4	3	4	4

These were converted to arbitrary absolute values by the equation quantity = power(10,log10(1/2)*Cq + 8) in Excel (blue shading). Values were then normalized to the known standards (red shading) by multiplying abs values by 10/1583 (plate 1) or 10/1469 (plate 2). Genomes per µl was calculated by multiplying by 7/8 (to account for adaptor mass) and then dividing by 3 pg/genome. The ligation efficiency was

calculated (20 ng/ligation & 1/100th measured = 200 pg into ligation), and the calculated efficiency indicated that ~5% conversion to library is about average. This was the same for libraries made without fill-in, which suggested that the fill-in reaction has rapid kinetics and can occur as the sample heats to 94°C in the first cycle.

Table 20: Library concentrations (genomes / µl)

	1	2	3	4	5	6	7	8
	10 pg/ul STD	1 pg/ul STD	F1	F2	F3	F4	F5	F6
	10 pg/ul STD	1 pg/ul STD	4	2	3	2	4	3
	F7	F8	F9	F10	F11	F12	F13	F14
	2	3	4	4	3	3	4	3
	F15	F16	M1	M2	M3	M4	M5	M6
	4	3	4	4	3	3	4	3
	1	2	3	4	5	6	7	8
	100 pg/ul STD	10 pg/ul STD	1 pg/ul STD	NTC	F15	M1	M2	M3
	100 pg/ul STD	10 pg/ul STD	1 pg/ul STD	NTC	F15	4	3	3
	M1	M2	M3	M4	M5	M6	M7	M8
	5	3	4	3	4	3	2	3
	M9	M10	M11	M12	M13	M14	M15	M16
	4	4	3	4	4	3	4	4

The goal of this experiment was to create ligation mixes containing gDNA library and to quantify genome equivalents per µl of ligation mix so that measured numbers of genomes can be amplified into microgram quantities of library material. Table 20 above shows the genomes per µl for each library that was made. The goal of Table 21, shown below, was to convert designated samples (picked by random drawing) into 10 copy, 20 copy, 40 copy, 80 copy, etc. libraries for downstream capture tests. The table transposes the genomes per µl into µl per PCR reaction to achieve the indicated depth of coverage. The table assumes a 200 µl PCR per sample and 40 µl of template input. These experiments may be used as guides where actual libraries are generated and purified.

Table 21: Calculation of the volume of each sample library needed for desired genome representation

ıle	genomes/ul		# genomes needed	ul to get	H2O	Sample	genomes/ul			# genomes needed	ul to get	H2O
	4		10.0	2.7	37.3	M1	4	ave				
	2	C	40.0	16.2	23.8	M2	3	ave				
	3					M3	3	ave	C	40.0	11.9	28.1
	2		80.0	44.8	-4.8	M4	3					
	4		20.0	4.9	35.1	M5	4					
	3		20.0	6.2	33.8	M6	3					
	2		40.0	25.0	15.0	M7	2					
	3					M8	3			80.0	23.3	16.7
	4					M9	4					
	4		40.0	10.1	29.9	M10	4					
	3					M11	3			40.0	15.0	25.0
	3					M12	4			10.0	2.8	37.2
	4					M13	4			20.0	4.8	35.2
	3					M14	3					
	4					M15	4			40.0	10.9	29.1
	3					M16	4			20.0	4.6	35.4

EXAMPLE 11

VALIDATION OF 8 NEW CAPTURE QPCR ASSAYS

PURPOSE

Validate the performance of eight new qPCR primer sets designed to follow capture efficiency of the expanded probe collection.

SUMMARY

All eight assays produced amplicons of the expected size when used to amplify human gDNA. Quantitative analysis of the chrX:154376051 region (4X in females, 2X in males) showed a surprisingly tight correlation between observed and expected copies.

METHODS

Eight segments for assay design that represent a sampling of the 49 probe target regions were chosen. To design assays, the DNA segment that is within 200 bp of the 5′ end of the probe was identified. The eight regions, as shown in Table 22 below, were chosen to be a more-or-less random selection of target regions. The 200 bp segments were submitted to PRIMER3 PCR primer picking in which we specified amplicons of 50-100 bp, primer Tm's of 65°C (optimal) and primer lengths of 24 nt (optimal). Table

22 below shows the region and unique genomic attributes, the forward (F) and reverse (R) primer sequences, the expected amplicon length, and the actual amplicon within the context of the genomic sequence.

Table 22: Genomic capture targets, and primers for their interrogation

SEQ ID NO:	Assay	Region	Feature	Name	Sequence	Product
122	9	CYP2D6	4X in males and females	CYP2D6_F	GGCTTCGACTGAACGTCTCCA	53 bp
123				CYP2D6_R	AGTGCTCCATGGCTGCTCAGTT	
124	10	chrX-154376051	4X in females, 2X in males	chrX-15_1_F	GAACCAGAGGAACGCTGTGGTAACT	52 bp
125				chrX-15_1_R	GACGTGTGCCTAGATGCGTTTTC	
126	11	chrX-154376051	4X in females, 2X in males	chrX-15_2_F	TGGCTGAAAAGTCTCCTTGAAACTG	53 bp
127				chrX-15_2_R	CTCAGTGGGTCTCCTTGAGAGAGGT	
128	12	chrX-6929264	6X in females, 5X in males (2 on chr19, one on chrX)	chrX-69_F	CTTTCCTCCAGTCACAAGCCATCTA	78 bp
129				chrX-69_R	CATGCTGATAGAAAGTCCCCTGGTA	
130	13	KRAS region 1	2X in females and males	KRAS_r1_F	TTTTCACACAGCCAGGAGTCTTTTC	66 bp
131				KRAS_r1_R	GGGAGATCCGACAATACAGATTGAA	
132	14	KRAS region 2	2X in females and males	KRAS_r2_F	CCTGTCTTGTCTTTGCTGATGTTTC	73 bp
133				KRAS_r2_R	ACAAAACAGGCTCAGGACTTAGCAA	
134	15	MYC region 2	2X in females and males	MYC_r2_1_F	CAACGTTAGCTTCACCAACAGGAAC	92 bp
135				MYC_r2_1_R	GCTGGTAGAAGTTCTCCTCCTCGTC	
136	16	MYC region 2	2X in females and males	MYC_r2_2_F	GTCTGCTCCACCTCCAGCTTGTA	93 bp
137				MYC_r2_2_R	GTTGAGAGGGTAGGGGAAGACCAC	

The performance of each primer pair was investigated by performing 100 µl PCR reactions that contained 200 ng (2 ng/µl) of female genomic DNA. The reaction mixes contained, per 100 µl, 50 µl water, 10 µl 10X STD Taq buffer, 10 µl 25 mM MgCl2, 10 µl of an F+R primer blend in which each primer was present at 10 µM, 10 µl of 20 ng/µl gDNA, 5 µl of DMSO, 5 µl of 10 mM dNTPs, and 1 µl of Taq polymerase. Reactions were set up on ice. Amplifications were performed for 30 cycles of 94°C-30sec, 60°C-30 sec, and 72°C-30sec, followed by a 2 min incubation at 72°C and hold at 10°C. Five µl of PCR product was examined on a 2% agarose gel.

PCR products were purified on Qiagen PCR purification columns by combining the remaining 95 µl of PCR product with 500 µl PB. The material was spun through the column at 6 KRPM for 30 sec and washed with 750 µl PE, spun at 13.2 KRPM. The products were eluted from the columns with 50 µl of EB and quantified by Qubit.

For qPCR analysis, the chrX-154376051 region (assays 10 & 11) was examined in more detail. The purified PCR products were diluted to 100 fg/µl, 10 fg/µl and 1 fg/µl. Genomic DNA was diluted to 10 ng/µl. Two microliters of either standards or gDNA was combined with 8 µl of PCR master mix per well of the 48-well Eco qPCR plate. Master mix contained, per 500 µl of final reaction volume (that accounts for the addition of template) 175 µl of water, 50 µl of 10X STD Taq buffer, 50 µl of 25 mM $MgCl_2$, 50 µl of F+R 10 µM primer blend, 25 µl of DMSO, 25 µl of 10 mM dNTPs, 12.5 µl EvaGreen, 10 µl of ROX and 5 µl of Taq polymerase. Thirty two µl of mix was distributed to 16 wells and 8 µl of templates were added. These were then distributed in quads to the qPCR plate. The plate layout is shown in Table 23 below.

Table 23: Plate layout for qPCR interrogation of chrX-154376051 region

	1	2	3	4	5	6	7	8
A	100 fg PCR prod		10 fg PCR prod		1 fg PCR prod		NTC	
B								
C	100 fg PCR prod		10 fg PCR prod		1 fg PCR prod		NTC	
D								
E	F-gDNA		M-gDNA		F-gDNA		M-gDNA	
F								

	Assay 10
	Assay 11

RESULTS AND DISCUSSION

Gel analysis of the PCR products amplified from genomic DNA showed that all eight PCR reactions produced unique products of the expected size (data not shown). The amplicons were sufficiently clean (no extra bands, no left-over primer) and were useful for generating standard curves for quantitative analysis. The amplicons were purified using Qiagen PCR spin columns and eluted products in 50 µl. The product yields were: Assay 9 – 18.4 ng/µl; Assay 10 – 26.1 ng/µl; Assay 11 – 13.9 ng/µl; Assay 12 – 26.6 ng/µl; Assay 13 – 7.9 ng/µl; Assay 14 – 19.2 ng/µl; Assay 15 – 23.1 ng/µl; and Assay 16 – 20.4 ng/µl.

Quantitative analysis was performed with assays 10 & 11 that correspond to a cryptic segmental duplication on chromosome X such that females have four copies and males have two copies.

The average Cq values are shown in Table 24 below. These were used to generate the standard curves shown. The two reactions were basically superimposable. Using these curves we calculated the absolute quantities in units of femtograms of both the STD curve wells and the genomic input wells. The data are shown in Table 24 below the standard curve data.

Table 24: Cq values of standard samples analyzed with assays 10 and 11

STD curve 10	13	17	20	31
STD curve 11	13	16	20	33
F then M samples	21	22	21	22

One point of this example was to emphasize the strength of quantitative molecular biology. In this experiment, 2 µl of STDs were added and sampled, meaning that the 1 fg/µl STD really had 2 fg in the qPCR reaction. This corresponds to 17,500 molecules of the 53 bp fragment of Assay 10. 20 ng of genomic DNA were put into the reaction. This corresponds to 6667 genomes worth of DNA. The genomic DNA was fragmented to an average size of 200 bp, meaning that only 75% of the target regions will remain intact. Hence, the gDNA had about 5000 "qPCR actionable" genome copies. Finally, in males the expected average of one copy of the duplicated X region

per genome, and in females the expected average was two. The expected versus observed values, broken out by numbers of molecules observed, turned out to be as follows: Expected males = 5000 copies; Observed males = 3500 copies; Expected females = 10000 copies; and Observed females = 7000 copies.

Table 25: Expected vs. Observed Values

STD curve 10	102.8004	9.421966	1.02996
F then M samples	0.447179	0.204926	
STD curve 11	98.27451	10.35326	0.983644
F then M samples	0.388559	0.174757	

EXAMPLE 12

FURTHER POST-CAPTURE PROCESSING STRATEGY

PURPOSE

An alternative method to achieve post-capture processing (see Figure 15) was developed.

SUMMARY

Post-capture processing steps performed with redesigned probes appeared to enhance the already robust capture by an additional 5 – 9-fold. Overall the test was very successful.

BACKGROUND

In other embodiments of the assay design, it was contemplated to use an exonuclease step at the 3′ end of clones prior to copying of the probe tail sequence that added a PCR priming site. The particular embodiments, it was further contemplated to shift from having the clone copy the probe to having the probe copy the clone. The reversal in polarity means that we use the 5′ end of the probe as both the pull-down sequence *and* the reverse PCR primer sequence. The 3′ end of the probe is left unmodified and can then copy the clone using DNA polymerase. Conceptually, there are some advantages to this approach. First, because there was a shift from a step that

required both exonuclease activity and polymerization to a simple polymerization step, this step can be done in concert with PCR. Moreover, this step can be done at 72°C with a thermostable polymerase enzyme, meaning potential secondary structures of single strand clones are less of an issue. Finally, the implication was that probes were shortened from 114 nt to 95 nt; and this provided a cost saving advantage.

Four well behaved qPCR assays (Example 11: Validation of eight new capture qPCR assays), assays 10, 14, 15 and 16 with probes that "point" at those assays were matched. While the probe and qPCR assays were within proximity to one another, their DNA sequences did not overlap with one another (See Figure 16). The sequences of the probes and corresponding assays are shown in Tables 26 and 27 below.

Table 26: Genomic capture targets and primers for their interrogation

SEQ ID NO:	Assay	Region	Feature	Name	Sequence	Product
138	10	chrX-154376051	4X in females, 2X in males	chrX-15_1_F	GAACCAGAGGAACGCTGTGGTAACT	52 bp
139				chrX-15_1_R	GACGTGTGCCTAGATGCGTTTTC	
140	14	KRAS region 2	2X in females and males	KRAS_r2_F	CCTGTCTTGTCTTTGCTGATGTTTC	73 bp
141				KRAS_r2_R	ACAAAACAGGCTCAGGACTTAGCAA	
142	15	MYC region 2	2X in females and males	MYC_r2_1_F	CAACGTTAGCTTCACCAACAGGAAC	92 bp
143				MYC_r2_1_R	GCTGGTAGAAGTTCTCCTCCTCGTC	
144	16	MYC region 2	2X in females and males	MYC_r2_2_F	GTCTGCTCCACCTCCAGCTTGTA	93 bp
145				MYC_r2_2_R	GTTGAGAGGGTAGGGGAAGACCAC	

Table 27: Capture probe sequences and genomic targets

SEQ ID NO:	Assay	Region	60 mer probe sequence
146	10	chrX-154376051_2x_chrX:154376979:region_1:1011nt:929:988:-	AGAATTCATTGCCAGCTATAAATCTGTGGAAACGCTGCCACACAATCTTAGCACACAAGA
147	14	KRAS_NM_033360_chr12:25378485:region_2:161nt:-62:-3:+	TAAATGACATAACAGTTATGATTTTGCAGAAAACAGATCTGTATTTATTTCAGTGTTACT
148	15	MYC_NM_002467_chr8:128750431:region_2:773nt:-62:-3:+	AGGTTTCCGCACCAAGACCCCTTTAACTCAAGACTGCCTCCCGCTTTGTGTGCCCCGCTC
149	16	MYC_NM_002467_chr8:128751122:region_2:773nt:630:689:-	AGAGCAGAGAATCCGAGGACGGAGAGAAGGCGCTGGAGTCTTGCGAGGCGCAGGACTTGG

METHODS

A gDNA library was remade from samples F13-F16 (Example 10) by combining 20 µl of each ligation mix into 80 µl total and amplifying in 800 µl total. Beads were cleaned to 400 µl and pool concentration at 32 ng/µl was measured by Qubit.

Oligos from IDT, listed below, were resuspended to 100 µM. ultramers come as 4 nmol, so these were suspended in 40 µl TEzero. Four 2 µl aliquots of the four test probes were combined with 8 µl of 100 µM universal tail sequence (derived from the first 35 bases of the full length reverse primer #9) to give a 50 µM tube of duplex. This duplex was diluted 10 µl into 990 µl of TEzero + Tween to give 500 nM, and 10 into 990 again to get 5 nM.

Combined 40 µl of gDNA was combined with 15 µl of 4X bind and 5 µl of capture duplex. The reaction mix was annealed and captured on 2 µls of washed MyOne strep coated beads. The reaction was washed four times with wash buffer and aspirated wash buffer from bead pellet. To measure capture alone, one bead pellet was resuspended in 100 µl PCR mix that contained single PCR primer ACA2. To measure capture + processing, another bead pellet was resuspended in 100 µl of PCR mix that contained the full-length ACA2 forward primer (oligo #8) and full-length CAC3 reverse primer (oligo #9). The latter sample was incubated for 72°C for 2 min. Both samples were amplified for 25 cycles of 94°C-30sec, 60°C – 30sec, and 72°C-60 sec.

After a hold at 72°C for 2min and cooling to RT, the PCR amplicons were purified on bead and resuspended in 50 µl of TEzero.

For qPCR, samples were assayed with assays 9 – 16 (assays 10, 14, 15, and 16 are targets), using EvaGreen as the reporter dye, ROX as the STD dye, and 3 step PCR of 94°C-30sec, 60°C-30sec and 72°C-60sec for 40 cycles. The original gDNA library was present at 2 ng/µl final concentration. The captured sample and capture+processed samples were present at 2 pg/µl final concentration (diluted in TEzero+0.05% Tween20).

RESULTS AND DISCUSSION

PCR yields of the capture only was 27.8 ng/µl and of the capture + processing was 40.4 ng/µl. These robust yields indicated that amplification went to completion. The 2% agarose gel image shows the starting input library, the captured library and the capture + processing library (Figure 17). If processing worked, the average insert size of the library should decrease, which it did. The fact that the bottom end of the library is more or less "a band" indicates that there may be some priming off of probe. Because the 3′ end of our probes are exposed in this format, it may be possible to eliminate residual, unbound probe with Exonuclease I, a ssDNA specific, 3′→5′ exonuclease.

An important metric in this experiment was qPCR measurement of capture sensitivity and specificity. qPCR data is shown in Table 28 below.

Table 28: qPCR analysis of capture sensitivity and specificity

Raw Cq	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA lib	20	19	20	19	20	19	20	20
1st cap	20	15	23	21	20	15	16	15
cap + proc	23	13	27	20	20	12	13	13

Abs value	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA lib	122	188	114	211	76	184	85	79
1st cap	72	3240	15	59	85	2305	1231	2197
cap + proc	11	16518	1	69	97	19504	11189	13275

Adj for dil	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA lib	122	188	114	211	76	184	85	79
1st cap	71606	3239810	14835	58862	84616	2304816	1230995	2196798
cap + proc	10793	16517508	713	69047	97429	19504350	11189209	13274986

Fold enric	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
1st cap	585	17261	131	279	1108	12530	14513	27753
cap + proc	88	88000	6	328	1276	106034	131921	167709
c+p/c	0	5	0	1	1	8	9	6

With respect to specificity, only those regions targeted (light gray highlight) exhibited significant enrichment. Moreover, the processed library had a significant increase in specific activity for all target regions relative to capture alone. These data indicated that this additional probe design embodiment could be used for efficient post-capture processing.

EXAMPLE 13

SEQUENCE ANALYSIS OF POST-CAPTURE PROCESSING STRATEGY

PURPOSE

The purpose of this experiment was to assess the enrichment and coverage of target regions in sequencing libraries.

SUMMARY

The level of enrichment and focusing of target sequences was dramatically improved by coupling hybridization-based capture with enzymatic processing, compared to capture alone.

BACKGROUND

Prior experiments disclosed herein have demonstrated that post-capture processing increases the target content and specific activity of enrichment libraries as measured by qPCR. In this experiment, next generation DNA sequencing was used to compare the representation and distribution of target sequences in libraries generated by capture alone or by the alternative processing method.

METHODS

Two enrichment library pools were constructed from an equal mix of male and female human genomic DNA using a set of 49 capture probes that target sites within specific genes (KRAS, MYC, PLP1, CYP2D6 and AMY1) and duplicated regions on the X chromosome. Probe sequences are shown in Table 29 below

Table 29: qPCR analysis of capture sensitivity and specificity

SEQ ID NO:	No.	Probe Name	Probe Sequence
150	1	CYP2D6_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AAGCACCTAGCCCCATTCCTGCTGAGCAGGAGGTG GCAGGTACCCCAGACTGGGAGGTAA
151	2	CYP2D6_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AGTCGGTGGGGCCAGGATGAGGCCCAGTCTGTTCA CACATGGCTGCTGCCTCTCAGCTCT
152	3	AMY1_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC ACCTGAGTAGCATCATTGTAGTTCTCGATATCTCCA CTTCCAGTTTTACATTTACCATCA
153	4	chrX_15_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CCTGGCCCTCAGCCAGTACAGAAAGTCATTTGTCA AGGCCTTCAGTTGGCAGACGTGCTC
154	5	chrX_15_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AGAATTCATTGCCAGCTATAAATCTGTGGAAACGC TGCCACACAATCTTAGCACACAAGA
155	6	chrX_477_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GACTTCAAAGAAATTACAAGTTGACATCTTGGACT CTACCCCTCGTACTTTATCTCCTAT
156	7	chrX_477_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TCTCTTTGGGGTCAAGAAAGAATCCCTAGTGGATT TGGGATTCTAGAGGAGGTGTTATAA
157	8	chrX_478_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TGCGATACCATGCTGAAGATGAGCTAACCCAACCA GCCAAGCAGGCAGGGCTGCGAAGGA

158	9	chrX_478_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GGGGTAGGTGGAAAACCCAAGTAATGTGATTTTGT AACATCCACTGCTGCATTTGTTTGC
159	10	chrX_69_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TTACTTCCCTCCAGTTTTGTTGCTTGCAAAACAACA GAATCTTCTCTCCATGAAATCATG
160	11	chrX_69_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CAGGGGTATCTATTATCCCCATTTTCTCACAAAGG AAACCAAGATAAAAGGTTTAAATGG
161	12	PLP1_ex1_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GAAATTCTCTTGTGAATTCCTGTGTCCTCTTGAATC TTCAATGCTAAAGTTTTTGAAACT
162	13	PLP1_ex2_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GGGTTTGAGTGGCATGAGCTACCTACTGGATGTGC CTGACTGTTTCCCCTTCTTCTTCCC
163	14	PLP1_ex2_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CTATCTCCAGGATGGAGAGAGGGAAAAAAAAAGAT GGGTCTGTGTGGGAGGGCAGGTACTT
164	15	PLP1_ex3_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GAAAGAAGCCAGGTCTTCAATTAATAAGATTCCCT GGTCTCGTTTGTCTACCTGTTAATG
165	16	PLP1_ex3_M	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CAGACTCGCGCCCAATTTTCCCCCACCCCTTGTTAT TGCCACAAAATCCTGAGGATGATC
166	17	PLP1_ex3_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TCTTTCTTCTTCCTTTATGGGGCCCTCCTGCTGGCT GAGGGCTTCTACACCACCGGCGCA
167	18	PLP1_ex4_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GTTTGTGTTTCTACATCTGCAGGCTGATGCTGATTT CTAACCACCCCATGTCAATCATTT
168	19	PLP1_ex4_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AACCAAATATATAGTGCTTCCATAGTGGGTAGGAG AGCCAAAGCACCCGTACCCTAACTC
169	20	PLP1_ex5_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AGTCTCCATGTGGCCCCGTAACTCCATAAAGCTTA CCCTGCTTGCTTTTTGTGTCTTACT
170	21	PLP1_ex5_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CCATGGGTGTAATTTGTATGGTATTAGCTACTCCCT TGTAAAATAACCCAAATAACCCAC
171	22	PLP1_ex6_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TTTACAGTGGAGCATATTACTGCTGTTGCAAGAAA CAGTTCTTCCTCTTTCATTTTCCTG
172	23	PLP1_ex6_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC ATAGCTGTACCCACACTATCTCAGGCCTATTTACTT GCCAAGATCATTCAAAGTCAACTC

173	24	PLP1_ex7_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GATTTGAGGAGGGAGTGCTTTCTTTTCTACTCTCAT TCACATTCTCTCTTCTGTTCCCTA
174	25	PLP1_ex7_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CAGCATTGTAGGCTGTGTGGTTAGAGCCTCGCTAT TAGAGAAAGGGGGATTTCTACGGGG
175	26	KRAS_ex1_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TGTTACCTTTAAAAGACATCTGCTTTCTGCCAAAAT TAATGTGCTGAACTTAAACTTACC
176	27	KRAS_ex1_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TTCCCAGTAAATTACTCTTACCAATGCAACAGACT TTAAAGAAGTTGTGTTTTACAATGC
177	28	KRAS_ex2_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TAAATGACATAACAGTTATGATTTTGCAGAAAACA GATCTGTATTTATTTCAGTGTTACT
178	29	KRAS_ex2_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GACAGGTTTTGAAAGATATTTGTGTTACTAATGAC TGTGCTATAACTTTTTTTTCTTTCC
179	30	KRAS_ex3_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC ACTCAAAAAATAAAAACTATAATTACTCCTTAATG TCAGCTTATTATATTCAATTTAAAC
180	31	KRAS_ex3_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AACACCTTTTTTGAAGTAAAAGGTGCACTGTAATA ATCCAGACTGTGTTTCTCCCTTCTC
181	32	KRAS_ex4_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GAAACCTTTATCTGTATCAAAGAATGGTCCTGCAC CAGTAATATGCATATTAAAACAAGA
182	33	KRAS_ex4_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GTGTATTAACCTTATGTGTGACATGTTCTAATATAG TCACATTTTCATTATTTTTATTAT
183	34	MYC_r1_F1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CCCCAGCCAGCGGTCCGCAACCCTTGCCGCATCCA CGAAACTTTGCCCATAGCAGCGGGC
184	35	MYC_r1_R1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CGACTCATCTCAGCATTAAAGTGATAAAAAAATAA ATTAAAAGGCAAGTGGACTTCGGTG
185	36	MYC_r2_F1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC CTGTGGCGCGCACTGCGCGCTGCGCCAGGTTTCCG CACCAAGACCCCTTTAACTCAAGAC
186	37	MYC_r2_F2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TTCTACTGCGACGAGGAGGAGAACTTCTACCAGCA GCAGCAGCAGAGCGAGCTGCAGCCC
187	38	MYC_r2_F3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC ACCGAGCTGCTGGGAGGAGACATGGTGAACCAGA GTTTCATCTGCGACCCGGACGACGAG

188	39	MYC_r2_F4	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GCCGCCGCCTCAGAGTGCATCGACCCCTCGGTGGT CTTCCCCTACCCTCTCAACGACAGC
189	40	MYC_r2_R1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GGCGGCTAGGGGACAGGGGCGGGGTGGGCAGCAG CTCGAATTTCTTCCAGATATCCTCGC
190	41	MYC_r2_R2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AGACGAGCTTGGCGGCGGCCGAGAAGCCGCTCCA CATACAGTCCTGGATGATGATGTTTT
191	42	MYC_r2_R3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AGGAGAGCAGAGAATCCGAGGACGGAGAGAAGGC GCTGGAGTCTTGCGAGGCGCAGGACT
192	43	MYC_r2_R4	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TAAGAGTGGCCCGTTAAATAAGCTGCCAATGAAAA TGGGAAAGGTATCCAGCCGCCCACT
193	44	MYC_r3_F1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TTGTATTTGTACAGCATTAATCTGGTAATTGATTAT TTTAATGTAACCTTGCTAAAGGAG
194	45	MYC_r3_F2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GAGGCCACAGCAAACCTCCTCACAGCCCACTGGTC CTCAAGAGGTGCCACGTCTCCACAC
195	46	MYC_r3_F3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC AGAGGAGGAACGAGCTAAAACGGAGCTTTTTTGCC CTGCGTGACCAGATCCCGGAGTTGG
196	47	MYC_r3_R1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC TCCAACTTGACCCTCTTGGCAGCAGGATAGTCCTT CCGAGTGGAGGGAGGCGCTGCGTAG
197	48	MYC_r3_R2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GCTTGGACGGACAGGATGTATGCTGTGGCTTTTTT AAGGATAACTACCTTGGGGGCCTTT
198	49	MYC_r3_R3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCAC GCATTTGATCATGCATTTGAAACAAGTTCATAGGT GATTGCTCAGGACATTTCTGTTAGA

The first library pool was generated as described for the ‘capture plus processing’ library in Example 12. The second pool was generated as described for the ‘capture only’ library in Example 12 except for the following modification. After capture, a second round of PCR was performed to convert single primer, ACA2-amplified libraries into dual primer, heterologous end libraries suitable for Illumina sequencing. To do this, libraries were diluted and re-amplified with the following primers: primer #55

AATGATACGGCGACCACCGAGATCTACACGTCATGCAGGACCAGAG (SEQ ID NO: 199) and primer #56 CAAGCAGAAGACGGCATACGAGATGTGACTGGCACGGGAGTTGAGAATTC GAATACA (SEQ ID NO: 200).

The 100 µl reaction mix contained 40 ng of library, 10 µl of 10X STD Taq buffer, 10 µl of 25 mM $MgCl_2$, 10 µl of 55 and 10 µl of 56 primer that were both at 10 uM, 5 µl of DMSO, 5 µl of dNTP and 1 µl of Taq DNA polymerase. The samples were amplified for 2 cycles of 94°C-30sec, 50°C-30 sec, 52.5°C-30sec, 55°C-30sec, 57.5°C-30sec, 60°C-30sec, 72°C-1min. They were then amplified for 8 cycles of 94C-30sec, 60C-30 sec and 72C-60 sec followed by 72°C for 2 min. PCR mixes were purified with beads and resuspended in 50 µl each.

RESULTS AND DISCUSSION

Both pools were analyzed using an Illumina MiSeq Personal Sequencer. The 50 nucleotide sequence reads from each library pool were trimmed to remove 4 base barcode sequences and mapped to the human genome reference sequence (version hg19) using the Bowtie sequence alignment program. Approximately 80% of reads in both libraries aligned unambiguously to the reference sequence. Further characterization of aligned reads revealed that coupling hybridization-based capture with enzymatic processing resulted in a 979,592-fold enrichment of the 4.9 kilobase target region relative to the input genomic DNA. This represented a 3-fold improvement in library content when compared to the unprocessed, 'capture only' approach. Overall, nearly 4 out of 5 sequences obtained by this alternative processing method mapped to genomic sites that were specifically targeted by capture probes.

A summary of alignment statistics for each library pool are shown in Table 30 below.

Table 30: Sequencing results

Enrichment Protocol	No. of Reads Aligning to Genome (hg19)	No. of Reads Aligning to Target Regions	% of Aligned Reads within Target Regions
Capture Only	4,122,637	1,031,370	25.0%
Capture + Processing	6,949,695	5,433,442	78.2%

Reads from each library pool were also displayed in the UCSC Genome Browser to assess local sequence coverage and distribution around target sites. A close-up view of two segments of the X chromosome shows that processed libraries produced sequence coverage that was more highly concentrated within targeted sites than 'capture only' libraries did (Figure 18). Moreover, the sequences mapping to target regions were more uniformly distributed in the processed libraries than the unprocessed controls. Taken together, these data indicated that the alternative processing method dramatically improved the quantity and quality of target sequences present in enriched libraries.

EXAMPLE 14

BIOINFORMATICS

OVERVIEW

Traditional Next Generation Sequencing (NGS) analysis is "vertical." The unique design of the inventive molecules contemplated herein enables a "horizontal" approach that will revolutionize the approach to clinical resequencing.

"Vertical" as used herein, with regard to sequence alignments, refers to approaches exemplified by Figure 19. Previous approaches to informatics analysis involve a first step in which short reads are aligned to a reference genome. Following alignment, overlapping reads are analyzed for base changes that may be indicative of a SNV (single nucleotide variant). The approach was nick-named here as "vertical" because it relies on alignments that are often depicted as vertical stacks of reads. Various programs make allowances for SNVs and Indels (insertions/deletions), but the core approach is alignment-recognition-based.

In contrast, the paired-end read data obtained by the methods contemplated herein will have DNA tagged sequence information in read 1 and probe ID information in read 2. The first step in data analysis is to match reads to probes. Step two is analyzing the sequence information connected "horizontally" to each probe. See, *e.g.*, Figure 20.

At sufficient read depth, horizontal, probe-based sequence association does not rely on alignment. Rather, reads can be assembled into contigs *de novo*. The advantage

of the method is that it is extremely robust to insertions/deletions and multiple sequence changes in a short stretch of sequence, both situations where conventional, alignment-based methods struggle and have the most difficulty detecting. Moreover, the combination of horizontal association with probes and tagging facilitates more accurate hypothesis generation (*i.e.*, determination if an observed sequence variant is likely to be true or false).

CNV AND STRUCTURAL VARIATION I

In large scale copy number variation (CNV) analyses, the method comprises determination of unique read numbers associated with captured sequence regions. The vast majority of observed CNV is "micro-CNV" that involves base insertions and deletions that are on the order of 2 - 100 bp in length. Vertical alignment methods struggle with micro insertions/deletions (indel) because they require relaxation of alignment stringency that promotes large numbers of false-positive hypotheses. Horizontal methods and *de* novo contig assembly require no such relaxation of alignment parameters and they demand that structural variation is accounted for.

Consider the simple case of a small insertion within one allele of an exon, as exemplified in Figure 21. In this example, horizontal alignment "forces" reads to be associated with probe 1 and probe 2. Assembly will generate two contigs, one with a wild-type exon structure and one with an insertion structure. Two principles emerge from this analysis: 1) Overlapping reads from adjacent probes will support or refute the hypothesis of an indel-containing allele of the captured exon; and 2) micro-CNV alleles outside of capture probes are readily detectable by horizontal methodologies.

CNV AND STRUCTURAL VARIATION II

Validation of CNV often involves vertical alignment methods. In these studies, typically perfect alignments to a reference sequence are demanded. Such methods are vulnerable to SNVs (such as common SNPs) where reads crossing SNVs that differ from the reference will be discarded. The net result will be chronic underestimation of copy number. The horizontal methods possible with the present invention methods should be used going forward.

HORIZONTAL HYPOTHESIS TESTING OF SNVS I

Vertical, alignment-based methods for SNV detection are difficult to analyze. Homozygous variant alleles involving a single base are fairly straightforward to identify, but these changes are rare. More commonly, SNVs are heterozygous, and variants may occur at several contiguous or closely spaced positions (error prone repair tends to lay down tracks where several bases are non-consensus). The heterozygous SNV hypotheses run the spectrum from true, high coverage detection where (strictly as an hypothetical example) 49 reads possess a SNV and 47 reads possess the wild-type, reference base. Calls become much more speculative when read depth thins and the numbers of SNV versus WT reads deviate significantly from 50/50 (*e.g.,* 10 total reads where 8 are WT and 2 are variant). Hypotheses nominated for orthogonal validation are invariably subject to an arbitrary cut-off.

In particular embodiments, where horizontal probe-based association is combined with tags, far greater granularity in SNV hypotheses is achieved. SNVs that reside on a single tag (tag = code + end-point), especially in cases where reads within the same tag are WT, are disregarded. See, *e.g.*, Figure 22.

HORIZONTAL HYPOTHESIS TESTING OF SNVS II

SNV hypotheses that arise on two different tags, even if the read start sites are identical (A), or that arise on different reads that horizontally associate with the same probe (B), or that arise from different probe associations at the same exon (C) are necessarily hypotheses that must be seriously considered. See, *e.g.,*, Figure 23.

EXAMPLE 15

MOLECULAR ANNOTATION

OVERVIEW

This example describes the interplay between "molecular annotation" of sequencing libraries (Figure 24) and the informatics used in subsequent steps to evaluate the resulting sequencing information. The reverse read from a probe has utility. Reverse read 2, which determines the DNA sequence of the probe region, has significant utility in all downstream analysis considerations. For example, utility can be

found in variant calling and the output from this in copy number determination. These two aspects of data analysis are described below.

READ_2 PROBE SEQUENCE

A probe set is a unique and known collection of sequences, which can include one or two probes or even tens of thousands of probes. This means that read_2 can be used to identify any and all of the probes within an experiment. This of course assumes that read 2 is of sufficient length and that probes are designed such that the region interrogated by read_2 constitutes a unique identifier. Table 31 describes a collection of 192 probes and the 10 nt read_2 sequence that serves as a unique identifier for each probe. Note that two probes (CYP2C19_r5_F and CYP2C9_r5_F) naturally share identical 10 nt 5′ DNA sequences, and a 2 nt code of "AG" or "CT" was added to distinguish between them (shaded).

Table 31: Collection of 192 probes

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
CYP2D6_F	AAGCACCTAGCCCCATTCCTGCTGAGCAGGAGGTGGCAGGTACCCCAGACTGGGAGGTAA	201	AAGCACCTAG	393
CYP2D6_R	AGTCGGTGGGGCCAGGATGAGGCCCAGTCTGTTCACACATGGCTGCTGCCTCTCAGCTCT	202	AGTCGGTGGG	394
AMY1_F	ACCTGAGTAGCATCATTGTAGTTCTCGATATCTCCACTTCCAGTTTTACATTTACCATCA	203	ACCTGAGTAG	395
chrX_15_F	CCTGGCCCTCAGCCAGTACAGAAAGTCATTTGTCAAGGCCTTCAGTTGGCAGACGTGCTC	204	CCTGGCCCTC	396
chrX_15_R	AGAATTCATTGCCAGCTATAAATCTGTGGAAACGCTGCCACACAATCTTAGCACACAAGA	205	AGAATTCATT	397
chrX_477_F	GACTTCAAAGAAATTACAAGTTGACA	206	GACTTCAAAG	398

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	TCTTGGACTCTACCCCTCGTACTTTAT CTCCTAT			
chrX_477_R	TCTCTTTGGGGTCAAGAAAGAATCCCT AGTGGATTTGGGATTCTAGAGGAGGT GTTATAA	207	TCTCTTTGGG	399
chrX_478_F	TGCGATACCATGCTGAAGATGAGCTA ACCCAACCAGCCAAGCAGGCAGGGCT GCGAAGGA	208	TGCGATACCA	400
chrX_478_R	GGGGTAGGTGGAAAACCCAAGTAATG TGATTTTGTAACATCCACTGCTGCATT TGTTTGC	209	GGGGTAGGTG	401
chrX_69_F	TTACTTCCCTCCAGTTTTGTTGCTTGCA AAACAACAGAATCTTCTCTCCATGAA ATCATG	210	TTACTTCCCT	402
chrX_69_R	CAGGGGTATCTATTATCCCCATTTTCT CACAAAGGAAACCAAGATAAAAGGTT TAAATGG	211	CAGGGGTATC	403
PLP1_ex1_F	GAAATTCTCTTGTGAATTCCTGTGTCC TCTTGAATCTTCAATGCTAAAGTTTTT GAAACT	212	GAAATTCTCT	404
PLP1_ex2_F	GGGTTTGAGTGGCATGAGCTACCTACT GGATGTGCCTGACTGTTTCCCCTTCTT CTTCCC	213	GGGTTTGAGT	405
PLP1_ex2_R	CTATCTCCAGGATGGAGAGAGGGAAA AAAAAGATGGGTCTGTGTGGGAGGGC AGGTACTT	214	CTATCTCCAG	406
PLP1_ex3_F	GAAAGAAGCCAGGTCTTCAATTAATA AGATTCCCTGGTCTCGTTTGTCTACCT GTTAATG	215	GAAAGAAGCC	407

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
PLP1_ex3_M	CAGACTCGCGCCCAATTTTCCCCCACCCCTTGTTATTGCCACAAAATCCTGAGGATGATC	216	CAGACTCGCG	408
PLP1_ex3_R	TCTTTCTTCTTCCTTTATGGGGCCCTCCTGCTGGCTGAGGGCTTCTACACCACCGGCGCA	217	TCTTTCTTCT	409
PLP1_ex4_F	GTTTGTGTTTCTACATCTGCAGGCTGATGCTGATTTCTAACCACCCCATGTCAATCATTT	218	GTTTGTGTTT	410
PLP1_ex4_R	AACCAAATATATAGTGCTTCCATAGTGGGTAGGAGAGCCAAAGCACCCGTACCCTAACTC	219	AACCAAATAT	411
PLP1_ex5_F	AGTCTCCATGTGGCCCCGTAACTCCATAAAGCTTACCCTGCTTGCTTTTTGTGTCTTACT	220	AGTCTCCATG	412
PLP1_ex5_R	CCATGGGTGTAATTTGTATGGTATTAGCTACTCCCTTGTAAAATAACCCAAATAACCCAC	221	CCATGGGTGT	413
PLP1_ex6_F	TTTACAGTGGAGCATATTACTGCTGTTGCAAGAAACAGTTCTTCCTCTTTCATTTTCCTG	222	TTTACAGTGG	414
PLP1_ex6_R	ATAGCTGTACCCACACTATCTCAGGCCTATTTACTTGCCAAGATCATTCAAAGTCAACTC	223	ATAGCTGTAC	415
PLP1_ex7_F	GATTTGAGGAGGGAGTGCTTTCTTTTCTACTCTCATTCACATTCTCTCTTCTGTTCCCTA	224	GATTTGAGGA	416
PLP1_ex7_R	CAGCATTGTAGGCTGTGTGGTTAGAGCCTCGCTATTAGAGAAAGGGGGATTT	225	CAGCATTGTA	417

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	CTACGGGG			
KRAS_ex1_F	TGTTACCTTTAAAAGACATCTGCTTTC TGCCAAAATTAATGTGCTGAACTTAA ACTTACC	226	TGTTACCTTT	418
KRAS_ex1_R	TTCCCAGTAAATTACTCTTACCAATGC AACAGACTTTAAAGAAGTTGTGTTTTA CAATGC	227	TTCCCAGTAA	419
KRAS_ex2_F	TAAATGACATAACAGTTATGATTTTGC AGAAAACAGATCTGTATTTATTTCAGT GTTACT	228	TAAATGACAT	420
KRAS_ex2_R	GACAGGTTTTGAAAGATATTTGTGTTA CTAATGACTGTGCTATAACTTTTTTTT CTTTCC	229	GACAGGTTTT	421
KRAS_ex3_F	ACTCAAAAAATAAAAACTATAATTAC TCCTTAATGTCAGCTTATTATATTCAA TTTAAAC	230	ACTCAAAAAA	422
KRAS_ex3_R	AACACCTTTTTTGAAGTAAAAGGTGC ACTGTAATAATCCAGACTGTGTTTCTC CCTTCTC	231	AACACCTTTT	423
KRAS_ex4_F	GAAACCTTTATCTGTATCAAAGAATG GTCCTGCACCAGTAATATGCATATTAA AACAAGA	232	GAAACCTTTA	424
KRAS_ex4_R	GTGTATTAACCTTATGTGTGACATGTT CTAATATAGTCACATTTTCATTATTTTT ATTAT	233	GTGTATTAAC	425
MYC_r1_F1	CCCCAGCCAGCGGTCCGCAACCCTTG CCGCATCCACGAAACTTTGCCCATAGC AGCGGGC	234	CCCCAGCCAG	426
MYC_r1_R1	CGACTCATCTCAGCATTAAAGTGATA	235	CGACTCATCT	427

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	AAAAAATAAATTAAAAGGCAAGTGGACTTCGGTG			
MYC_r2_F1	CTGTGGCGCGCACTGCGCGCTGCGCCAGGTTTCCGCACCAAGACCCCTTTAACTCAAGAC	236	CTGTGGCGCG	428
MYC_r2_F2	TTCTACTGCGACGAGGAGGAGAACTTCTACCAGCAGCAGCAGCAGAGCGAGCTGCAGCCC	237	TTCTACTGCG	429
MYC_r2_F3	ACCGAGCTGCTGGGAGGAGACATGGTGAACCAGAGTTTCATCTGCGACCCGGACGACGAG	238	ACCGAGCTGC	430
MYC_r2_F4	GCCGCCGCCTCAGAGTGCATCGACCCCTCGGTGGTCTTCCCCTACCCTCTCAACGACAGC	239	GCCGCCGCCT	431
MYC_r2_R1	GGCGGCTAGGGGACAGGGGCGGGGTGGGCAGCAGCTCGAATTTCTTCCAGATATCCTCGC	240	GGCGGCTAGG	432
MYC_r2_R2	AGACGAGCTTGGCGGCGGCCGAGAAGCCGCTCCACATACAGTCCTGGATGATGATGTTTT	241	AGACGAGCTT	433
MYC_r2_R3	AGGAGAGCAGAGAATCCGAGGACGGAGAAGGCGCTGGAGTCTTGCGAGGCGCAGGACT	242	AGGAGAGCAG	434
MYC_r2_R4	TAAGAGTGGCCCGTTAAATAAGCTGCCAATGAAAATGGGAAAGGTATCCAGCCGCCCACT	243	TAAGAGTGGC	435
MYC_r3_F1	TTGTATTTGTACAGCATTAATCTGGTAATTGATTATTTTAATGTAACCTTGCTAAAGGAG	244	TTGTATTTGT	436

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
MYC_r3_F2	GAGGCCACAGCAAACCTCCTCACAGC CCACTGGTCCTCAAGAGGTGCCACGT CTCCACAC	245	GAGGCCACAG	437
MYC_r3_F3	AGAGGAGGAACGAGCTAAAACGGAG CTTTTTTGCCCTGCGTGACCAGATCCC GGAGTTGG	246	AGAGGAGGAA	438
MYC_r3_R1	TCCAACTTGACCCTCTTGGCAGCAGGA TAGTCCTTCCGAGTGGAGGGAGGCGC TGCGTAG	247	TCCAACTTGA	439
MYC_r3_R2	GCTTGGACGGACAGGATGTATGCTGT GGCTTTTTTAAGGATAACTACCTTGGG GGCCTTT	248	GCTTGGACGG	440
MYC_r3_R3	GCATTTGATCATGCATTTGAAACAAGT TCATAGGTGATTGCTCAGGACATTTCT GTTAGA	249	GCATTTGATC	441
AMY1A_r_9	CATCATTGTTGAAAACAATGAATCCTC TGTTTCCTCTCCCAAAAGCCACTTGGT TGCTCC	250	CATCATTGTT	442
AMY1A_r_10	TTTATCTCCAGAAATGACATCACAGTA TGTGCCAGCAGGAAGACCAGTTTGCA AAGTTAA	251	TTTATCTCCA	443
AMY1A_r_8	CTATTAGAGGACATGTCTAAATACAT ATTCTCACCTTATTTGGCGCCATCGAT GTTCACA	252	CTATTAGAGG	444
AR_r1_F	TCTGAGCAAGAGAAGGGGAGGCGGG GTAAGGGAAGTAGGTGGAAGATTCAG CCAAGCTCA	253	TCTGAGCAAG	445
AR_r1_R	CCAGAACACAGAGTGACTCTGCCCTG GGCCGAAAGGCGACATTTCTGGAAGG	254	CCAGAACACA	446

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	AAAAACTT			
AR_r2_F	CTTCACTTGCCTATTTCTGCCATTCAGTGACATGTGTTGCATTGGTTTTTTGTGTCTTTC	255	CTTCACTTGC	447
AR_r2_R	GTGTCTCTCTCTGGAAGGTAAAGGAGAAAGGGAAAGAGAAGTGCATGTGCAAGACCCTTT	256	GTGTCTCTCT	448
AR_r3_F	CCGAAGAAAGAGACTCTGGAAACTCATTATCAGGTCTATCAACTCTTGTATTTGTTCTCC	257	CCGAAGAAAG	449
AR_r3_R	ACTAGAAAATGAGGGAGAAGGGGGAGAGAGGAAGGAGGAGGAAGAGAAAGAAAAGTATCT	258	ACTAGAAAAT	450
AR_r4_F	GTAGTTGCATTGTGTGTTTTTGACCACTGATGATAAATTCAAGTCTCTCTTCCTTCCCAA	259	GTAGTTGCAT	451
AR_r4_R	CATAGGAGCGTTCACTAAATATGATCCCCCTTATCTCATGCTCCCACTTCCCTTTTCCTT	260	CATAGGAGCG	452
AR_r5_F	CTCAGACTTAGCTCAACCCGTCAGTACCCAGACTGACCACTGCCTCTGCCTCTTCTTCTC	261	CTCAGACTTA	453
AR_r5_R	CACCAACCAGGTCTGGCCAAGCTGCTGTATTTTAGTGAGGTCTGGGCCCCAGGAGCACTT	262	CACCAACCAG	454
ATR_r44_F	GGAAGATACAGTTGTTGAGAAAAGGAAATTGAGAGAAAACACAATTAGTAAGAGTAACTC	263	GGAAGATACA	455
ATR_r44_R	TTTTAGATTTATATTGGCCTCATATGT	264	TTTTAGATTT	456

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	ATATGGATATTTCATAGGCATTGTGTT TCTTTT			
ATR_r45_F	TGTAGGGGCCAATAATTATATTCGAG GTTACTGTTAAATTATTTACAAAGTAT AGGTGAT	265	TGTAGGGGCC	457
ATR_r45_R	TTTGAGTAAAGATTTTTAAATTCTAAC ATTGTTAGTTTGTAATAAAATGTATTG TTTCTA	266	TTTGAGTAAA	458
ATR_r46_F	CATATCAAGTTCATTTGTAGAGATGAG GACTACAGCCCATATCAAGCTATACCT TCTACT	267	CATATCAAGT	459
ATR_r46_R	ATGTCAGCAAGATTTCTTCTTGCAAAG ATAACATCATACCATAATATTTGTTTC AATTTT	268	ATGTCAGCAA	460
ATR_r47_F	CAACCACAGATTCATACCAAATGCAT TACTTTTAGATTATTAACATATTCTTTT ACATAA	269	CAACCACAGA	461
ATR_r47_R	AAGAAAGGTAGTAATTCCAAATTATT AACATCTGTTTTTGGTTTTATGTTTCTT CTTTTT	270	AAGAAAGGTA	462
C4A_r20_F	GGCCTATGTGTGGCCACCCCAGTCCA GCTCCGGGTGTTCCGCGAGTTCCACCT GCACCTC	271	GGCCTATGTG	463
C4A_r24_R	AGGCGTGGCCTCCCTCTTGAGGCTTCC TCGAGGCTGTGGGGAGCAAACCATGA TCTACTT	272	AGGCGTGGCC	464
C4A_r1_F	CCTAGCTTGGCCAGAAGGTAGCAGAC AGACAGACGGATCTAACCTCTCTTGG ATCCTCCA	273	CCTAGCTTGG	465

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
C4A_r1_R	TGAATCGGGTCCCGATGCCAGCCCTG CCCCAATCCAAGCACCCAGCATCCCG CCTCCAGG	274	TGAATCGGGT	466
C4A_r2_F	AGGGAGAGCAGGGGTGGAGGTGTCAG AGCGAAGTCTGACTGCTGATCCTGTCT GTTCTCC	275	AGGGAGAGCA	467
C4A_r2_R	TGACACTTACAAGACAGATGGGAACA GGGCAGGAGGCCCCCACAAGCAGCAG GAGGGCAT	276	TGACACTTAC	468
C4A_r3_F	CCTTTTTGGTCAGCTGTCTCTTGCTCTG TGACCCGCTCCCTCTCCCTCTCCCTCT CCTGA	277	CCTTTTTGGT	469
C4A_r3_R	CAAACTCATCCTGAGAGGGCTCGGAG GGGGTTAAAGGTTGAGGCCCTGGGGC TGAGACTC	278	CAAACTCATC	470
C4A_r4_F	AGTTTGACCCACCCTCCCCTTGCACAT GGACCCCTGCTCACCTCTCTCCTCCTC CACTCC	279	AGTTTGACCC	471
C4A_r4_R	AAGGGGAGAAGTGCTCACAGGCAGGA GGTCACATCAGTGGCCAGGATCAGGA AGGCCAGA	280	AAGGGGAGAA	472
CCL4_r1_F	GTTCTGAAGCTTCTGAGTTCTGCAGCC TCACCTCTGAGAAAACCTCTTTGCCAC CAATAC	281	GTTCTGAAGC	473
CCL4_r2_F	GGCAGTGTTGATCTCACCCTGGCCTTT CCTTTCAGTGGGCTCAGACCCTCCCAC CGCCTG	282	GGCAGTGTTG	474
CCL4_r2_R	ACCACAGCTGGCTGGGAGCAGAGGCT GCTGGTCTCATAGTAATCTACCACAAA	283	ACCACAGCTG	475

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	GTTGCGA			
CCL4_r3_F	TCAGGTGACCTTCCCTGAAGACTTCCTGTCTCTGAGCAGCTCAGTTCAGTTCCAGGTCAT	284	TCAGGTGACC	476
CYP2C19_r1_F	GGAGCATATAGTGGGCCTAGGTGATTGGCCACTTTATCCATCAAAGAGGCACACACACTT	285	GGAGCATATA	477
CYP2C19_r1_R	CCTTTCAAAGTATTTTACTTTACAATGATCTCTTGTAACATTGTACCTCTAGGGATATAC	286	CCTTTCAAAG	478
CYP2C19_r3_F	ATGGGGAGGATGGAAAACAGACTAGCAGAGCTTCTCGGGCAGAGCTTGGCCCATCCACAT	287	ATGGGGAGGA	479
CYP2C19_r3_R	GATCTGGCCACCCCTGAAATGTTTCCAAGAATGTCAGTAGAGAGGAGAGCAGTCCAGAAA	288	GATCTGGCCA	480
CYP2C19_r5_F	AGCAACCAGAGCTTGGCATATTGTATCTATACCTTTATTAAATGCTTTTAATTTAATAAATT	289	AGCAACCAGA	481
CYP2C19_r5_R	CAAAACTAGTCAATGAATCACAAATACGCAAGCAGTCACATAACTAAGCTTTTGTTAACA	290	CAAAACTAGT	482
CYP2C9_r1_F	AAGGAGCATATAGTGGACCTAGGTGATTGGTCAATTTATCCATCAAAGAGGCACACACCG	291	AAGGAGCATA	483
CYP2C9_r1_R	AGCCTTTCAAAGTATTTTACTTTACCATTACCTCTTGTAACATGTACCTCTAGGGATACA	292	AGCCTTTCAA	484
CYP2C9_r3_F	GGGGAGGATGGAAAACAGAGACTTAC	293	GGGGAGGATG	485

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	AGAGCTCCTCGGGCAGAGCTTGGCCC ATCCACAT			
CYP2C9_r3_R	GATATGGCCACCCCTGAAATGTTTCCA AGAATGTCAGTAGAGAAGATAGTAGT CCAGTAA	294	GATATGGCCA	486
CYP2C9_r5_F	CTCAACCAGAGCTTGGTATATGGTATG TATGCTTTTATTAAAATCTTTTAATTTA ATAAATT	295	CTCAACCAGA	487
CYP2C9_r5_R	CAGAACTAGTCAACAAATCACAAATT CACAAGCAGTCACATAACTAAGCTTTT GTTTACA	296	CAGAACTAGT	488
CYP2D6_r1_F	GGAAGTCCCCCAAACCTGCTTCCCCTT CTCAGCCTGGCTTCTGGTCCAGCCTGT GGTTTC	297	GGAAGTCCCC	489
CYP2D6_r1_R	GCCTGGTGGGGTGGGGGTGCCAGGTG TGTCCAGAGGAGCCCATTTGGTAGTG AGGCAGGT	298	GCCTGGTGGG	490
CYP2D6_r2_F	GCCCAGCTCGGACTACGGTCATCACC CACCCGGGTCCCACGGAAATCTGTCTC TGTCCCC	299	GCCCAGCTCG	491
CYP2D6_r2_R	AATAGGACTAGGACCTGTAGTCTGGG GGGATCCTGGCTTGACAAGAGGCCCT GACCCTCC	300	AATAGGACTA	492
CYP2D6_r5_F	AAGTTCATGGGCCCCCGCCTGTACCCT TCCTCCCTCGGCCCCTGCACTGTTTCC CAGATG	301	AAGTTCATGG	493
CYP2D6_r5_R	GGGCTGACAGGTGCAGAATTGGAGGT CATTTGGGGGCTACCCCGTTCTGTCCC GAGTATG	302	GGGCTGACAG	494

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
CYP2D6_r8_F	CATTGCTTTATTGTACATTAGAGCCTC TGGCTAGGGAGCAGGCTGGGGACTAG GTACCCC	303	CATTGCTTTA	495
CYP2D6_r8_ R	TGGAGTCTTGCAGGGGTATCACCCAG GAGCCAGGCTCACTGACGCCCCTCCC CTCCCCAC	304	TGGAGTCTTG	496
DCC_r1_F	AGTGCATGTGTGTGAGTGCTGCCGCTG CCCGCGACCCCTGGCCCCGAAGGTGT TGGCTGA	305	AGTGCATGTG	497
DCC_r1_R	AATGAGAAGGGAAGTGGGGTACGGA AGGGGGTGCGACGAGAAGAAAGGAA AGAGCCACTT	306	AATGAGAAGG	498
DCC_r2_F	GGAATCTAAGCCTGAGATTTATTTGAA TACATGAACATATTTCCCTGTGCTCTC TTGTTC	307	GGAATCTAAG	499
DCC_r2_R	AAGATGGCATTCATCTGGCCTTGGGA ATGGATGAAAGGAAGCAGCAACTTTC AAATGGGT	308	AAGATGGCAT	500
DCC_r2	GGTAAAGTCCCTCATCTGGCTTGTGGT GTCTGGAATGAAGTATGTTTTGTATCA GCAGAG	309	GGTAAAGTCC	501
DCC_r3_F	TTTATTGGCGATTATTGTGCTTTATTTG GAAGACTTATTCTTCCTTCTTTGTTTTT CTCC	310	TTTATTGGCG	502
DCC_r3_R	GAAAAAAATTGTTTTTCAACTTATTCC AGAGAATATCATTCTGAAGGCAACAA AGAGCAT	311	GAAAAAAATT	503
DCC_r4_F	ATATATCATTTATCTTTGCAATGTTTTT CATATATCATATGATACTGTGTTTTCC	312	ATATATCATT	504

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	CCTCA			
DCC_r4_R	ATTAGAGAAATATAAATAGCAGCATA TACCATACAAAAGTACACTTTACAAA AAAGTCTT	313	ATTAGAGAAA	505
EP300_r18_F	ATACTCCATCTCCCGTAAAAATAGTGA GACTTGAGTAATGTTTGATGTCACTTG TCTTTC	314	ATACTCCATC	506
EP300_r18_R	CAGTCACCACTATATTATTCTAGGTAT CCCAGAAAAGTTAAAGTCAAATCTGA AACACAT	315	CAGTCACCAC	507
EP300_r19_F	TTCTTACTGTTCTAGCTTGTCCTTAAG GCCTCTGTGCTTTTTAACAAATGGTTT CTTTTG	316	TTCTTACTGT	508
EP300_r19_R	TCCGCATGCACTCCCTGGACATGTGGA CACATGGACCATGGTCCACACCTGGC CAAGCTT	317	TCCGCATGCA	509
EP300_r20_F	CTTGGCTTGGGCTGTGTTGTGTGAACG GAACAGTTCACCCCAGTATGGCCTTCT TGCCGA	318	CTTGGCTTGG	510
EP300_r20_R	GCTGTGCATAATCACTGGACAACAAA CTAATTAGCACTTTTCAAAATAATGCA GTTACTT	319	GCTGTGCATA	511
EP300_r21_F	GAACAGCAGTCAGATTGCTCATCTCTA TCACTTTTTCTCATTGTGTCCCTTTTCT CTCCT	320	GAACAGCAGT	512
EP300_r21_R	AGAGAATGAAAGGGAAAAAGAAAAG CCAAAGCGTACTGACTATTCAAGGGG ATCGTACTT	321	AGAGAATGAA	513
EP300_r22_F	TATTGCAAGTTTTCATTTGGTTAAGGT	322	TATTGCAAGT	514

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	TTGGGGTTAATTTTGGAATTGGCTCTG CTCTTC			
EP300_r22_R	AAAACTTGTTAATATTCACGATAAAG AAAAATTCCAGAGAAAGTAACAACGT TAAGACTT	323	AAAACTTGTT	515
PTEN_r1_F	GCAGAAGAAGCCCCGCCACCAGCAGC TTCTGCCATCTCTCTCCTCCTTTTTCTT CAGCCA	324	GCAGAAGAAG	516
PTEN_r1_R	CTACTCCCACGTTCTAAGAGAGTGAC AGAAAGGTAAAGAGGAGCAGCCGCA GAAATGGAT	325	CTACTCCCAC	517
PTEN_r2_F	AGTATTCTTTTAGTTTGATTGCTGCAT ATTTCAGATATTTCTTTCCTTAACTAA AGTACT	326	AGTATTCTTT	518
PTEN_r2_R	AATGAAAACACAACATGAATATAAAC ATCAATATTTGAAATAGAAAATCAAA GCATTCTT	327	AATGAAAACA	519
PTEN_r3_F	GTAATTTCAAATGTTAGCTCATTTTTG TTAATGGTGGCTTTTTGTTTGTTTGTTT TGTTT	328	GTAATTTCAA	520
PTEN_r3_R	CTTTCACTTAATAGTTGTTTTAGAAGA TATTTGCAAGCATACAAATAAGAAAA CATACTT	329	CTTTCACTTA	521
PTEN_r4_F	GTACTTTTTTTTCTTCCTAAGTGCAAA AGATAACTTTATATCACTTTTAAACTT TTCTTT	330	GTACTTTTTT	522
PTEN_r4_R	ACAGTAAGATACAGTCTATCGGGTTT AAGTTATACAACATAGTACAGTACAT TCATACCT	331	ACAGTAAGAT	523

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
PTEN_r5_F	TACTTGTTAATTAAAAATTCAAGAGTTTTTTTTCTTATTCTGAGGTTATCTTTTTACCA	332	TACTTGTTAA	524
PTEN_r5_R	CCAAAATCTGTTTTCCAATAAATTCTCAGATCCAGGAAGAGGAAAGGAAAAACATCAAAA	333	CCAAAATCTG	525
RHD_r1_F	TCAAGTAGGTGTTGGAGAGAGGGGTGATGCCTGGTGCTGGTGGAACCCCTGCACAGAGAC	334	TCAAGTAGGT	526
RHD_r2_F	CCTGTCCTTTCGGGGTCCATTCCCTCTATGACCCAGAAGTGATCCAGCCACCATCCCAAT	335	CCTGTCCTTT	527
RHD_r5_F	AACCCCTCGAGGCTCAGACCTTTGGAGCAGGAGTGTGATTCTGGCCAACCACCCTCTCTG	336	AACCCCTCGA	528
RHD_r5_R	CATAAATATGTGTGCTAGTCCTGTTAGACCCAAGTGCTGCCCAAGGGCAGCGCCCTGCTC	337	CATAAATATG	529
RHD_r6_F	TTGCAGCAAGATGGTGTTCTCTCTCTACCTTGCTTCCTTTACCCACACGCTATTTCTTTG	338	TTGCAGCAAG	530
RHD_r7_F	GAGATCAAGCCAAAATCAGTATGTGGGTTCATCTGCAATAAAAATGTTTGTTTTGCTTTT	339	GAGATCAAGC	531
RHD_r7_R	GCAACAGTGAGAGGAAGTTGTCTTGTTTTTGAACAGGCCTTGTTTTTCTTGGATGCTTTT	340	GCAACAGTGA	532
RUNX1_r1_F	CTGCCATTTCATTACAGGCAAAGCTGAGCAAAAGTAGATATTACAAGACCAGC	341	CTGCCATTTC	533

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	ATGTACT			
RUNX1_r1_R	AAGGTAAAAGAAATCATTGAGTCCCCGCCTTCAGAAGAGGGTGCATTTTCAGGAGGAAG	342	AAGGTAAAAG	534
RUNX1_r3_F	GCGGATCTCCCCCGGCCTCGCCGGCCTCCGCCTGTCCTCCCACCACCCTCTCCGGGCCAG	343	GCGGATCTCC	535
RUNX1_r3_R	CTGGTAGGAGCTGTTTGCAGGGTCCTAACTCAATCGGCTTGTTGTGATGCGTATCCCCGT	344	CTGGTAGGAG	536
RUNX1_r4_F	TTTTGAAATGTGGGTTTGTTGCCATGAAACGTGTTTCAAGCATAGTTTTGACAGATAACG	345	TTTTGAAATG	537
RUNX1_r4_R	TGCCCTAAAAGTGTATGTATAACATCCCTGATGTCTGCATTTGTCCTTTGACTGGTGTTT	346	TGCCCTAAAA	538
RUNX1_r5_F	GTATACCAGCCTGGAGGGTGTACCAGCCTGGAGGGTGTACCAGCCCCAAGTGGATGCACT	347	GTATACCAGC	539
RUNX1_r5_R	TTCAACAGATATGTTCAGGCCACCAACCTCATTCTGTTTTGTTCTCTATCGTGTCCCCAC	348	TTCAACAGAT	540
SKI_r4_F	AGGATGTGTCTGGGTGGTGCTTGGGGACAGAGGCACCTTCCCGACACCCGCCTGCCCCTC	349	AGGATGTGTC	541
SKI_r4_R	CCTCAGCCAGTGCCACCCCCACAGCCCACAGGGAGGAGGCACAGAAAGCGACTCACACGT	350	CCTCAGCCAG	542
SKI_r5_F	CTGGTGTGGAGCTGCCGGGCACTTCC	351	CTGGTGTGGA	543

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	ATGACTTTGTTTCTGTCTCTGCTTCCTC CTCAGT			
SKI_r5_R	CTGGTGCAGGCTGTGCTCACTGCCCGT GCCCTGGACCTCCCAGCACCACTCGCC CCGCTC	352	CTGGTGCAGG	544
SKI_r6_F	GTCATGGTGAGGGGTGTGCTGGGACC GGCTGGGCAGTGACCCCGAGCCGCCT CCGGCCCC	353	GTCATGGTGA	545
SKI_r6_R	CCGGGGCCCACGGCGGCTCCACGCCC ACCGTGCTGCGTGCCTCAGTCTCCCCA CCCGCAT	354	CCGGGGCCCA	546
SRY_r1_F	CTGTAAGTTATCGTAAAAAGGAGCAT CTAGGTAGGTCTTTGTAGCCAATGTTA CCCGATT	355	CTGTAAGTTA	547
SRY_r1_M1	AGTAAAATAAGTTTCGAACTCTGGCA CCTTTCAATTTTGTCGCACTCTCCTTGT TTTTGA	356	AGTAAAATAA	548
SRY_r1_M2	GAAGCAAACTGCAATTCTTCGGCAGC ATCTTCGCCTTCCGACGAGGTCGATAC TTATAAT	357	GAAGCAAACT	549
SRY_r1_M3	AATGGCCATTCTTCCAGGAGGCACAG AAATTACAGGCCATGCACAGAGAGAA ATACCCGA	358	AATGGCCATT	550
SRY_r1_M4	CTTGCGCCTCTGATCGCGAGACCACAC GATGAATGCGTTCATGGGTCGCTTCAC TCTATC	359	CTTGCGCCTC	551
SRY_r1_R	GAAAGCTGTAACTCTAAGTATCAGTG TGAAACGGGAGAAAACAGTAAAGGC AACGTCCAG	360	GAAAGCTGTA	552

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
TNFRSF14_r1_F	AGTTCCTCTGCTGGAGTTCATCCTGCTAGCTGGGTTCCCGAGCTGCCGGTCTGAGCCTGA	361	AGTTCCTCTG	553
TNFRSF14_r1_R	TCTGTGGGAGGCCCTGGGGTCAGAACTGGGATCTGCGAGCAGACGGAGAGGAGGCTCGGG	362	TCTGTGGGAG	554
TNFRSF14_r2_F	CAAGCCTGGCAGAGCCCACAGGGCAGCCAGGGCATCTCCCAATGCCTGTCCTGACCCCCT	363	CAAGCCTGGC	555
TNFRSF14_r2_R	TGTCTGGGGCAGAAGGGGGCAAGAGTGTCTGCCCTCGGCCCACAGAGCTGGCCCGCCAAA	364	TGTCTGGGGC	556
TNFRSF14_r3_F	TGATGGGTGGGCTCCCGAAGGGGCCTCCCGCAGACTTGCGAAGTTCCCACTCTCTGGGCG	365	TGATGGGTGG	557
TNFRSF14_r3_R	CAGGGTGCGGGGGCATCCAGGCTGCCCAAGCGGAGGCTGGGCCGGCTGTGCTGGCCTCTT	366	CAGGGTGCGG	558
UGT1A1_rP_P	CTCCACCTTCTTTATCTCTGAAAGTGAACTCCCTGCTACCTTTGTGGACTGACAGCTTTT	367	CTCCACCTTC	559
UGT1A1_r1_F	TCGATCCAAAGTAATACATCTGAAAGAATATACGCATGTAAAAGTCCCACTCCAATACAC	368	TCGATCCAAA	560
UGT1A1_r1_M1	CTGCAGCAGAGGGGACATGAAATAGTTGTCCTAGCACCTGACGCCTCGTTGTACATCAGA	369	CTGCAGCAGA	561
UGT1A1_r1_M2	ACTCTTTCACATCCTCCCTTTGGAATGGCACAGGGTACGTCTTCAAGGTGTAA	370	ACTCTTTCAC	562

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
	AATGCTC			
UGT1A1_r1_M3	GCGTGTGATCAAAACATACAAGAAAATAAAAAAGGACTCTGCTATGCTTTTGTCTGGCTG	371	GCGTGTGATC	563
UGT1A1_r1_M4	ACATCAAAGCTGCTTTCTGCCAGGGAGGCCATGAGCTCCTTGTTGTGCAGTAAGTGGGAA	372	ACATCAAAGC	564
UGT1A1_r1_M5	CTCTGCCCACTGTATTCTTCTTGCATGCACTGCCATGCAGCCTGGAATTTGAGGCTACCC	373	CTCTGCCCAC	565
UGT1A1_r1_M6	TCATGTGATCTGAATGAGAGGAGAGAGGCCTGGGCACGTAGGAGAATGGGTTGGGGCACT	374	TCATGTGATC	566
UGT1A1_r1_M7	TGCGACGTGGTTTATTCCCCGTATGCAACCCTTGCCTCAGAATTCCTTCAGAGAGAGGTG	375	TGCGACGTGG	567
UGT1A1_r1_R	CTTCACAAAGTCACTTCTAAACAGCCAGACAGATGCAGAGCTCAATAGGTCCTGGACAGT	376	CTTCACAAAG	568
UGT1A1_r2_F	ACTGTATGTAGTCATCAAAGAATATGAGAAAAAATTAACTGAAAATTTTTCTTCTGGCTC	377	ACTGTATGTA	569
UGT1A1_r2_R	ATTAATCTGGAAGCTGGAAGTCTGGGATTAGCGCTCCTGTGAAAATAGATATGAGGCCAT	378	ATTAATCTGG	570
UGT1A1_r3_F	CAGATTTGTTTTCTAATCATATTATGTTCTTTCTTTACGTTCTGCTCTTTTTGCCCCTCC	379	CAGATTTGTT	571
UGT1A1_r3_	GCTTAAGCCATTTTCTTAATTTGACCC	380	GCTTAAGCCA	572

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
R	TGGTTTGACCTATACATCCAATCCGCC CAACAT			
UGT1A1_r4_ F	TGTGTCCAGCTGTGAAACTCAGAGAT GTAACTGCTGACATCCTCCCTATTTTG CATCTCA	381	TGTGTCCAGC	573
UGT1A1_r4_ R	ATTTGAAACAATTTTATCATGAATGCC ATGACCAAAGTATTCTTCTGTATCTTC TTTCTT	382	ATTTGAAACA	574
UGT1A1_r5_ F	ATAAAGAGAGGATTGTTCATACCACA GGTGTTCCAGGCATAACGAAACTGTC TTTGTGTT	383	ATAAAGAGAG	575
UGT1A1_r5_ R	TTTTCAAGTTTGGAAATGACTAGGGA ATGGTTCAAAATTTTACCTTATTTCCC ACCCACT	384	TTTTCAAGTT	576
VHL_r1_F	CGCCCCGCGTCCGACCCGCGGATCCC GCGGCGTCCGGCCCGGGTGGTCTGGA TCGCGGAG	385	CGCCCCGCGT	577
VHL_r1_M1	TAGAGGGGCTTCAGACCGTGCTATCG TCCCTGCTGGGTCGGGCCTAAGCGCC GGGCCCGT	386	TAGAGGGGCT	578
VHL_r1_M2	GGCGCCGAGGAGGAGATGGAGGCCG GGCGGCCGCGGCCCGTGCTGCGCTCG GTGAACTCG	387	GGCGCCGAGG	579
VHL_r1_R	CCATACGGGCAGCACGACGCGCGGAC TGCGATTGCAGAAGATGACCTGGGAG GGCTCGCG	388	CCATACGGGC	580
VHL_r2_F	GGTGTGGGCCACCGTGCCCAGCCACC GGTGTGGCTCTTTAACAACCTTTGCTT GTCCCGA	389	GGTGTGGGCC	581

Name	Sequence	SEQ ID NO:	10 nt read_2	SEQ ID NO:
VHL_r2_R	AAGTGGTCTATCCTGTACTTACCACAACAACCTTATCTTTTTAAAAAGTAAAACGTCAGT	390	AAGTGGTCTA	582
VHL_r3_F	CTTGTTCGTTCCTTGTACTGAGACCCTAGTCTGCCACTGAGGATTTGGTTTTTGCCCTTC	391	CTTGTTCGTT	583
VHL_r3_R	ATCAAGACTCATCAGTACCATCAAAAGCTGAGATGAAACAGTGTAAGTTTCAACAGAAAT	392	ATCAAGACTC	584

In paired-end sequencing experiments, read_1 and read_2 are derived from the same DNA clone. This implies that the read_1 genomic sequence (parts (3) and (4) in Figure 24) is present because it was associated with a particular probe (part (5) in Figure 24). Taken in total, this data indicates that each DNA sequence present in a collection of next-generation sequences can be associated with the probe sequence that targeted it. All DNA sequences associated with a particular probe can be retrieved.

The present paradigm for next-generation resequencing analysis (targeted or otherwise) is to align reads back to a reference genome. Knowledge of the targeting probe association affords a novel workflow in which reads are first sorted by probe and then analyzed by either alignment-based methods, *de novo* assembly methods, or both. As described in Example 14, probe-associated-read-scaffold-assembly (PARSAR) solves one of the more complex and difficult issues in variant discovery, which is that the most interesting variants are those that deviate most significantly from the reference sequence, yet these are the very sequences that will be most refractile to conventional sequence based alignment (Figure 25). Using probe-association followed by *de novo* local assembly, such variants are easily identified.

Probe-based read grouping is used in conjunction with other aspects of molecular design to identify variants with a high-degree of first-pass confidence. As shown in Figure 26, probes are generally designed to bracket target regions. The overlapping aspect of reads allows potential variant sites to be queried by independent

reads in both orientations. In addition, this dual-probe design ensures that adjacent probe binding sites themselves are sequenced. This is an important feature where probe capture performance may be in question. As an example, variant alleles where single nucleotide variants underlie one of the capture probe sequences are identified by this molecular design and can be accounted for in downstream informatics analysis.

An additional aspect of flow of information from molecular annotation to downstream variant analysis involves sequence "tags", defined as the combination of a three base sequence label and ragged sequence start sites ((1)+(3) in Figure 24). Sequence tags define the uniqueness of each sequencing clone. As illustrated in Figure 27, variants that occur within a collection of sibling clones that share identical sequence tags are likely to be false positives. In contrast, variants are shared among sequences with different tags – even if they occur at low frequency – have a higher probability of being true positive variants. This system of tagging sequences and using tags to assign confidence predictions to variant calling has the prospect of substantially decreasing the burden of downstream variant validation (which can be costly and time consuming). Molecular annotation is described in more detail in the document Example 16, which describes the molecular technology sequencing platform.

In summary, one of the distinguishing features of the technology platforms contemplated herein is the fact that all "annealing probe" events are copied into DNA clones that also possess additional molecular annotation. Sequences are segregated by probe and by sample labels into a collection belonging to a specific target region of a specific input sample. Combinations of alignment and *de novo* assembly can then be used for variant detection. Finally, redundancy in the appearance of candidate variants can be used to assign confidence in variant calling. In addition to variant analysis, methods for copy number determination were also provided. These two elements are tightly coupled, specifically because copy number determination is dependent on high confidence sequencing reads. The overall schema for determining copy number from sequence information is shown in Figure 28.

EXAMPLE 16

MOLECULAR TECHNOLOGY SEQUENCING PLATFORM

OVERVIEW

The genomic sequencing platforms contemplated herein provide methods to: (1) Address genomic samples from multiple individuals in a single sequencing run; (2) Detect single (and/or multiple) nucleotide variants (SNVs) and single (and/or multiple) nucleotide insertions and deletions (SNIDs) with high confidence; (3) Detect large and small scale copy number variations (CNVs) in all queried gene environments; (4) Detect micro-scale translocations, inversions and insertion/deletion events in queried gene environments; (5) Develop a technological system that is scalable from ≥ exome-scale investigation (≥ 1-2% of the overall human genome sequence) to ≤ single gene-scale validation; (6) Achieve high specificity (low false-negative rate) and high sensitivity (low false-positive rate) in genomic variation tests; (7) Create a molecular and bioinformatics technology that is simple, portable and scalable in its execution; and (8) Provide molecular methods that are readily amenable to quality control measurements.

The overall schematic of genomic sequencing read is shown in Figure 29. A description of each element is as follows:

(1) The "sequence label" is a set of (contiguous*) nucleotides (*i.e.* a unique set of 3 mers) that is used in conjunction with the read start position (3) to establish the uniqueness of each sequencing read. In founding documents, the combination of this label and the read start point were referred to as the "unique sequence tag." Because the sequence label is the first set of bases encountered and in sequencing-by-synthesis (SBS) chemistry all four DNA bases must be equally represented at each read position, the constraints on the sequencing label are not only uniqueness, but also that the collection of bases used in the collective set of sequence labels must have all four bases present at all positions sequenced. The use of unique sequence tags to determine local CNVs is described in the bioinformatics section of this document.

(2) The "sample label" is a set of (contiguous*) nucleotide codes that uniquely identify a particular sample within a set of multiplexed samples. As with the sequencing label, the collection of sample labels must also contain all four bases to

satisfy the requirements of SBS sequence base calling. The sample codes are intentionally positioned next to genomic DNA fragments. The driver for this design is ligation bias, meaning that there are base preferences for DNA ligation efficiency in the ~2 bases upstream and the 1-2 bases downstream of the ligation junction. By placing the sample code at the ligation junction, all fragments within a specific sample experience the ligation influences/biases.

* - Without wishing to be bound to any particular theory, it is contemplated that the sequence labels and sample labels could be created as inter-digitated nucleotide sequences.

(3) The "read start point" within genomic fragments is one of two key elements that define a "unique sequence read." As discussed in section (1) above, the unique identifying "tag" for each read is comprised of the sequencing label AND the read start point. As considered in more detail below, the collection of unique [(1)+(3)] sequence tags is essential for determination of large scale CNV. Here "large scale CNV" is defined as any CNV that involves the entirety of at least one probe binding region plus some adjacent sequence. Large scale CNV can be as large as gain or loss of entire chromosomes.

[(1) + (2)] The sequence label and sample label are embedded in adaptor sequences that are ligated to end-repaired genomic fragments in the initial stage of library construction process where a whole genome library is created.

(4) The sequencing read. The sequence information from genomic fragments is, of course, a central focus of the genomic assays. Each read is considered in the context of multiple, overlapping reads produced within the same assay.

(5) The probe level – "genomic indexing." The overall genomics assay strategy is to combine multiple sequence labels into a composite "molecular annotation" that places each sequencing read into a larger framework of genomic analysis. Within this operational paradigm, Read 1 reveals elements (1-4) of each annotated clone. Read 2 reveals the probe sequence that retrieved each clone by hybridization based capture and subsequent enzymatic processing. The probe sequence information is central to the genomics strategy because all reads are initially clustered according to the probe that

captured them. This clustering of information on a probe-by-probe basis is termed "genomic indexing" because each read is indexed to a genomic probe prior to analysis.

One of the interesting features of the probe label is that the constellation of all probe sequences within a capture reaction is well-defined (we know which probes went into the capture reaction). This implies that Read 2 does not necessarily need to cover the entire 60 nt probe sequence. Rather, Read 2 only needs to be of sufficient length to enable unambiguous identification of all probes within a specific reaction. As one non-limiting example, the probe set discussed in Example 15 consists of 192 probes that can be differentiated based on only 7 nt of 5′ probe sequence (two of the probes with identical 7 nt 5′ termini were tagged with dinucleotide codes so they could be informatically differentiated).

(6) The capture label. The composition of the libraries is determined by the intimate molecular interaction between probes and target sequences. The performance of each unique probe sequence can be monitored using the capture label, which can be as simple as a string of several (4 – 6) random bases. The diversity and statistical distribution of capture labels detected in sequencing is a direct measure of probe performance. By way of example, imagine a case where very few sequences are associated with a particular probe sequence. It may be tempting to attribute this deficit of sequences to poor probe performance, and therefore to initiate iterative cycles of probe redesigns. However, sequence under-representation may also be a consequence of sequences that do not ligate well to adaptors and or sequences that do not amplify well with the particular PCR regimen that is used. The use of capture labels allows differentiation of these failure modes. With poor probe performance, the very few capture events that do occur will manifest as very few capture labels that show up multiple times. In contrast, poor representation for reasons upstream of the actual capture reaction (ligation, PCR, end-repair, etc.) will result in a large constellation of capture labels that will be, by and large, uniquely represented. In particular embodiments, as one transitions into automated designs of thousands of probes, the ability to informatically QC probe performance will become increasingly important.

EXAMPLE 17

PROBE SELECTION AND IMPLEMENTATION

SUMMARY

Probe sequence selection and the methods to use them have, necessarily, been developed in concert. This example describes probe selection criterion in Section I and the laboratory methods that make them most effective in Section II. See, *e.g.*, Figure 30.

SECTION I. SELECTION OF TARGETING PROBES.

In the most general terms, target enrichment probes are 60 nt in length. Probes are generally directional, meaning they capture sequences on one side (generally the 3′ side) of their position. Tail sequences that add additional functionality (*e.g.,* PCR primer binding site, binding site for complementary oligo that enables biotin pull-out, *etc*) are added in addition to the core targeting 60-mer. Sixty nucleotide targeting sequences are chosen with the following constraints and criterion: (1) The probe is positioned from -100 to +50 nt relative to the start of the target sequence. In Figure 30, the "start" of the target sequence is the intron:exon junction; (2) Probes are designed with redundancy, as illustrated, such that the resulting sequences from a pair of probes are overlapping in opposite orientations; (3) Probes are selected (where possible) to possess GC content not less than 33% (>20 G's or C's per 60 mer) and not more than 67% (<40 G's or C's per 60 mer); (4) Probes are selected to avoid repeats wherever possible. This is done with the help of REPEATMASKER and/or unique alignability criterion, both of which can be viewed on the UCSC genome browser; and (5) In case the position requirement, GC requirement, and the uniqueness requirement cannot be met, selection rules are relaxed in the following order (GC >position>uniqueness). In other words, GC and positioning are flexible; the uniqueness criterion is not.

SECTION II. LABORATORY METHODS.

The input to target enrichment is probes, a gDNA library, and buffers, which have been described elsewhere herein. The first step in targeted enrichment is melting of the gDNA library, which begins in a form as double-stranded PCR fragments. This

is achieved by denaturation of the gDNA, preferably at a concentration of 100 ng/μl in a total volume of 10 μl, at 98°C for 2 min followed by immediate transfer to ice. The gDNA library is suspended in a low salt buffer that contains 10 mM Tris pH 8.0 and 0.1 mM EDTA. The second step is to add 5 μl of concentrated binding buffer (4M NaCl, 40 mM Tris pH 8.0, 0.4 mM EDTA and 0.4% Tween20). While these conditions are specific, the overarching concept is that the concentration of salt must be increased to 2N osmolarity to achieve rapid kinetic association of complementary DNA strands. Five microliters of probe is also added such that the final concentration of probe is 250 pM in each probe. The mixture of gDNA library, buffer and probe is heated to 98°C for 2 min and cooled in 1°C increments for four min each down to 68°C. In the third step, probe:gDNA complexes (the probe has a biotin associated with it) are bound to magnetic beads that are coated with streptavidin. In the fourth step, stringent washing is used to remove unwanted associations between probe and non-target sequences that may occur because of, for example, short matches of nucleotide sequence between probe and gDNA. Stringency is achieved by using low-salt, high-formamide wash buffer as, for example, a buffer containing 30%-35% formamide (v/v), 10 mM Tris pH 8.0, 0.1 mM EDTA and 0.5% Tween 20). Several washes of the beads are used to achieve the desired purity of target sequence (*e.g.*, four). The washed beads possess target sequences bound to probe that are processed, amplified, and sequenced. In summary, low salt melting of the gDNA library, high salt probe annealing, and high formamide washes are used in concert with probe designs to achieve high levels of target sequence enrichment.

EXAMPLE 18

EXEMPLARY SEQUENCES

OVERVIEW

Exemplary genome tags, sample codes and library information are shown in Tables 32-34 below.

Table 32: Exemplary Genome tags

Genome tags			
AAC	CAA	GAT	TAG
ACA	CCC	GCG	TCT
AGT	CGG	GGC	TGA
ATG	CTT	GTA	TTC

Table 33: Exemplary Sample Codes

Sample codes			
GAA	TCA	CGA	ATA
TAC	GCC	AGC	CTC
AAG	CCG	TGG	GTG
CAT	ACT	GGT	TTT

Table 34: Exemplary Library

Genome tags	Sample codes	Sample ID	Input
AAC	ATA	NA18917	50 genomes
ACA	CCG	NA18917	100 genomes
AGT	GGT	NA18917	200 genomes
ATG	TAC	NA18917	400 genomes
CAA	AGC	NA12878	50 genomes
CCC	CAT	NA12878	100 genomes
CGG	GTG	NA12878	200 genomes
CTT	TCA	NA12878	400 genomes
GAT			
GCG			
GGC			
GTA			
TAG			
TCT			
TGA			
TTC			

EXAMPLE 19

CONSTRUCTION OF TAGGED, TARGETED, GENOMIC LIBRARY

SUMMARY

Several ways to construct tagged, targeted genomic sequencing libraries are contemplated herein. In this embodiment, DNA repair is used to attach probe-associated sequences to captured genomic fragments. This approach worked well for creating sequence-ready targeted genomic libraries.

CONCEPT

An important principle of the library construction is that the sequence ready clones are comprised of DNA sequences derived from both genomic fragments and capture probes. This "recombination" of parts greatly enriches for those genomic fragments that are in direct contact with probe, and it enables focused sequencing reads on one side of a probe sequence. In this design, the tripartite complex formed between the target genomic library fragment, the capture probe and the common partner oligo possesses a structure reminiscent of a DNA replication fork. Such forks occur during normal DNA replication, but they also occur during DNA repair processes. In the latter case, it is often necessary to trim 5′ displaced strands to enable joining of the newly polymerized strand to the adjacent 3′ sequence. This repair process requires two enzymes and three enzymatic activities. DNA polymerase holoenzymes like *E. coli* DNA polymerase or *Bst* DNA polymerase possess two of these activities, a 5′ to 3′ endonuclease activity that removes these 5′ displaced flaps and, of course, DNA polymerase activity.

In particular embodiments, *Bst* polymerase is preferred because it also lacks 3′ to 5′ nuclease activity that is often associated with DNA polymerase holoenzymes. See, *e.g.,* Figure 31. This feature is useful because it suggests that the single-stranded 3′ DNA protrusions of the target genomic clones do not require protection. The other enzyme and activity required is a nick-closing DNA ligase such as the NAD^+-requiring *Taq* DNA ligase. Following processing, processed fragments are amplified by PCR to allow for size selection and quantification prior to sequencing.

PROOF OF PRINCIPLE OLIGONUCLEOTIDES

For this experiment, eight target regions were chosen that correspond to eight genomic regions for which we have qPCR assays. The forward and reverse primers for these eight regions are shown in Table 35. The capture probes are the exact reverse complement of capture probes that were used and validated elsewhere herein. These probes span a %GC range from 22% to 73% as noted in Table 37.

Table 35: Proof of principle oligonucleotides

Table 1. Proof of principle oligonucleotides			
SEQ ID NO:	**qPCR Assay**	**Name**	**Sequence**
585	17	SRY_F	CTGGTGCTCCATTCTTGAGTGTGT
586		SRY_R	GGACAACAGGTTGTACAGGGATGA
587	18	VHL_F	TACAGGAGACTGGACATCGTCAGG
588		VHL_R	CTTTCTGCACATTTGGGTGGTCTT
589	19	UGT_F	GGTGATCAGATGGACAATGCAAAG
590		UGT_R	TCATTTCCAGAACATTCAGGGTCA
591	20	TNF_F	ACCTCAATGGCCTAAGCAAGTGTC
592		TNF_R	GCCTCTTACCTGGGTCACACATTT
593	21	RUNX_F	ATCTTGCAACCTGGTTCTTCATGG
594		RUNX_R	GCTGGCAATGATGAAAACTACTCG
595	22	RHD_F	CCAAGTTTCAACTCTGCTCTGCTG
596		RHD_R	GTTGAACACGGCATTCTTCCTTTC
597	23	PTEN_F	GGAAAGGGACGAACTGGTGTAATG
598		PTEN_R	AAATCTAGGGCCTCTTGTGCCTTT
599	24	EP300_F	AGCCCCATGGATCTTTCTACCATT
600		EP300_R	ATACTGCCAGGGCTCCTGATACTG
	Capture probe	**Name**	**Sequence**
601	17	SRY_r1f_V3	AATCGGGTAACATTGGCTACAAAGACCTACCTAGATGCTCCTTTTTACGATAACTTACAGGTGAAAACCAGGATCAACTCCCGTGCCAGTCACATCTCGT
602		SRY_r1r_V3	ATTATAAGTATCGACCTCGTCGGAAGGCGAAGATGCTGCCGAAGAATTGCAGTTTGCTTCGTGAAAACCAGGATCAACTCCCGTGCCAGTCACATCTCGT

Probes were synthesized as ultramers by IDT, rehydrated to 100 uM, and pooled; each probe in the pool is present at 6.25 uM. To create a 100X stock solution in which each probe was present at 100 nM, 10 uM of pool and 10 µl of 100 uM common, biotinylated partner oligo were combined in 605 µl of TEzero + 0.05% Tween 20 (TT). The 100X stock was further diluted 100-fold (10 µl into 990 µl TT) to give a working solution in which each probe is present at a concentration of 1 nM.

CAPTURE/PROCESSING PROTOCOLS

One objective of the proof of principle study was to validate probe performance and test the efficiency of processing on sequence-ready library yield. The genomic library pool was derived from a 16 sample set library. For probe annealing, four of 10 µl library aliquots in separate PCR strip tubes were heated to 98°C for 2 min and cooled on ice. Five µl of 4X bind buffer and five µl of probe were added to each tube, and the solution was annealed using a 1°C step for four min thermal cycler program from 98°C to 69°C. Annealed complexes were bound to streptavidin-coated magnetic beads, washed four times with 25% formamide-containing wash buffer and one time with TEzero. The final complexes were suspended in 2 µl of TEzero.

Four treatments of the four complexes were investigated: (1) No processing, amplification with ACA2 primer alone to determine raw capture efficiency; (2) No processing, amplification with AF and CR to determine unprocessed amplification and capture efficiency; (3) PreCR processing in 10 µl prior to AF and CR amplification to explore low volume processing; and (4) PreCR processing in 50 µl prior to AF and CR amplification to establish high volume processing effects.

PreCR processing was accomplished by adding the manufacturer's recommended solution that contained per 100 µl:

- 82 µl of water
- 10 µl of Thermopol buffer
- 1 µl of 100X NAD^+
- 1 µl of 10 mM dNTPs
- 2 µl of PreCR enzyme mix

Ten µl of PreCR cocktail was added to tube 3 and 50 µl was added to tube 4. These were incubated at 37C for 20 min.

Following PreCR treatment, all four samples were resuspended to 50 µl by the addition of TEzero, and Q5 PCR cocktail with the appropriate PCR primers was added to a final volume of 250 µl. Each aliquot of PCR cocktail contained:

- 125 µl water
- 50 µl 5X Q5 reaction buffer
- 25 µl of 10 uM primer (either ACA2 or a 1:1 blend of AF and CR)
- 5 µl of 10 mM dNTPs
- 2.5 µl of Q5 hot start enzyme

50 µl of each PCR reaction mix was aliquoted to a tube containing 1.25 µl EvaGreen and 1 µl ROX dyes, mixed, and quadruplicate 10 µl aliquots were added to a qPCR optical PCR plate. The remaining 200 µl was split into to 100 µl aliquots. Both the qPCR and conventional PCR reaction were cycled as:

- 98°C – 30 sec
- 98°C – 10sec, 69°C – 10 sec, 72°C-10sec for 40 cycles (qPCR) and plateau cycle (conventional).

The real-time PCR reaction was monitored to determine the optimal stopping point for the conventional PCR reactions. For the ACA2 reaction, the stopping point was at 21 cycles. For the remaining reactions, the stopping point was at 28 cycles. These qPCR reactions are further described in the Results section, *infra*.

Ten µl of raw PCR was collected for gel analysis and a remaining aliquot of 100 µl was purified 1:1 with beads. The purified PCR product was eluted with 50 µl TEzero and quantified by Qubit. The DNA yields were: (1) 7.44 ng/µl; (2) 10.6 ng/µl; (3) 12.1 ng/µl; and (4) 15.7 ng/µl.

QPCR ANALYSIS OF CAPTURE/PROCESSING

A single Eco qPCR plate containing an array of eight assays – Assays 17 – 24 (Table 37) by six samples was used to assess capture efficiency. The six samples were:

1. 10 ng/µl of original gDNA library
2. NTC
3. 0.01 ng/µl of sample 1
4. 0.01 ng/µl of sample 2
5. 0.01 ng/µl of sample 3

6. 0.01 ng/μl of sample 4

The Q5 hot start assay mixture contained:

- 237.5 μl H_2O
- 100 μl 5X Q5 reaction buffer
- 10 μl dNTPs
- 12.5 μl EvaGreen
- 10 μl ROX
- 5 μl Q5 hot start enzyme

This cocktail was distributed in 48 μl aliquots and 3 μl of Assay primer (10 uM in both F and R primer) was added. This was distributed in columns. Two μl of sample was added in rows and the plate was cycled as described above.

RESULTS

Amplification of complexes. While the fluorescence profile of amplifying complexes is used primarily to identify the amplification plateau (which occurs much sooner for single primer than dual primer amplicons), the Cq value can be used to look at the content of amplicon between samples. In this experiment, the observed Cq values were:

Sample	Conditions	Cq
1	no PreCR, ACA2 single primer	15
2	no PreCR, AF + CR	21
3	10 μl PreCR, AF + CR	20
4	50 μl PreCR, AF + CR	19

These data demonstrated that PreCR treatment increased the abundance of P1 + P2 (AF + CR) amplicons.

Gel images of the post-processing PCR products shown in Figure 33 show that PreCR treatment supported the amplification of a larger size distribution of clones. The untreated, sample 2 amplicon is primarily a cluster of small size fragments. Sample 3 and to a greater extent sample 4 are more broadly distributed smears.

The qPCR results showing target enrichment are shown in Table 36 below. The raw sequence capture in sample 1 was surprisingly high. At least two factors may have

contributed to such an unexpected improvement over historical data sets: (1) The core annealing process (pre-melt, higher temps, low salt binding to strep beads) has been optimized; and (2) A longer partner oligo (40 nt vs 35 nt) was used.

Even with no PreCR treatment, P1+P2 (AF+CR) amplifiable material was made (sample 2) and substantial enrichment of target signal over gDNA (and/or NTC) was observed.

PreCR treated complex also yielded enrichment levels comparable to unprocessed (sample 1) control. This is a fantastic demonstration of the fact that PreCR processing can stimulate the recombination of the probe-based partner oligo with the genomic library-based target clones. While the levels of enrichment are not remarkable, the majority of clone material is small and falls outside the range of the qPCR assay. As noted elsewhere herein, judicious bead enrichment can dramatically increase the proportion of the library that covers the qPCR site.

In addition, the results indicated that more PreCR is not necessarily better. Sample 3 (10 µl PreCR treatment) outperformed Sample 4 (50 µl PreCR treatment) with respect to enrichment specific activity in 6 of 8 assays.

Table 36. qPCR enrichment data

Table 36. qPCR enrichment data for V3 experimental samples								
Cqs	Assay 17	Assay 18	Assay 19	Assay 20	Assay 21	Assay 22	Assay 23	Assay 24
gDNA lib	24	21	21	22	22	22	22	22
NTC	35	31	34	26	33	N/A	30	29
sample 1	16	13	13	12	14	13	15	14
sample 2	21	18	19	16	18	18	22	20
sample 3	16	14	15	13	14	15	17	15
sample 4	16	14	15	13	15	15	17	15
Abs value	Assay 17	Assay 18	Assay 19	Assay 20	Assay 21	Assay 22	Assay 23	Assay 24
gDNA lib	5	40	36	32	20	22	34	29
NTC	0	0	0	1	0		0	0
sample 1	1918	14374	9372	20243	8444	10189	2820	8133
sample 2	59	382	172	1526	295	403	34	87
sample 3	1085	5090	3051	10275	5213	4144	1068	2822
sample 4	1492	5381	2751	8770	3866	2777	777	2233
Adj value	Assay 17	Assay 18	Assay 19	Assay 20	Assay 21	Assay 22	Assay 23	Assay 24
gDNA lib	5	40	36	32	20	22	34	29
NTC	0	0	0	1	0	0	0	0

sample 1	1918016	14374082	9372333	20243011	8443797	10188837	2820166	8132920
sample 2	59292	381694	171590	1526000	295038	403467	33575	87344
sample 3	1085442	5089739	3050781	10275412	5212989	4143563	1068184	2821950
sample 4	1491501	5380882	2751029	8770152	3865764	2777330	777035	2232604
Fold enrich	Assay 17	Assay 18	Assay 19	Assay 20	Assay 21	Assay 22	Assay 23	Assay 24
sample 1	378673	356798	262412	641805	415341	453405	84090	281573
sample 2	11706	9475	4804	48382	14513	17954	1001	3024
sample 3	214298	126339	85418	325782	256421	184389	31850	97700
sample 4	294466	133566	77025	278058	190152	123592	23169	77296

DISCUSSION

The capture and processing methods disclosed in this example performed well using untreated complexes. Without wishing to be bound to any particular theory, it is contemplated that one reason that the untreated complexes performed so well was because both the capture probe and genomic fragments possess primer binding sites.

APPENDIX TO EXAMPLE 19

The primer and amplicon designs for this example are shown below in Table 37.

Table 37. Sequences used to perform Example 19

SEQ ID NO:	Target	Name	Sequence	%GC
603	SRY_r1, Assay 17	F qPCR primer	CTGGTGCTCCATTCTTGAGTGTGT	
604		R qPCR primer	GGACAACAGGTTGTACAGGGATGA	
605		qPCR Amplicon	CTGGTGCTCCATTCTTGAGTGTGTGGCTTTCGTA CAGTCATCCCTGTACAACCTGTTGTCC	
606		F-probe	CTGTAAGTTATCGTAAAAAAGGAGCATCTAGGTA GGTCTTTGTAGCCAATGTTACCCGATT	40%
607		R-probe	GAAGCAAACTGCAATTCTTCGGCAGCATCTTCG CCTTCCGACGAGGTCGATACTTATAAT	47%

608		F-probe reverse complement	AATCGGGTAACATTGGCTACAAAGACCTACCTA GATGCTCCTTTTTACGATAACTTACAG	
609		R-probe reverse complement	ATTATAAGTATCGACCTCGTCGGAAGGCGAAGA TGCTGCCGAAGAATTGCAGTTTGCTTC	
610	VHL_r3 Assay 18	F qPCR primer	TACAGGAGACTGGACATCGTCAGG	
611		R qPCR primer	CTTTCTGCACATTTGGGTGGTCTT	
612		qPCR Amplicon	TACAGGAGACTGGACATCGTCAGGTCGCTCTAC GAAGATCTGGAAGACCACCCAAATGTGCAGAA AG	
613		F-probe	CTTGTTCGTTCCTTGTACTGAGACCCTAGTCTGC CACTGAGGATTTGGTTTTTGCCCTTC	48%
614		R-probe	ATCAAGACTCATCAGTACCATCAAAAGCTGAGA TGAAACAGTGTAAGTTTCAACAGAAAT	35%
615		F-probe reverse complement	GAAGGGCAAAAACCAAATCCTCAGTGGCAGAC TAGGGTCTCAGTACAAGGAACGAACAAG	
616		R-probe reverse complement	ATTTCTGTTGAAACTTACACTGTTTCATCTCAGC TTTTGATGGTACTGATGAGTCTTGAT	
617	UGT1A 1_r4, Assay 19	F qPCR primer	GGTGATCAGATGGACAATGCAAAG	
618		R qPCR primer	TCATTTCCAGAACATTCAGGGTCA	

619		qPCR Amplicon	GGTGATCAGATGGACAATGCAAAGCGCATGGA GACTAAGGGAGCTGGAGTGACCCTGAATGTTCT GGAAATGA	
620		F-probe	TGTGTCCAGCTGTGAAACTCAGAGATGTAACTG CTGACATCCTCCCTATTTTGCATCTCA	45%
621		R-probe	ATTTGAAACAATTTTATCATGAATGCCATGACC AAAGTATTCTTCTGTATCTTCTTTCTT	28%
622		F-probe reverse complement	TGAGATGCAAAATAGGGAGGATGTCAGCAGTTA CATCTCTGAGTTTCACAGCTGGACACA	
623		R-probe reverse complement	AAGAAAGAAGATACAGAAGAATACTTTGGTCAT GGCATTCATGATAAAATTGTTTCAAAT	
624	TNFRSF14_r3, Assay 20	F qPCR primer	ACCTCAATGGCCTAAGCAAGTGTC	
625		R qPCR primer	GCCTCTTACCTGGGTCACACATTT	
626		qPCR Amplicon	ACCTCAATGGCCTAAGCAAGTGTCTGCAGTGCC AAATGTGTGACCCAGGTAAGAGGC	
627		F-probe	TGATGGGTGGGCTCCCGAAGGGGCCTCCCGCAG ACTTGCGAAGTTCCCACTCTCTGGGCG	67%
628		R-probe	CAGGGTGCGGGGGCATCCAGGCTGCCCAAGCG GAGGCTGGGCCGGCTGTGCTGGCCTCTT	73%
629		F-probe reverse complement	CGCCCAGAGAGTGGGAACTTCGCAAGTCTGCGG GAGGCCCCTTCGGGAGCCCACCCATCA	
630		R-probe reverse complement	AAGAGGCCAGCACAGCCGGCCCAGCCTCCGCTT GGGCAGCCTGGATGCCCCCGCACCCTG	

631	RUNX_r4, Assay 21	F qPCR primer	ATCTTGCAACCTGGTTCTTCATGG	
632		R qPCR primer	GCTGGCAATGATGAAAACTACTCG	
633		qPCR Amplicon	AATCTTGCAACCTGGTTCTTCATGGCTGCGGTAGCATTTCTCAGCTCAGCCGAGTAGTTTTCATCATTGCCAGC	
634		F-probe	TTTTGAAATGTGGGTTTGTTGCCATGAAACGTGTTTCAAGCATAGTTTTGACAGATAACG	37%
635		R-probe	TGCCCTAAAAGTGTATGTATAACATCCCTGATGTCTGCATTTGTCCTTTGACTGGTGTTT	40%
636		F-probe reverse complement	CGTTATCTGTCAAAACTATGCTTGAAACACGTTTCATGGCAACAAACCCACATTTCAAAA	
637		R-probe reverse complement	AAACACCAGTCAAAGGACAAATGCAGACATCAGGGATGTTATACATACACTTTTAGGGCA	
638	RHD_r5 Assay 22	F qPCR primer	CCAAGTTTCAACTCTGCTCTGCTG	
639		R qPCR primer	GTTGAACACGGCATTCTTCCTTTC	
640		qPCR Amplicon	CCAAGTTTCAACTCTGCTCTGCTGAGAAGTCCAATCGAAAGGAAGAATGCCGTGTTCAAC	
641		F-probe	AACCCCTCGAGGCTCAGACCTTTGGAGCAGGAGTGTGATTCTGGCCAACCACCCTCTCTG	58%
642		R-probe	CATAAATATGTGTGCTAGTCCTGTTAGACCCAAGTGCTGCCCAAGGGCAGCGCCCTGCTC	55%

643		F-probe reverse complement	CAGAGAGGGTGGTTGGCCAGAATCACACTCCTG CTCCAAAGGTCTGAGCCTCGAGGGGTT	
644		R-probe reverse complement	GAGCAGGGCGCTGCCCTTGGGCAGCACTTGGGT CTAACAGGACTAGCACACATATTTATG	
645	PTEN_r5, Assay 23	F qPCR primer	GGAAAGGGACGAACTGGTGTAATG	
646		R qPCR primer	AAATCTAGGGCCTCTTGTGCCTTT	
647		qPCR Amplicon	GGAAAGGGACGAACTGGTGTAATGATATGTGCA TATTTATTACATCGGGGCAAATTTTTAAAGGCA CAAGAGGCCCTAGATTT	
648		F-probe	TACTTGTTAATTAAAAATTCAAGAGTTTTTTTTT CTTATTCTGAGGTTATCTTTTTACCA	22%
649		R-probe	CCAAAATCTGTTTTCCAATAAATTCTCAGATCCA GGAAGAGGAAAGGAAAAACATCAAAA	33%
650		F-probe reverse complement	TGGTAAAAAGATAACCTCAGAATAAGAAAAAA AAACTCTTGAATTTTTAATTAACAAGTA	
651		R-probe reverse complement	TTTTGATGTTTTTCCTTTCCTCTTCCTGGATCTGA GAATTTATTGGAAAACAGATTTTGG	
652	EP300_r18, Assay 24	F qPCR primer	AGCCCCATGGATCTTTCTACCATT	
653		R qPCR primer	ATACTGCCAGGGCTCCTGATACTG	

654		qPCR Amplicon	AGCCCCATGGATCTTTCTACCATTAAGAGGAAG TTAGACACTGGACAGTATCAGGAGCCCTGGCAG TAT	
655		F-probe	ATACTCCATCTCCCGTAAAAATAGTGAGACTTG AGTAATGTTTGATGTCACTTGTCTTTC	37%
656		R-probe	CAGTCACCACTATATTATTCTAGGTATCCCAGA AAAGTTAAAGTCAAATCTGAAACACAT	33%
657		F-probe reverse complement	GAAAGACAAGTGACATCAAACATTACTCAAGTC TCACTATTTTTACGGGAGATGGAGTAT	
658		R-probe reverse complement	ATGTGTTTCAGATTTGACTTTAACTTTTCTGGGA TACCTAGAATAATATAGTGGTGACTG	
659	Addition al oligos	Tail sequence for capture probes	GTGAAAACCAGGATCAACTCCCGTGCCAGTCAC ATCTCGT	
660		Common partner oligo sequence	/5BioTEG/ACGAGATGTGACTGGCACGGGAGTTG ATCCTGGTTTTCAC	
661		Forward amplification primer AF	AATGATACGGCGACCACCGAGATCTACACGTCA TGCAGGACCAGAGAATTCGAATACA	
662		Reverse amplification primer CR	CAAGCAGAAGACGGCATACGAGATGTGACTGG CACGGGAGTTGATCCTGGTTTTCAC	

EXAMPLE 20

LIBRARY-FREE TARGETED GENOMIC ANALYSIS

SUMMARY

This example demonstrates a library-free genomic analysis. The goals were to identify the most useful parameters for implementing such methods in a reliable, reproducible, low-cost, and high-throughput format. In particular, it was discovered that T4 polymerase can copy many and diverse genomic sequences provided it is supplemented with T4 gene 32 protein in the presence of PEG8000 – a molecular crowding agent. In addition, it was found that suppression PCR just upstream of sequence library construction is a powerful method to enrich for long insert sequencing clones.

BACKGROUND

The molecular concepts behind library-free methods include:

(1) fragmenting gDNA to ~400bp or performing 1st strand cDNA synthesis with random 15-mers in the presence of ddNTPs (Figure 33);

(2) melting the gDNA or cDNA with labeled capture probes and purify the end-repaired gDNA/cDNA. For gDNA, the genomic sequence is repaired with a sequence tag that comprises a random hexamer sequence contained within the tail portion (Figure 33);

(3) processing the DNA complex in a single reaction at 20°C. The Buffer used is NEB CutSmart (NEB#4 and BSA), ATP, dNTPs, and PEG8000. The complex is processed with T4 DNA polymerase, T4 gene 32 protein (SB), and T4 DNA ligase. The adapter ligation strand is 5′ phosphorylated and the partner strand comprises a 3′ ddC. The opposite end of the adapter is staggered and can be blocked. A blunt configuration makes no self-dimer, is extremely efficiency and attaches the P1 containing ligation strand to the P2 containing target. (Figure 34);

(4) PCR amplification to add flow cell compatible sequences and to introduce sample-specific index sequence to each reaction (Figure 35); and

(5) DNA sequencing (Figure 35).

One potential artifact that may occur in particular embodiments is associated with the abundance of unoccupied probes. The 3′ to 5′ exonuclease activity of T4 DNA polymerase is capable of generating a blunt end on these molecules, which then becomes a substrate for ligation to the P1 adaptor sequence (Figure 36). These short "oligo-dimer" products will, without intervention, overwhelm the subsequent PCR reaction. To circumvent the potential artifact, a suppressive PCR design was used, in which a 25 nt segment of P2 was included in the P1 adaptor. Following suppression PCR amplification with this segment, forward and reverse primers with P1 or P2-specific extensions are used to add the index sequence and the flow cell-compatible extensions.

The oligonucleotides that enable post-processing suppressive PCR, full-length amplification and sequencing are shown in the Table 38 below.

Table 38.

SEQ ID NO:	Name	Sequence	Description
663	LF_part strand	AGTTGATCCTGGTTATACA/3ddC/	Adaptor partner strand
664	LF_lig strand	/5Phos/GTGTATAACCAGGATCAACTCCC GTGCCAGT	Adaptor ligation strand
665	LF_I1P	GTGAAAACCAGGATCAACTCCCGTGCCA GTCAC	Index 1 sequencing primer
666	LF_FSP	GTCATGCAGGAGTTGATCCTGGTTATAC AC	Library-free Forward sequencing primer
667	LF_Single primer sequence	ACTGGCACGGGAGTTGATCCTGGTT	Post-processing amplification primer
668	LF_FLFP	AATGATACGGCGACCACCGAGATCTAC ACGTCATGCAGGAGTTGATCCTGGTTAT ACAC	Library-free forward amplification primer
669	LF_FLRP_ N701	CAAGCAGAAGACGGCATACGAGATTCG CCTTAGTGACTGGCACGGGAGTTGATCC TGGTTTTCAC	Index N701 reverse primer
670	LF_FLRP_ N702	CAAGCAGAAGACGGCATACGAGATCTA GTACGGTGACTGGCACGGGAGTTGATCC TGGTTTTCAC	Index N702 reverse primer
671	LF_FLRP_ N703	CAAGCAGAAGACGGCATACGAGATTTC TGCCTGTGACTGGCACGGGAGTTGATCC TGGTTTTCAC	Index N703 reverse primer
672	LF_FLRP_ N704	CAAGCAGAAGACGGCATACGAGATGCT CAGGAGTGACTGGCACGGGAGTTGATC CTGGTTTTCAC	Index N704 reverse primer

MATERIALS

Genomic DNA samples were collected from 4 subjects and purified using the Oragene saliva collection kit. The samples that were sequenced in this study were:

Genomic DNA samples and Illumina Indexes used in this study		
Coriel ID #	Sample description	Illumina Index ID
NA19240	Yoruba female	Index N701 reverse primer
GM03623	Aneuploid XXX and chr18 trisomy	Index N702 reverse primer
GM11226	XXXX	Index N703 reverse primer
GM18917	Yoruban/Nigerian - harbors deletion variants 1,2,4,9	Index N704 reverse primer

The probes used in these experiments are provided in Table 39 below. Hexamer tags are required to establish independent capture events with the same sequencing start site from sibling clones that arise during post-capture amplification.

Table 39.

SEQ ID NO:	Name	Sequence
673	CYP2D6_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAAGCACCTAGCCCCATTCCTGCTGAGCAGGAGGTGGCAGGTACCCCAGACTGGGAGGTAA
674	CYP2D6_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGTCGGTGGGGCCAGGATGAGGCCCAGTCTGTTCACACATGGCTGCTGCCTCTCAGCTCT
675	AMY1_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNACCTGAGTAGCATCATTGTAGTTCTCGATATCTCCACTTCCAGTTTTACATTTACCATCA
676	chrX_15_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCCTGGCCCTCAGCCAGTACAGAAAGTCATTTGTCAAGGCCTTCAGTTGGCAGACGTGCTC
677	chrX_15_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGAATTCATTGCCAGCTATAAATCTGTGGAAACGCTGCCACACAATCTTAGCACACAAGA
678	chrX_477_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGACTTCAAAGAAATTACAAGTTGACATCTTGGACTCTACCCCTCGTACTTTATCTCCTAT
679	chrX_477_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTCTCTTTGGGGTCAAGAAAGAATCCCTAGTGGATTTGGGATTCTAGAGGAGGTGTTATAA
680	chrX_478_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTGCGATACCATGCTGAAGATGAGCTAACCCAACCAGCCAAGCAGGCAGGGCTGCGAAGGA

681	chrX_478_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGGGGTAGGTGGAAAACCCAAGTAATGTGATTTTGTAAC ATCCACTGCTGCATTTGTTTGC
682	chrX_69_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTTACTTCCCTCCAGTTTTGTTGCTTGCAAAACAACAGAA TCTTCTCTCCATGAAATCATG
683	chrX_69_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NCAGGGGTATCTATTATCCCCATTTTCTCACAAAGGAAAC CAAGATAAAAGGTTTAAATGG
684	PLP1_ex1_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGAAATTCTCTTGTGAATTCCTGTGTCCTCTTGAATCTTCA ATGCTAAAGTTTTTGAAACT
685	PLP1_ex2_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGGGTTTGAGTGGCATGAGCTACCTACTGGATGTGCCTGA CTGTTTCCCCTTCTTCTTCCC
686	PLP1_ex2_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NCTATCTCCAGGATGGAGAGAGGGAAAAAAAAAGATGGGT CTGTGTGGGAGGGCAGGTACTT
687	PLP1_ex3_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGAAAGAAGCCAGGTCTTCAATTAATAAGATTCCCTGGT CTCGTTTGTCTACCTGTTAATG
688	PLP1_ex3_M	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NCAGACTCGCGCCCAATTTTCCCCCACCCCTTGTTATTGC CACAAAATCCTGAGGATGATC
689	PLP1_ex3_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTCTTTCTTCTTCCTTTATGGGGCCCTCCTGCTGGCTGAGG GCTTCTACACCACCGGCGCA
690	PLP1_ex4_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGTTTGTGTTTCTACATCTGCAGGCTGATGCTGATTTCTA ACCACCCCATGTCAATCATTT
691	PLP1_ex4_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NAACCAAATATATAGTGCTTCCATAGTGGGTAGGAGAGC CAAAGCACCCGTACCCTAACTC
692	PLP1_ex5_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NAGTCTCCATGTGGCCCCGTAACTCCATAAAGCTTACCCT GCTTGCTTTTTGTGTCTTACT
693	PLP1_ex5_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NCCATGGGTGTAATTTGTATGGTATTAGCTACTCCCTTGT AAAATAACCCAAATAACCCAC
694	PLP1_ex6_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTTTACAGTGGAGCATATTACTGCTGTTGCAAGAAACAGT TCTTCCTCTTTCATTTTCCTG
695	PLP1_ex6_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NATAGCTGTACCCACACTATCTCAGGCCTATTTACTTGCC AAGATCATTCAAAGTCAACTC

696	PLP1_ex7_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGATTTGAGGAGGGAGTGCTTTCTTTTCTACTCTCATTCA CATTCTCTCTTCTGTTCCCTA
697	PLP1_ex7_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NCAGCATTGTAGGCTGTGTGGTTAGAGCCTCGCTATTAGA GAAAGGGGGATTTCTACGGGG
698	KRAS_ex1_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTGTTACCTTTAAAAGACATCTGCTTTCTGCCAAAATTAA TGTGCTGAACTTAAACTTACC
699	KRAS_ex1_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTTCCCAGTAAATTACTCTTACCAATGCAACAGACTTTAA AGAAGTTGTGTTTTACAATGC
700	KRAS_ex2_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTAAATGACATAACAGTTATGATTTTGCAGAAAACAGAT CTGTATTTATTTCAGTGTTACT
701	KRAS_ex2_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGACAGGTTTTGAAAGATATTTGTGTTACTAATGACTGTG CTATAACTTTTTTTTCTTTCC
702	KRAS_ex3_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NACTCAAAAAATAAAAACTATAATTACTCCTTAATGTCAG CTTATTATATTCAATTTAAAC
703	KRAS_ex3_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NAACACCTTTTTTGAAGTAAAAGGTGCACTGTAATAATCC AGACTGTGTTTCTCCCTTCTC
704	KRAS_ex4_F	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGAAACCTTTATCTGTATCAAAGAATGGTCCTGCACCAGT AATATGCATATTAAAACAAGA
705	KRAS_ex4_R	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGTGTATTAACCTTATGTGTGACATGTTCTAATATAGTCA CATTTTCATTATTTTTATTAT
706	MYC_r1_F1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NCCCCAGCCAGCGGTCCGCAACCCTTGCCGCATCCACGA AACTTTGCCCATAGCAGCGGGC
707	MYC_r1_R1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN Ncgactcatctcagcattaaagtgataaaaaaataaattaaaaggcaagtggacttcggtg
708	MYC_r2_F1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN Nctgtggcgcgcactgcgcgctgcgccaggtttccgcaccaagacccctttaactcaagac
709	MYC_r2_F2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTTCTACTGCGACGAGGAGGAGAACTTCTACCAGCAGCA GCAGCAGAGCGAGCTGCAGCCC
710	MYC_r2_F3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NACCGAGCTGCTGGGAGGAGACATGGTGAACCAGAGTTT CATCTGCGACCCGGACGACGAG

711	MYC_r2_F4	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGCCGCCGCCTCAGAGTGCATCGACCCCTCGGTGGTCTTC CCCTACCCTCTCAACGACAGC
712	MYC_r2_R1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGGCGGCTAGGGGACAGGGGCGGGGTGGGCAGCAGCTC GAATTTCTTCCAGATATCCTCGC
713	MYC_r2_R2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NAGACGAGCTTGGCGGCGGCCGAGAAGCCGCTCCACATA CAGTCCTGGATGATGATGTTTT
714	MYC_r2_R3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NAGGAGAGCAGAGAATCCGAGGACGGAGAGAAGGCGCT GGAGTCTTGCGAGGCGCAGGACT
715	MYC_r2_R4	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN Ntaagagtggcccgttaaataagctgccaatgaaaatgggaaaggtatccagccgcccact
716	MYC_r3_F1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN Nttgtatttgtacagcattaatctggtaattgattattttaatgtaaccttgctaaaggag
717	MYC_r3_F2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGAGGCCACAGCAAACCTCCTCACAGCCCACTGGTCCTC AAGAGGTGCCACGTCTCCACAC
718	MYC_r3_F3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NAGAGGAGGAACGAGCTAAAACGGAGCTTTTTTGCCCTG CGTGACCAGATCCCGGAGTTGG
719	MYC_r3_R1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NTCCAACTTGACCCTCTTGGCAGCAGGATAGTCCTTCCGA GTGGAGGGAGGCGCTGCGTAG
720	MYC_r3_R2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGCTTGGACGGACAGGATGTATGCTGTGGCTTTTTTAAGG ATAACTACCTTGGGGGCCTTT
721	MYC_r3_R3	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNN NGCATTTGATCATGCATTTGAAACAAGTTCATAGGTGATT GCTCAGGACATTTCTGTTAGA

METHODS, RESULTS AND DISCUSSION

PART I. Four gDNAs (F, S, C and L) were diluted to 20 ng/µl in 150 µl final volume. The samples were sonicated to 500 bp and 125 µl was purified with 125 µl of beads. The starting material and purified, fragmented gDNA are shown in Figure 37. The concentrations of gDNA were: (1) F - 137 ng/µl; (2) S - 129 ng/µl; (3) C - 153 ng/µl; and (4) L - 124 ng/µl.

For capture, 10 µl of gDNA sample was heated to 98°C for 2 min (to achieve strand dissociation) and cooled on ice. 5 µl of 4X bind and 5 µl of the 49 probe pool (SEQ ID NOs: 150-198) (1 nM in each probe combined with 50 nM universal oligo 61, were added and the mix was annealed (98°C 2 min followed by 4 min incubations at successive 1°C lower temperatures down to 69°C). The complexes were bound to 2 µl of MyOne strep beads that were suspended in 180 µl TEzero (total volume 200 µl) for 30 min, washed four times, 5 min each with 25% formamide wash, washed once with TEzero, and the supernatants were withdrawn from the bead complexes.

For processing and adaptor ligation, 100 µl of T4 mix was made that contained: 60 µl water; 10 µl NEB "CutSmart" buffer; 15 µl 50% PEG8000; 10 µl 10 mM ATP; 1 µl 1 mM dNTP blend; 1 µl T4 gene 32 protein (NEB); and 0.5 µl T4 DNA polymerase (NEB). 25 µl of mix was added to each of the four samples and incubated at 20°C for 15 min followed by a 70°C incubation for 10 min to heat inactivate the T4 polymerase. Following the inactivation step, 1.25 µl of adaptor (10 µM) and 1.25 µl of HC T4 DNA ligase were added. This mixture was further incubated at 22°C for 30 min and 65°C for 10 min.

One attractive feature of the library free methods is that processed complexes are still attached to beads. Beads were pulled from the ligation buffer and washed once with 200 µl of TEzero. The complexes were then resuspended in 2 µl. For amplification, single primer amplification in a 20 µl volume was used to both amplify target fragments and to enrich for long genomic fragments over probe "stubs". Following the amplification, a larger volume PCR reaction with full length primers were used to create a "sequence-ready" library.

A Q5-based single primer PCR amplification buffer was made by combining 57 µl water, 20 µl 5X Q5 reaction buffer, 10 µl of single primer 117 (see Table 38), 2 µl of 10 mM dNTPs, and 1 µl of Q5 hot start polymerase. Eighteen µl was added to each tube followed by amplification for 20 cycles (98°C – 30sec; 98°C – 10 sec, 69°C – 10 sec, 72°C – 10 sec for 20 cycles; 10°C hold). Following the PCR, the beads were pulled out and the 20 µl of pre-amp supernatant was transferred to 280 µl of PCR mix that contained 163.5 µl water, 60 µl 5X Q5 buffer, 15 µl of forward primer 118 (10 uM), 15 uM of reverse primer 119 (10 uM), 6 µl of 10 mM dNTPs, 13.5 µl of EvaGreen+ROX

dye blend (1.25 parts EG to 1 part ROX), and 3 µl of Q5 hot start polymerase (adding the dye to all reactions was unintended). Two 100 µl aliquots were amplified by conventional PCR (98°C- 10sec, 69°C – 10 sec, 72°C – 10 sec) and quadruplicate ten µl aliquots were amplified under qPCR conditions. Figure 38 shows the amplification plot that was observed for all four samples. The reaction seems to go through an inflection/plateau reminiscent of PCR and the conventional reactions were stopped at 20 cycles (now 40 total cycles of PCR). Figure 39 shows a 2% agarose gel containing the products of these amplification reactions. Figure 40 shows a 2% agarose gel containing the amplification products following bead purification.

The library-free samples were assayed to determine if gene specific targets were captured and selectively amplified using the well-validated qPCR capture assays described elsewhere herein. The target regions for Assays 1 – 16 are shown in Table 40.

Table 40. Target regions for assays 1-16.

Assay	Sequence
Assay 1	PLP1 exon 2
Assay 2	PLP1 exon 2
Assay 3	PLP1 exon 2
Assay 4	PLP1 upstream of exon 2
Assay 5	PLP1 downstream of exon 2
Assay 6	PLP1 200 bp downstream of exon 2
Assay 7	PLP1 exon 3
Assay 8	Chr 9, off target
Assay 9	CYP2D6
Assay 10	Chr X: 154376051
Assay 11	Chr X: 154376051
Assay 12	Chr X: 6929264
Assay 13	KRAS Region 1
Assay 14	KRAS Region 2
Assay 15	Myc Region 2
Assay 16	Myc Region 2

For qPCR analysis, genomic DNA from sample F at 10 ng/μl (2 μl is added to 8 μl of PCR mix to give a final volume and concentration of 10 μl and 2 ng/μl, respectively) was used as control. Purified processed material from the F and S samples was diluted to 0.01 ng/μl = 10 pg/μl and 2 μl was added to each 8 μl PCR reaction to give a final concentration of 2 pg/μl. The results are shown in Table 41.

Table 41. qPCR results

Qty	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA	23	22	25	22	22	28	23	19
F-Rep	13	13	13	13	13	30	12	35
S-Rep	14	14	13	13	14	20	13	N/A
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA	21	21	22	22	22	21	21	21
F-Rep	11	13	13	16	18	17	14	13
S-Rep	11	13	14	17	17	35	13	12

Qty	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA	16	24	4	17	22	15	8	146
F-Rep	12150	13296	8374	16739	13728	79	23495	0
S-Rep	6142	7704	10410	10893	6425	133	14548	
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA	60	39	18	30	17	43	49	37
F-Rep	45447	11671	11034	1288	440	353	6373	16448
S-Rep	68935	12477	7634	678	1077	1303	10171	19305

Qty	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
gDNA	16	24	4	17	22	15	8	146
F-Rep	12150333	13296288	8374203	16739290	13737864	79482	23495321	2
S-Rep	6141637	7703565	10410406	10893356	6424647	131376	14548060	0
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
gDNA	60	39	18	30	17	43	49	37
F-Rep	45447322	11670722	11034289	1288163	440236	352319	6373326	16447972
S-Rep	68934509	12476684	7634492	677562	1077493	1303428	10170993	19304903

Qty	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7	Assay 8
F-Rep	778820	562525	3993851	987542	627702	5247	3657854	0
S-Rep	393671	325914	2808248	595466	393551	8706	1730754	0
	Assay 9	Assay 10	Assay 11	Assay 12	Assay 13	Assay 14	Assay 15	Assay 16
F-Rep	757299	300823	627284	42799	25665	19716	130461	439562
S-Rep	1348823	321597	4340103	22512	62817	27819	208198	515912

The qPCR data indicated that the library-free technology is very effective at retrieving the targeted genomic regions and at leaving off-target regions behind (*e.g.*, Assays 6, 8). The fold purifications, often >500,000-fold, were directly comparable to data from earlier experiments generated with libraries as disclosed elsewhere herein.

Part II – Leave-one-out analysis: The enzymatic requirements for complex processing were evaluated: the design of experiment is shown in Table 42 below.

Table 42. Experimental design

EXPERIMENT:	1	2	3	4	5
T4 DNA polymerase	no	no	yes	yes	yes
T4 gene 32 protein	no	yes	no	yes	yes
T4 DNA ligase	no	yes	yes	no	yes

To make capture complexes for analysis, twelve identical reactions were created. Ten μl of 135 ng/μl sonicated gDNA was melted, annealed with a tagged, bound to streptavidin coated beads, washed and resuspended in TEzero as described *supra*. Five hundred μl of processing master mix was prepared by combining 270 μl water, 50 μl 10X CutSmart buffer, 50 μl of 10 mM ATP, 75 μl of 50% PEG8000, and 5 μl of 10 mM dNTPs. This buffer was divided into 10 90 μl aliquots (duplicate tests were performed) and enzyme was added in the amounts described above (per 90 μl of master mix was added 1 μl of T4 gene 32 protein, 0.5 μl of T4 polymerase, 5 μl of adaptor and/or 5 μl of HC T4 ligase). Following T4 fill-in and ligation as described *supra*, the complexes were washed free of processing mix in TEzero and resuspended in 2 μl TEzero. Complexes were resuspended in 20 μl final volume each of single primer amplification mix and amplified for 20 cycles as described *supra*. The beads were then pulled aside using a magnet and the 20 μl clarified amplification was diluted into 180 μl of full-length F+R (118+119) PCR amplification mix. Fifty μl was pulled aside for qPCR analysis and the remaining 150 μl was split in two and amplified by conventional PCR. The 50 μl qPCR samples were mixed with 2.5 μl of dye blend and 10 μl aliquots were monitored by fluorescence change. The traces of this experiment are shown in Figure 41.

One of the two conventional PCR aliquots was pulled at 10 cycles and the other at 16 cycles of PCR. Aliquots of these raw PCR reactions (5 μl of each reaction) were analyzed on 2% agarose gels. The results are shown in Figure 42. The surprising result is that all three enzymes are required for the efficient production of amplifiable library material. A more subtle observation is that the size distribution of all-three-enzyme-material at 10 cycles is significantly larger than the size distribution of P+L alone that appears at 16 cycles.

These data together with the qPCR from the initial investigation support the interpretation that T4 DNA polymerase in conjunction with T4 gene 32 protein in the

presence of the molecular crowding agent PEG8000 (the latter contribution has not been evaluated) is capable of efficiently copying captured genomic material onto capture probes.

Part III - Generation of a library-free sequencing library: The methods described above were used to produce a DNA sequencing library with the four Coriel samples shown in the "Materials" section of this report. Each one of the four samples was coded with an individual index code in the final PCR step. The final library constituents (shown separately prior to pooling) are shown in the gel image in Figure 43. The "normal" library smear usually stretches from 175 bp upward. Here, the smallest fragments are > 300 bp. Similarly, the largest fragments appear to be 750 bp or larger. Larger fragments do not give rise to optimal libraries. These samples were all twice purified on 80% bead:sample ratios. These samples were pooled into a 16.9 ng/μl pool that, with an estimated average insert size of 400 bp, is ~65nM. The samples were sequenced.

The library-free methods worked well for CNV analyses. Unique read counts for the X-linked genePLP1 were normalized to the autosomal loci KRAS and MYC and the plot of these data is shown in Figure 44. The data illustrate that absolute copy number is lost with the library-free procedure (the "copies" of KRAS relative to MYC are no longer comparable). However, relative copy number (the change of PLP1 relative to the autosomal normalizers) is robustly detected. The sequencing results also showed striking features related to read start sites relative to probe. Figure 45 shows that reads are detected as far as 900 bp from the probe; and between coordinates 1100 and 1300 every single start point is used multiple times. These data indicated that reads start at every single possible base position and that there is little ligation/processing bias. In addition, there are very few reads that start within 100 bp of the probe, consistent with the very large size distribution of the library that was observed on gels.

EXAMPLE 20

TARGETED GENE EXPRESSION ANALYSIS

OVERVIEW

This example demonstrates the development of targeted gene expression libraries. The input is RNA, not DNA, and therefore double-strand cDNA synthesis step is required. A preferred method is 1st strand synthesis using an RNAse H- reverse transcriptase or a kit that exhibits RNAse H¯like activity (*e.g.*, Promega's GoScript) and priming with random hexamers. A preferred method for 2nd strand synthesis is to use a kit that includes *E. coli* DNA polymerase holoenzyme, NAD^+-dependent ligase and RNAse H (*e.g.*, New England Biolabs 2nd strand cDNA synthesis module).

Because there is a very wide range of transcript copies, there must be a correspondingly wide range of random tags that are introduced on adaptors to sheared and end-repaired cDNA. Accordingly, random 8-mers (65,536 possible sequences) were used. The adaptor was engineered with a random 8-mer sequence followed by 10-12 fixed bases that can both serve as an annealing site for a complementary 10-12 that facilitates ligation and that is used as a sample identifier in the case of multiplexed samples.

The actual number of unique versus duplicated reads – in other words the statistical distribution of reads - is one important factor in determining expression levels. One potential source of error are reads that are duplicated after the capture event. To identify these errors, a random tag was added to the capture probe such that each capture event is labeled.

The treatment and sequencing of targeted RNA-seq libraries follows the same procedure as treatment of genomic libraries.

The informatics analysis starts with the removal of post-capture duplicate reads and alignment to the target transcriptome. The unique read counts among aligning reads are then determined. While the data can then be fit to a statistical distribution, it was found that the raw unique read counts are a very close approximation to actual expression levels.

PURPOSE

The purpose of these experiments was to make targeted expression sequencing libraries from heart and liver total RNAs and to make both total RNA and targeted RNA libraries from the same starting material a direct comparison could be made.

SUMMARY

The RNA counts from total RNA libraries and from targeted RNA libraries showed good agreement along two parameters. First, the expression ratios between heart vs liver samples were well correlated. Second, the measurement of absolute abundance of different transcripts within a given sample was in good agreement when total RNA counts were compared to targeted RNA counts. These first-pass data indicated that quantitative targeted nucleic acid methods can be extended beyond genomic DNA into the analysis of cDNA libraries.

STRATEGY

To create a reasonable total RNA library that is depleted of rRNA, dT priming was used. To create targeted RNA libraries, total RNA samples were initially primed with IDT-supplied random hexamers. Random hexamer priming likely provides the most comprehensive coverage of the transcriptome. The total RNA libraries were sequenced after amplification with PCR primers that introduce P1 and P2 flow cell sequences. For targeted analysis, the capture, washing, processing and amplification steps were performed as contemplated elsewhere herein. The targeted clones were then sequenced.

METHODS

Oligonucleotides: For total RNA libraries the poly-dT primer: TTTTTTTTTTTTTTTTTTVN (SEQ ID NO: 722) was used. For targeted RNA-seq, an adaptor design was created in which the first 8 bases were random and the next 12 served as a "code" and an anchor sequence for a 12 mer partner strand oligo that could therefore form a ligatable duplex.

The sequences of these adaptors were:

SEQ ID NO:	Name	Sequence
723	RNA_L_1	TGCAGGACCAGAGAATTCGAATACANNNNNNNNGACTCCGATCCC
724	RNA_P_1	GGGATCGGAGTC
725	RNA_L_2	TGCAGGACCAGAGAATTCGAATACANNNNNNNNCGGAACTCGGAG
726	RNA_P_2	CTCCGAGTTCCG

cDNA library construction: The following methods were used to synthesize the four following cDNA libraries: (1) Heart total RNA (dT primed); (2) Heart targeted RNA (N_6 primed); (3) Liver total RNA; and (4) Liver targeted RNA (N_6 primed). One μg/μl total RNA was diluted10-fold to 100 ng/μl in TEz. The following components were combined in a total volume of 10 μl, heated to 65°C and transferred to ice: 2 μl of diluted total RNA (100 ng); 2 μl of 5 uM poly-dTVN primer or 2 μl of 50 uM N6 (IDT); and 6 μl of water.

The mix was combined with 10 μl of 1st strand cocktail (4 μl 5X GoScript buffer; 1.6 μl 25 mM Mg^{++} (2 mM final concentration); 1.0 μl 10 mM dNTPs (500 uM final concentration); 1.0 μl GoScript enzyme; and 2.4 μl water) and incubated at 42°C for 30 min, then 70°C for 10 min. Sixty microliters of 2nd strand synthesis reagents (48 μl water, mix; 8 μl 10X second strand synthesis buffer; 4 μl second strand enzyme mix) were added to each reaction and incubated at 16°C for 2 hours.

Following the second strand synthesis 55 μl TEz was added to each reaction and reactions were transferred to glass Covaris sonication tubes and sonicated to about 500bp. 125 μl of the sonicated sample was transferred to a PCR strip tube and 125 μl beads were added. Following purification, the sample was resuspended to a final volume of 20 μl

End repair was carried out for 19 μl of the reaction using the methods contemplated herein. The end repaired fragments were then ligated to adaptors at 22°C for 30 min and heat inactivated at 65°C for 10 min. Ligation was carried out in a 40 μl final volume: 25 μl of repaired fragments; 2 μl of 10 uM adaptor (10 uM in L strand, 20 uM in P strand); 4 μl 10X buffer; 6 μl 50% PEG8000; 1 μl water; and 2 μl HC T4 ligase. 60 μl TEz and 100 μl beads were added to each reaction and the sample was purified to a final volume of 20 μl.

Library amplification was monitored using qPCR and each library was amplified by PCR by combining 20 μl of purified ligation mix with 130 μl PCR mix (75 μl 2X NEBNext master mix, 15 μl ACA2-20, 40 μl water). 50 μl was aliquoted into a well that contains 2.5 μl EvaGreen+ROX and further aliquoted to10 μl in qPCR plate. The remaining 100 μl was kept in PCR strip tube. The PCR amplification was carried out at 72°C – 30 sec, 98°C – 30 sec, and variable cycles of 98°C – 10sec, 60°C – 10 sec, 72°C – 10 sec.

For dT libraries, 100 μl PCR reactions were purified with 120 μl beads. ACA2-20 (a 20 nt PCR primer) amplified material was diluted 20-fold – 5 μl into 95 μl of PCR mix – that contains 50 μl 2X NEBNext master mix, 5 μl of F primer, 5 μl of R-primer and 35 μl of water. The F primer is oligo #8, ACA2_FLFP AATGATACGGCGACCACCGAGATCTACACGTCATGCAGGACCAGAGAATTC GAATACA (SEQ ID NO: 69) and the reverse primer is oligo #63, exome CAC3_FLRP CAAGCAGAAGACGGCATACGAGATGTGACTGGCACGGGACCAGAGAATTC GAATACA (SEQ ID NO: 74). Amplification was carried out for 8 cycles. This step was included to grow the shorter, 20 bp ACA2 terminal sequences into longer P1 and P2, flow cell-compatible and sequencable sequences. Constructs that pick up the two different primers will amplify while those that have only one sequence will be suppressed. The resulting DNA was purified by adding 100 μl beads to the 100 μl PCR rxn and resuspended in a final volume of 50 μl.

DNA was quantified by Qubit and examined by gel electrophoresis. The DNA was sequenced using the forward primer oligo #7, ACA2_FSP ACACGTCATGCAGGACCAGAGAATTCGAATACA (SEQ ID NO: 68) and the reverse primer oligo #62, exome CAC3_RSP GTGACTGGCACGGGACCAGAGAATTCGAATACA (SEQ ID NO: 73). dT-primed RNA was sequenced in runs_48 & 49.

The DNA gel of samples is shown in Figure 46. The large fragment size distribution of dT-primed total RNA libraries was somewhat surprising.

For targeted RNA sequencing, N_8-primed libraries were resuspended in 40 μl of TEz. Fragment content in heart and liver libraries was quantified: 153 fg/μl of heart

sample cDNA and 760 fg/μl of liver sample cDNA. Based on these data, 40 μl of the heart ligation sample and 8 μl of the liver ligation sample were carried into downstream PCR amplification.

Progress of library amplification was monitored using qPCR and the library was amplified by PCR combining 40 μl of purified ligation mix (heart) or 8 μl of ligation mix + 32 μl of TEz (liver) with 210 μl PCR mix (125 μl 2X NEBNext master mix, 25 μl ACA2, 60 μl water). 50 μl was aliquoted into a well that contains 2.5 μl EvaGreen+ROX and further aliquoted into10 μl aliquots in a qPCR plate. The remaining 100 μl was placed in a PCR strip tube. The PCR amplification was carried out at 72°C – 30 sec, 98°C – 30 sec, and variable cycles of 98°C – 10sec, 60°C – 10 sec, 72°C – 10 sec. 200 μl of PCR products were purified with 200 μl beads and resuspended in a final volume of 25 μl. The concentration of PCR products was 41 ng/μl for the heart library and 42 ng/μl for the liver library.

For capture, heart and liver samples were combined and two of "2x" capture reactions were performed with tagged RNA-seq-specific probes (see Appendix, *infra* for sequences), washed, processed C+P (final yield = 40 μl of 23 ng/μl), and size selected 240 – 600 bp fragments on a Pippin automated DNA size selector. 5.4 ng/μl of fragments were recovered from the Pippin = 20.8 nM. A flow cell was loaded with the fragments and 51 nt first reads and 24 nt second reads were collected.

RESULTS AND DISCUSSION

To determine useful RNA-seq data, a heart versus liver sample was chosen for comparison. 21 transcripts were parsed out of transcripts reported in the RNA-seq Atlas (medicalgenomics.org/rna_seq_atlas) based on their absolute abundance in one or the other tissue (RPKM values of about 100, 10, 1 *etc*. in heart or liver) and on their ratios between tissues (again about 100, 10, 1, 0.1, 0.01 for heart vs liver ratios). The list of candidate transcripts and their reported RPKM values are shown in Table 43.

Table 43. Candidate RNA-seq transcripts

hgnc_symbol	transcript	transcript_length	heart	liver	h/l	l/h
MYH7	NM_000257	6030	2137.51	0.007	305358.6	0.0
NEBL	NM_006393	9213	115.617	0.097	1191.9	0.0
MGP	NM_000900	661	1059.49	11.155	95.0	0.0
DKK3	NM_013253	2746	118.464	1.136	104.3	0.0
BVES	NM_147147	5505	11.27	0.11	102.5	0.0
PDE4DIP	NM_001002812	4824	108.053	10.053	10.7	0.1
HAND2	NM_021973	2368	10.069	0.992	10.2	0.1
RP9P	NR_003500	1297	1.279	0.125	10.2	0.1
SOD2	NM_000636	1593	492.028	500.782	1.0	1.0
ATP5E	NM_006886	417	101.927	93.268	1.1	0.9
TRAPPC1	NM_021210	819	10.197	9.646	1.1	0.9
TAF1A	NM_005681	1879	1.101	1.035	1.1	0.9
MGST1	NM_145764	910	43.368	429.307	0.1	9.9
PDIA4	NM_004911	2952	14.701	154.615	0.1	10.5
STARD10	NM_006645	1988	1.163	11.147	0.1	9.6
TMEM14A	NM_014051	1014	0.11	1.119	0.1	10.2
APOB	NM_000384	14121	5.822	765.522	0.0	131.5
SERPINF2	NM_001165921	2092	1.034	102.91	0.0	99.5
TFR2	NM_003227	2888	0.142	13.762	0.0	96.9
FGL1	NM_147203	1337	1.102	1305.36	0.0	1184.5
FGA	NM_000508	3655	24.14	9012.08	0.0	373.3

The targeted RNA-seq library was compared to an untargeted, total RNA library made from the same total RNA sample. Poly dT priming was used to convert total RNA, which is primarily rRNA, into non-rRNA transcript libraries. For targeted RNA-seq, random hexamers were used. For the dT primed total RNA libraries, reads can be derived along the entire length of a transcript, some of which are quite long. As an example, the distribution of reads along MYH7 in heart was examined and reads derived from near the 5′ end of this long transcript were found. To compare one (long) transcript to another (short) transcript, counts were normalized by transcript length (often referred to as the reads per million per Kb or RPKM method). Following this 1st degree normalization, counts were also normalize between total and targeted samples. The final read count data set is shown in Table 44.

Table 44. Read Count Data for Figures 49 and 50.

HEART	Atlas	Total	Targeted	LIVER	Atlas	Total	Targeted
MYH7	50188	71792	40164	MYH7	0	374	721
NEBL	2715	936	3460	NEBL	1	27	24
MGP	24876	1315	13162	MGP	90	42	393
DKK3	2781	18092	20763	DKK3	9	36	211
BVES	265	179	650	BVES	1	5	62
PDE4DIP	2537	850	5029	PDE4DIP	81	88	294
HAND2	236	479	1052	HAND2	8	42	279
RP9P	30	121	63	RP9P	1	20	12
SOD2	11553	780	5437	SOD2	4031	1503	8802
ATP5E	2393	1607	2363	ATP5E	751	451	2173
TRAPPC1	239	536	1970	TRAPPC1	78	80	397
TAF1A	26	22	34	TAF1A	8	7	48
MGST1	1018	1312	2862	MGST1	3455	5514	9281
PDIA4	345	603	1310	PDIA4	1244	1197	2144
STARD10	27	908	1214	STARD10	90	1857	3338
TMEM14A	3	74	57	TMEM14A	9	110	74
APOB	137	56	107	APOB	6162	5243	17030
SERPINF2	24	119	89	SERPINF2	828	7474	7795
TFR2	3	20	11	TFR2	111	3395	3489
FGL1	26	50	44	FGL1	10507	21763	11420
FGA	567	131	160	FGA	72538	50772	32014

Visual inspection revealed a good correlation between all three types of data (Atlas, Total and Targeted). One important comparison was between that total RNA-seq sample prepared herein and the targeted RNA-seq sample prepared herein, because both data sets were derived from the same total RNA samples. Two important points of comparison include: (1) The correlation of the actual heart versus liver expression ratio; and (2) the correlation of the absolute abundances of transcripts within a specific sample between total and targeted counts.

The first point addresses the preservation of expression profiles, but ignores the actual magnitude of counts being compared. An expression ratio heart versus liver comparison plot for total versus targeted expression ratios is shown in Figure 47. This plot shows an exceptional correlation ($r^2 = 0.95$) between "expression profiles" generated by the two methods.

The second point can be more rigorous because it asks for an absolute comparison between the two methods. A comparison of absolute expression levels as

measured in total RNA-seq or targeted RNA-seq is shown in Figure 48, where log10(count) values are plotted against one another. This comparison was sensitive not only to targeting, but also to the fact that the RNA-seq libraries were prepared by fundamentally different methods (dT priming for total, random hexamer priming for targeted). Despite the different methods of preparation there was an excellent correlation between the two methods.

This study demonstrated that the core methods of labeling with random tags combined with sequence-specific capture can produce target-specific RNA transcript data that preserves absolute expression abundance, reveals transcript-specific sequence information, and dramatically reduces the complexity of transcriptome data.

APPENDIX: PROBE SEQUENCES USED FOR RNA-SEQ ANALYSIS.

SEQ ID NO:	Name	Sequence
727	APOB_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGCCGAAGAGGAAATGCTGGAAAATGTCAGCCTGGTCTGTCCAAAAGATGCGACCCGATTC
728	APOB_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCAGCTGACCTCATCGAGATTGGCTTGGAAGGAAAAGGCTTTGAGCCAACATTGGAAGCT
729	ATP5E_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTACTGGAGACAGGCTGGACTCAGCTACATCCGATACTCCCAGATCTGTGCAAAAGCAGTG
730	ATP5E_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCTGAAGACAGAATTCAAAGCAAATGCTGAGAAGACTTCTGGCAGCAACGTAAAAATTGTG
731	BVES_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNACAGAGTCCAGCCCATTGAGAGAATCAACTGCCATAGGTTTTACACCTGAGTTAGAAAGT
732	BVES_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGCCAGCTCCAGTGACAGTGACGACGGCTTGCACCAGTTTCTTCGGGGTACCTCCAG

		CA
733	DKK3_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGACACGC AGCACAAATTGCGCAGCGCGGTGGAAGAGATGGAGGCAGAAGAAGCT GCTGC
734	DKK3_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTGGTGTAT GTGTGCAAGCCGACCTTCGTGGGGAGCCGTGACCAAGATGGGGAGATC CTG
735	FGA_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATGAGGA TCGTCTGCCTGGTCCTAAGTGTGGTGGGCACAGCATGGACTGCAGATAG TGGT
736	FGA_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATTTTTTC TGTTTATTGCGATCAAGAGACCAGTTTGGGAGGATGGCTTTTGATCCAG CA
737	FGL1_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGTGTTCAG TTTCATCCTTGTTACCACCGCTCTGACAATGGGCAGGGAAATTTCGGCG CTC
738	FGL1_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTGCAAACC TGAATGGTGTATACTACAGCGGCCCCTACACGGCTAAAACAGACAATG GGA
739	HAND2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCATCCCCA ACGTACCCGCCGACACCAAACTCTCCAAAATCAAGACCCTGCGCCTGG CCA
740	HAND2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCGACCAG AATGGCGAGGCGGAGGCCTTCAAGGCAGAGATCAAGAAGACCGACGT GAAAG
741	MGP_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCTGATCCT TCTTGCCATCCTGGCCGCCTTAGCGGTAGTAACTTTGTGTTATGAATCAC AT
742	MGP_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCGAGCTCA

		ATAGGGAAGCCTGTGATGACTACAGACTTTGCGAACGCTACGCCATGGTTT
743	MGST1_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAAGAAGTATCTTCGAACAGATGACAGAGTAGAACGTGTACGCAGAGCCCACCTGAATGA
744	MGST1_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTATTCCTTGAGTGGTCCCGACCCCTCTACAGCCATCCTGCACTTCAGACTATTTGTCGG
745	MYH7_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCAGACCAGGCCTTTTGACCTCAAGAAGGATGTCTTCGTGCCTGATGACAAACAGGAGTTT
746	MYH7_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGAGCTCTTCAAACTCAAGAACGCCTATGAGGAGTCCCTGGAACATCTGGAGACCTTCA
747	NEBL_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCAGGTCTTCTATAAGCCTGTTATTGAAGACTTAAGCATGGAATTGGCCAGAAAATGCACG
748	NEBL_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAAAGAGCAAAACTACAAGGCCACTCCGGTAAGCATGACCCCGGAGATAGAGAGAGTGAG
749	PDE4DIP_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGGATATCGCACTCTGTCCCAGCACCTCAATGACCTGAAGAAGGAGAACTTCAGCCTCAAG
750	PDE4DIP_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGAAAGCCAAGCTGCTGCAGAGAAGTTGGTGCAAGCCTTAATGGAAAGAAATTCAGAATT
751	PDIA4_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCCGGACGAGGATTCTTCTAACAGAGAAAATGCCATTGAGGATGAAGAGGAGGAGGAGGAG

752	PDIA4_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCGTCAAGGTCGTGGTGGGAAAGACCTTTGACTCCATTGTGATGGACCCCAAGAAGGACG
753	RP9P_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGATCACGAGTTATGAAAAACCTCCTCCTGGGCTTATCAAGGTTGGCATTGCAAATGGT
754	RP9P_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAAAGTTTCTTCATCGTTGTCCTCCCTGCTGGTCACATGAGTTTACGATTCCTTAGAAGTG
755	SERPINF2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCCCGGCGCGTTCCGACTGGCTGCCAGGATGTACCTGCAGAAAGGATTTCCCATCAAAGA
756	SERPINF2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGCCTGAAAGGCTTCCCCCGCGGAGACAAGCTTTTCGGCCCTGACTTAAAACTTGTGCCC
757	SOD2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAACAACCTGAACGTCACCGAGGAGAAGTACCAGGAGGCGTTGGCCAAGGGAGATGTTAC
758	SOD2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGGAGAAGCTGACGGCTGCATCTGTTGGTGTCCAAGGCTCAGGTTGGGGTTGGCTTGGT
759	STARD10_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGACCAAGACTTTCGCAGCTTCCGGTCAGAGTGTGAGGCTGAGGTGGGCTGGAACCTGACC
760	STARD10_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGATGTACAAGGCGTGCCTCAAGTACCCCGAGTGGAAACAGAAGCACCTGCCTCACTTCA
761	TAF1A_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTTCAGTGAAGAATTAAAAGGGCCTGTGACAGATGATGAAGAAGTGGAAACATCTGT

		GCTC
762	TAF1A_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGAAACCA CCTTGCGTGGGTTCAAGAAGAGTGGAACTCCAGGAAAAACTGGTGGCC AGGC
763	TFR2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCTTTGGGG TCTATTCCAGAGAGCGCAACAACTGTCCCCAAGATCCTCTCAGACCGTC TAC
764	TFR2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGGCCTAC CCATTCCTGCACACAAAGGAGGACACTTATGAGAACCTGCATAAGGTG CTG
765	TMEM14A_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATCGGTTT TGGTTATGCAGCCCTCGTGACATTTGGAAGCATTTTTGGATATAAGCGG AGA
766	TMEM14A_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGCTTTCTT CCTGGCTACCATAATGGGTGTGAGATTTAAGAGGTCCAAGAAAATAAT GCC
767	TRAPPC1_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCACAACCT GTACCTGTTTGACCGGAATGGAGTGTGTCTGCACTACAGCGAATGGCAC CGC
768	TRAPPC1_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATCCGAG ATGTGCTGCACCACATCTACAGTGCGCTGTATGTGGAGCTGGTGGTGAA GAA
769	APOB_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGCCGAAG AGGAAATGCTGGAAAATGTCAGCCTGGTCTGTCCAAAAGATGCGACCC GATTC
770	APOB_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCAGCTGAC CTCATCGAGATTGGCTTGGAAGGAAAAGGCTTTGAGCCAACATTGGAA GCT
771	ATP5E_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTACTGGAG

		ACAGGCTGGACTCAGCTACATCCGATACTCCCAGATCTGTGCAAAAGC AGTG
772	ATP5E_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCTGAAGA CAGAATTCAAAGCAAATGCTGAGAAGACTTCTGGCAGCAACGTAAAAA TTGTG
773	BVES_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNACAGAGT CCAGCCCATTGAGAGAATCAACTGCCATAGGTTTTACACCTGAGTTAGA AAGT
774	BVES_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGCCAGCT CCAGTGACAGTGACGACGGCTTGCACCAGTTTCTTCGGGGTACCTCCAG CA
775	DKK3_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGACACGC AGCACAAATTGCGCAGCGCGGTGGAAGAGATGGAGGCAGAAGAAGCT GCTGC
776	DKK3_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTGGTGTAT GTGTGCAAGCCGACCTTCGTGGGGAGCCGTGACCAAGATGGGGAGATC CTG
777	FGA_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATGAGGA TCGTCTGCCTGGTCCTAAGTGTGGTGGGCACAGCATGGACTGCAGATAG TGGT
778	FGA_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATTTTTTC TGTTTATTGCGATCAAGAGACCAGTTTGGGAGGATGGCTTTTGATCCAG CA
779	FGL1_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGTGTTCAG TTTCATCCTTGTTACCACCGCTCTGACAATGGGCAGGGAAATTTCGGCG CTC
780	FGL1_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTGCAAACC TGAATGGTGTATACTACAGCGGCCCCTACACGGCTAAAACAGACAATG GGA

781	HAND2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCATCCCCA ACGTACCCGCCGACACCAAACTCTCCAAAATCAAGACCCTGCGCCTGG CCA
782	HAND2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCGACCAG AATGGCGAGGCGGAGGCCTTCAAGGCAGAGATCAAGAAGACCGACGT GAAAG
783	MGP_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCTGATCCT TCTTGCCATCCTGGCCGCCTTAGCGGTAGTAACTTTGTGTTATGAATCAC AT
784	MGP_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCGAGCTCA ATAGGGAAGCCTGTGATGACTACAGACTTTGCGAACGCTACGCCATGG TTT
785	MGST1_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAAGAAGT ATCTTCGAACAGATGACAGAGTAGAACGTGTACGCAGAGCCCACCTGA ATGA
786	MGST1_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTATTCCTT GAGTGGTCCCGACCCCTCTACAGCCATCCTGCACTTCAGACTATTTGTC GG
787	MYH7_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCAGACCA GGCCTTTTGACCTCAAGAAGGATGTCTTCGTGCCTGATGACAAACAGGA GTTT
788	MYH7_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGAGCTCT TCAAACTCAAGAACGCCTATGAGGAGTCCCTGGAACATCTGGAGACCT TCA
789	NEBL_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCAGGTCTT CTATAAGCCTGTTATTGAAGACTTAAGCATGGAATTGGCCAGAAAATGC ACG
790	NEBL_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAAAGAGC AAAACTACAAGGCCACTCCGGTAAGCATGACCCCGGAGATAGAGAGAG

		TGAG
791	PDE4DIP_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGGATATCG CACTCTGTCCCAGCACCTCAATGACCTGAAGAAGGAGAACTTCAGCCTC AAG
792	PDE4DIP_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGAAAGCC AAGCTGCTGCAGAGAAGTTGGTGCAAGCCTTAATGGAAAGAAATTCAG AATT
793	PDIA4_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCCGGACG AGGATTCTTCTAACAGAGAAAATGCCATTGAGGATGAAGAGGAGGAGG AGGAG
794	PDIA4_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCGTCAAG GTCGTGGTGGGAAAGACCTTTGACTCCATTGTGATGGACCCCAAGAAG GACG
795	RP9P_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGATCAC GAGTTATGAAAAACCTCCTCCTGGGCTTATCAAGGTTGGCATTGCAAAT GGT
796	RP9P_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAAAGTTTC TTCATCGTTGTCCTCCCTGCTGGTCACATGAGTTTACGATTCCTTAGAAG TG
797	SERPINF2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCCCGGCGC GTTCCGACTGGCTGCCAGGATGTACCTGCAGAAAGGATTTCCCATCAAA GA
798	SERPINF2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGCCTGAA AGGCTTCCCCCGCGGAGACAAGCTTTTCGGCCCTGACTTAAAACTTGTG CCC
799	SOD2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAACAACCT GAACGTCACCGAGGAGAAGTACCAGGAGGCGTTGGCCAAGGGAGATGT TAC
800	SOD2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGGAGAA

		GCTGACGGCTGCATCTGTTGGTGTCCAAGGCTCAGGTTGGGGTTGGCTTGGT
801	STARD10_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGACCAAGACTTTCGCAGCTTCCGGTCAGAGTGTGAGGCTGAGGTGGGCTGGAACCTGACC
802	STARD10_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGATGTACAAGGCGTGCCTCAAGTACCCCGAGTGGAAACAGAAGCACCTGCCTCACTTCA
803	TAF1A_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNTTCAGTGAAGAATTAAAAGGGCCTGTGACAGATGATGAAGAAGTGGAAACATCTGTGCTC
804	TAF1A_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGAAACCACCTTGCGTGGGTTCAAGAAGAGTGGAACTCCAGGAAAAACTGGTGGCCAGGC
805	TFR2_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCTTTGGGGTCTATTCCAGAGAGCGCAACAACTGTCCCCAAGATCCTCTCAGACCGTCTAC
806	TFR2_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNAGGCCTACCCATTCCTGCACACAAAGGAGGACACTTATGAGAACCTGCATAAGGTGCTG
807	TMEM14A_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATCGGTTTTGGTTATGCAGCCCTCGTGACATTTGGAAGCATTTTTGGATATAAGCGGAGA
808	TMEM14A_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNGCTTTCTTCCTGGCTACCATAATGGGTGTGAGATTTAAGAGGTCCAAGAAAATAATGCC
809	TRAPPC1_1	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNCACAACCTGTACCTGTTTGACCGGAATGGAGTGTGTCTGCACTACAGCGAATGGCACCGC

810	TRAPPC1_2	ATGTGACTGGCACGGGAGTTGATCCTGGTTTTCACNNNNNNATCCGAG ATGTGCTGCACCACATCTACAGTGCGCTGTATGTGGAGCTGGTGGTGAA GAA

In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

1. A method for generating a tagged genomic library comprising:

(a) treating fragmented genomic DNA with end-repair enzymes to generate fragmented end-repaired genomic DNA; and

(b) ligating a random nucleic acid tag sequence, and optionally a sample code sequence and/or a PCR primer sequence to the fragmented end-repaired genomic DNA to generate the tagged genomic library.

2. The method of any of the preceding claims, wherein the random nucleic acid tag sequence is from about 2 to about 100 nucleotides.

3. The method of any of the preceding claims, wherein the random nucleic acid tag sequence is from about 2 to about 6 nucleotides.

4. The method of any of the preceding claims, wherein the fragmented end-repaired genomic DNA contains blunt ends.

5. The method of any of the preceding claims, wherein the blunt ends are further modified to contain a single base pair overhang.

6. The method of any of the preceding claims, wherein the ligating comprises ligating a multifunctional adaptor module to the fragmented end-repaired genomic DNA to generate the tagged genomic library, wherein the multifunctional adaptor molecule comprises:

(i) a first region comprising a random nucleic acid tag sequence;

(ii) a second region comprising a sample code sequence; and

(iii) a third region comprising a PCR primer sequence.

7. The method of any of the preceding claims, further comprising hybridizing a tagged genomic library with a multifunctional capture probe module to form a complex,

wherein the multifunctional capture probe module hybridizes to a specific genomic target region in the genomic library.

8. The method of any of the preceding claims, further comprising isolating the tagged genomic library-multifunctional capture probe module complex.

9. The method of any of the preceding claims, further comprising 3′-5′ exonuclease enzymatic processing of the isolated tagged genomic library-multifunctional capture probe module complex to remove the single stranded 3′ ends.

10. The method of any of the preceding claims wherein the enzyme for use in the 3′-5′ exonuclease enzymatic processing is T4 DNA polymerase.

11. The method of any of the preceding claims, further comprising performing PCR on the 3′-5′ exonuclease enzymatically processed complex from the preceding claims, wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence.

12. A method for targeted genetic analysis comprising:

(a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific genomic target region in the genomic library;

(b) isolating the tagged genomic library-multifunctional capture probe module complex from a);

(c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends;

(d) performing PCR on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a

hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and

(e) performing targeted genetic analysis on the hybrid nucleic acid molecule from d).

13. The method of claim 12, wherein steps a) through d) are repeated at least about twice and the targeted genetic analysis of e) comprises a sequence alignment of the hybrid nucleic acid molecule sequences obtained from the at least two d) steps.

14. The method of claim 13, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

15. The method of claim 14, wherein at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

16. A method for determining copy number of a specific genomic target region comprising:

(a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module complex selectively hybridizes to a specific genomic target region in the genomic library;

(b) isolating the tagged genomic library-multifunctional capture probe module complex from a);

(c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends;

(d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule

comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence;

(e) performing PCR amplification of the hybrid nucleic acid molecule in d); and

(f) quantitating the PCR reaction in e), wherein the quantitation allows for a determination of copy number of the specific genomic target region.

17. The method of claim 16, further comprising obtaining the sequences of the hybrid nucleic acid molecules from step e).

18. The method of claim 17, wherein steps a) through e) are repeated at least about twice and a sequence alignment is performed using the hybrid nucleic acid molecule sequences obtained from the at least two e) steps.

19. The method of claim 18, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

20. The method of claim 19, wherein at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

21. A method for determining copy number of a specific genomic target region comprising:

(a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module complex selectively hybridizes to a specific genomic target region in the genomic library;

(b) isolating the tagged genomic library-multifunctional capture probe module complex from a);

(c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends;

(d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and

(e) performing PCR amplification of the hybrid nucleic acid molecule in d).

22. The method of claim 21, further comprising obtaining the sequences of the hybrid nucleic acid molecules from step e).

23. The method of claim 22, wherein steps a) through e) are repeated at least about twice and a sequence alignment is performed using the hybrid nucleic acid molecule sequences obtained from the at least two e) steps.

24. The method of claim 23, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

25. The method of claim 24, wherein at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

26. A method for determining copy number of a specific genomic target region comprising:

(a) hybridizing a tagged genomic library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module comprises selectively hybridizes to a specific genomic target region in the genomic library;

(b) isolating the tagged genomic library-multifunctional capture probe module complex from a);

(c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged genomic library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends;

(d) performing a PCR reaction on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is replicated in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the genomic target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence;

(e) performing PCR amplification of the hybrid nucleic acid molecule in d); and

(f) performing targeted genetic analysis on the hybrid nucleic acid molecule from e).

27. The method of claim 26, wherein steps a) through e) are repeated at least about twice and the targeted genetic analysis of f) comprises performing a sequence alignment of the hybrid nucleic acid molecule sequences from the at least two e) steps.

28. The method of claim 27, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

29. The method of claim 28, wherein at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

30. A method for targeted genetic analysis comprising:

(a) hybridizing a tagged genomic library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific genomic target region in the genomic library;

(b) isolating the tagged genomic library-multifunctional capture probe hybrid module complex from a);

(c) performing 5′ to 3′ DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured, tagged genomic target region that is 3′ of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged genomic target region that is located in the 3′ direction from the location where the multifunctional capture probe hybrid module hybridizes to the genomic target region; and

(d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

31. The method of claim 30, wherein steps a) through c) are repeated at least about twice and the targeted genetic analysis of d) comprises a sequence alignment of the hybrid nucleic acid molecule sequences obtained from the at least two d) steps.

32. The method of claim 31, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

33. The method of claim 32, wherein at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

34. A method for determining copy number of a specific genomic target region comprising:

(a) hybridizing a tagged genomic library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific genomic target region in the genomic library;

(b) isolating the tagged genomic library-multifunctional capture probe hybrid module complex from a);

(c) performing 5' to 3' DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured tagged genomic target region that is 3' of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of a region of the tagged genomic target region that is located in the 3'direction from the location where the multifunctional capture probe hybrid module hybridizes to the genomic target region; and

(d) performing PCR amplification of the hybrid nucleic acid molecule in c); and

(e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific genomic target region.

35. The method of claim 34, further comprising obtaining the sequences of the hybrid nucleic acid molecules from step d).

36. The method of claim 35, wherein steps a) through d) are repeated at least about twice and a sequence alignment of the hybrid nucleic acid molecules from the at least two d) steps.

37. The method of claim 36, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

38. The method of claim 37, wherein at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

39. The method of any of the preceding claims wherein the targeted genetic analysis is sequence analysis.

40. The method of any of the preceding claims wherein the tagged genomic library is amplified by PCR to generate an amplified tagged genomic library.

41. The method of any of the preceding claims wherein the genomic DNA is from a biological sample selected from the group consisting of blood, skin, hair, hair follicles, saliva, oral mucous, vaginal mucous, sweat, tears, epithelial tissues, urine, semen, seminal fluid, seminal plasma, prostatic fluid, pre-ejaculatory fluid (Cowper's fluid), excreta, biopsy, ascites, cerebrospinal fluid, lymph, and tissue extract sample or biopsy sample.

42. A tagged genomic library comprising tagged genomic sequences, wherein each tagged genomic sequence comprises:

(a) fragmented end-repaired genomic DNA;

(b) a random nucleotide tag sequence;

(c) a sample code sequence; and

(d) a PCR primer sequence.

43. A hybrid tagged genomic library comprising hybrid tagged genomic sequences for use in targeted genetic analysis, wherein each hybrid tagged genomic sequence comprises:

(a) fragmented end-repaired genomic DNA;

(b) a random nucleotide tag sequence;

(c) a sample code sequence;

(d) a PCR primer sequence;

(e) a genomic target region; and

(f) a multifunctional capture probe module tail sequence.

44. A multifunctional adaptor module comprising:

(a) a first region comprising a random nucleotide tag sequence;

(b) a second region comprising a sample code sequence; and

(c) a third region comprising a PCR primer sequence.

45. A multifunctional capture probe module comprising:

(a) a first region capable of hybridizing to a partner oligonucleotide;

(b) a second region capable of hybridizing to a specific genomic target region; and

(c) a third region comprising a tail sequence.

46. The multifunctional capture probe module of any of the preceding claims wherein the first region is bound to a partner oligonucleotide.

47. A multifunctional adaptor probe hybrid module comprising:

(a) a first region capable of hybridizing to a partner oligonucleotide and capable of functioning as a PCR primer and

(b) a second region capable of hybridizing to a specific genomic target region.

48. The multifunctional capture probe hybrid module of any of the preceding claims, wherein the first region is bound to a partner oligonucleotide.

49. The method of any of the preceding claims, wherein the partner oligonucleotide is chemically modified.

50. A composition comprising a tagged genomic library, a multifunctional adaptor module and a multifunctional capture probe module.

51. A composition comprising a hybrid tagged genomic library according to any of the preceding claims.

52. A reaction mixture for performing the method of any one of the preceding claims.

53. A reaction mixture capable of generating a tagged genomic library comprising:

(a) fragmented genomic DNA; and

(b) DNA end-repair enzymes to generate fragmented end-repaired genomic DNA.

54. The reaction mixture of any of the preceding claims, further comprising a multifunctional adaptor module.

55. The reaction mixture of any of the preceding claims, further comprising a multifunctional capture probe module.

56. The reaction mixture of any of the preceding claims, further comprising an enzyme with 3′-5′ exonuclease activity and PCR amplification activity.

57. A method for DNA sequence analysis comprising:

(a) obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence;

(b) performing a paired end sequencing reaction on the one or more clones and obtaining one or more sequencing reads; and

(c) ordering or clustering the sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

58. A method for DNA sequence analysis comprising:

(a) obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence;

(b) performing a sequencing reaction on the one or more clones in which a single long sequencing read of greater than about 100 nucleotides is obtained, wherein the read is sufficient to identify both the first DNA sequence and the second DNA sequence; and

(c) ordering or clustering sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

59. The method of claim 57 or claim 58, wherein the sequences of the one or more clones are compared to one or more human reference DNA sequences.

60. The method of claim 59, wherein sequences that do not match the one or more human reference DNA sequences are identified.

61. The method of claim 60, wherein non-matching sequences are used to create a *de novo* assembly from the non-matching sequence data.

62. The method of claim 61, wherein the *de novo* assemblies are used to identify novel sequence rearrangements associated with the capture probe.

63. A method for genomic copy number determination analysis comprising:

(a) obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises a random nucleotide tag sequence and a targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence;

(b) performing a paired end sequencing reaction on the one or more clones and obtaining one or more sequencing reads; and

(c) ordering or clustering the sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

64. A method for genomic copy number determination analysis comprising:

(a) obtaining one or more clones, each clone comprising a first DNA sequence and a second DNA sequence, wherein the first DNA sequence comprises a random nucleotide tag sequence and a targeted genomic DNA sequence and the second DNA sequence comprises a capture probe sequence;

(b) performing a sequencing reaction on the one or more clones in which a single long sequencing read of greater than about 100 nucleotides is obtained, wherein the read is sufficient to identify both the first DNA sequence and the second DNA sequence; and

(c) ordering or clustering sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

65. The method of claim 63 or claim 64, wherein the random nucleotide tag sequence is about 2 to about 50 nucleotides in length.

66. The method of claim 63 or claim 64, further comprising analyzing all sequencing reads associated with a second read sequence by:

(a) determining the distributions of unique and redundant sequencing reads;

(b) counting the number of times a unique read is encountered;

(c) fitting a frequency distribution of the unique reads to a statistical distribution;

(d) inferring a total number of unique reads; and

(e) normalizing the total number of inferred unique read to an assumption that most human genetic loci are generally diploid.

67. The method of claim 66, wherein an inferred copy number of one or more targeted loci are determined.

68. The method of claim 67, wherein the one or more target loci that deviate from an expected copy number value are determined.

69. The method of claim 67,wherein the one or more targeted loci of a gene are grouped together in a collection of loci and the copy number measurements from the collection of targeted loci are averaged and normalized.

70. The method of claim 67, wherein the inferred copy number of a gene is represented by the normalized average of all the target loci representing that gene.

71. A method for generating a tagged RNA expression library comprising:

(a) fragmenting a cDNA library;

(b) treating the fragmented cDNA library with end-repair enzymes to generate fragmented end-repaired cDNA; and

(c) ligating a multifunctional adapter molecule to the fragmented end-repaired c DNA to generate a tagged RNA expression library.

72. A method for generating a tagged RNA expression library comprising:

(a) preparing a cDNA library from the total RNA of one or more cells;

(b) fragmenting the cDNA library;

(c) treating the fragmented cDNA with end-repair enzymes to generate fragmented end-repaired cDNA; and

(d) ligating a multifunctional adapter molecule to the fragmented end-repaired c DNA to generate a tagged RNA expression library.

73. The method of claim 71 or claim 72, wherein the cDNA library is an oligo-dT primed cDNA library.

74. The method of claim 71 or claim 72, wherein the cDNA library is primed by random oligonucleotides comprising about 6 to about 20 random nucleotides.

75. The method of claim 71 or claim 72, wherein the cDNA library is primed by random hexamers or random octamers.

76. The method of claim 71 or claim 72, wherein the cDNA library is fragmented to a size of about 250 bp to about 750 bp.

77. The method of claim 71 or claim 72, wherein the cDNA library is fragmented to a size of about 500 bp.

78. The method of any one of claims 71 to 77, wherein the multifunctional adaptor module comprises:

(i) a first region comprising a random nucleic acid tag sequence, and optionally;

(ii) a second region comprising a sample code sequence, and optionally

(iii) a third region comprising a PCR primer sequence.

79. The method of any one of claims 71 to 78, wherein the multifunctional adaptor module comprises a first region comprising a random nucleic acid tag sequence, a second region comprising a sample code sequence, and a third region comprising a PCR primer sequence.

80. The method of any one of claims 71 to 78, further comprising hybridizing a tagged cDNA library with a multifunctional capture probe module to form a complex, wherein the multifunctional capture probe module hybridizes to a specific target region in the cDNA library.

81. The method of any one of claims 71 to 78, further comprising isolating the tagged cDNA library-multifunctional capture probe module complex.

82. The method of any one of claims 71 to 78, further comprising 3′-5′ exonuclease enzymatic processing of the isolated tagged cDNA library-multifunctional capture probe module complex to remove the single stranded 3′ ends.

83. The method of claim 82, wherein the enzyme for use in the 3′-5′ exonuclease enzymatic processing is T4 DNA polymerase.

84. The method of claim 82 or claim 83, further comprising performing PCR on the 3′-5′ exonuclease enzymatically processed complex, wherein a tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the cDNA target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence.

85. A method for targeted gene expression analysis comprising

(a) hybridizing a tagged RNA expression library with a multifunctional capture probe module complex, wherein the multifunctional capture probe module selectively hybridizes to a specific target region in the tagged RNA expression library;

(b) isolating the tagged RNA expression library-multifunctional capture probe module complex from a);

(c) performing 3′-5′ exonuclease enzymatic processing on the isolated tagged RNA expression library-multifunctional capture probe module complex from b) using an enzyme with 3′-5′ exonuclease activity to remove the single stranded 3′ ends;

(d) performing PCR on the enzymatically processed complex from c) wherein the tail portion of the multifunctional capture probe molecule is copied in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the target region capable of hybridizing to the multifunctional capture probe module and the complement of the multifunctional capture probe module tail sequence; and

(e) performing targeted gene expression analysis on the hybrid nucleic acid molecule from d).

86. A method for targeted gene expression analysis comprising:

(a) hybridizing a tagged RNA expression library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the RNA expression library;

(b) isolating the tagged RNA expression library-multifunctional capture probe hybrid module complex from a);

(c) performing 5’ to 3’ DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured, tagged target region that is 3’ of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of the tagged target region that is located in the 3’direction from the location where the multifunctional capture probe hybrid module hybridizes to the target region; and

(d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

87. A method for targeted gene expression analysis comprising:

(a) hybridizing a tagged cDNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the cDNA library;

(b) isolating the tagged cDNA library-multifunctional capture probe hybrid module complex from a);

(c) performing 5' to 3' DNA polymerase extension of the multifunctional capture probe on the complex from b) to replicate a region of the captured, tagged target region in the cDNA library that is 3' of the multifunctional capture probe in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the multifunctional capture probe hybrid module and the complement of the tagged target region in the cDNA library that is located in the 3'direction from the location where the multifunctional capture probe hybrid module hybridizes to the target region; and

(d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

88. The method of any one of claims 85 to 87, wherein at least two different multifunctional capture probe modules are used in the at least two (a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

89. The method of claim 88, wherein at least one multifunctional capture probe module hybridizes downstream of the target region and at least one multifunctional capture probe module hybridizes upstream of the target region.

90. A method for cDNA sequence analysis comprising:

(a) obtaining one or more clones, each clone comprising a first cDNA sequence and a second cDNA sequence, wherein the first cDNA sequence comprises targeted

genomic cDNA sequence and the second cDNA sequence comprises a capture probe sequence;

(b) performing a paired end sequencing reaction on the one or more clones and obtaining one or more sequencing reads; and

(c) ordering or clustering the sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

91. A method for cDNA sequence analysis comprising:

(a) obtaining one or more clones, each clone comprising a first cDNA sequence and a second cDNA sequence, wherein the first cDNA sequence comprises targeted genomic DNA sequence and the second cDNA sequence comprises a capture probe sequence;

(b) performing a sequencing reaction on the one or more clones in which a single long sequencing read of greater than about 100 nucleotides is obtained, wherein the read is sufficient to identify both the first cDNA sequence and the second cDNA sequence; and

(c) ordering or clustering sequencing reads of the one or more clones according to the probe sequence of the sequencing reads.

92. The method of claim 90 or claim 91, further comprising analyzing all sequencing reads associated with a second read sequence by:

(a) determining the distributions of unique and redundant sequencing reads;

(b) counting the number of times a unique read is encountered;

(c) fitting a frequency distribution of the unique reads to a statistical distribution;

(d) inferring a total number of unique reads; and

(e) converting unique read counts into transcript abundance using normalization to the total reads collected within each cDNA library sample.

93. A method for targeted genetic analysis comprising:

(a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the DNA library;

(b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a);

(c) performing a concerted enzymatic processing of the tagged DNA library-multifunctional capture probe hybrid module complex from b) that includes 5′ FLAP endonuclease activity, 5′ to 3′ DNA polymerase extension, and nick closure by a DNA ligase to join the complement of the multifunctional capture probe to the target region that is 5′ of the multifunctional capture probe binding site in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the complement of the multifunctional capture probe hybrid module and a region of the tagged target region that is located 5′ of the location where the multifunctional capture probe hybrid module hybridizes to the genomic target region; and

(d) performing targeted genetic analysis on the hybrid nucleic acid molecule from c).

94. The method of claim 93, wherein steps a) through c) are repeated at least about twice and the targeted genetic analysis of d) comprises a sequence alignment of the hybrid nucleic acid molecule sequences obtained from the at least two d) steps.

95. The method of claim 94, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

96. The method of claim 95, wherein at least one multifunctional capture probe module hybridizes downstream of the target region and at least one multifunctional capture probe module hybridizes upstream of the target region.

97. A method for determining copy number of a specific target region comprising:

(a) hybridizing a tagged DNA library with a multifunctional capture probe hybrid module complex, wherein the multifunctional capture probe hybrid module selectively hybridizes to a specific target region in the genomic library;

(b) isolating the tagged DNA library-multifunctional capture probe hybrid module complex from a);

(c) performing a concerted enzymatic processing of the tagged DNA library-multifunctional capture probe hybrid module complex from b) that includes 5′ FLAP endonuclease activity, 5′ to 3′ DNA polymerase extension, and nick closure by a DNA ligase to join the complement of the multifunctional capture probe to the target region that is 5′ of the multifunctional capture probe binding site in order to generate a hybrid nucleic acid molecule, wherein the hybrid nucleic acid molecule comprises the complement of the multifunctional capture probe hybrid module and a region of the tagged target region that is located 5′ of the location where the multifunctional capture probe hybrid module hybridizes to the target region; and

(d) performing PCR amplification of the hybrid nucleic acid molecule in c); and

(e) quantitating the PCR reaction in d), wherein the quantitation allows for a determination of copy number of the specific target region.

98. The method of claim 97, further comprising obtaining the sequences of the hybrid nucleic acid molecules from step d).

99. The method of claim 98, wherein steps a) through d) are repeated at least about twice and a sequence alignment of the hybrid nucleic acid molecules from the at least two d) steps.

100. The method of claim 99, wherein at least two different multifunctional capture probe modules are used in the at least two a) steps, wherein the at least two a) steps employ one multifunctional capture probe module each.

101. The method of claim 100, wherein at least one multifunctional capture probe module hybridizes downstream of the genomic target region and at least one multifunctional capture probe module hybridizes upstream of the genomic target region.

102. The method of any one of claims 93 to 101, wherein the targeted genetic analysis is sequence analysis.

103. The method of any one of claims 93 to 102, wherein the target region is a genomic target region and the DNA library is a genomic DNA library.

104. The method of any one of claims 93 to 103, wherein the target region is a cDNA target region and the DNA library is a cDNA library.

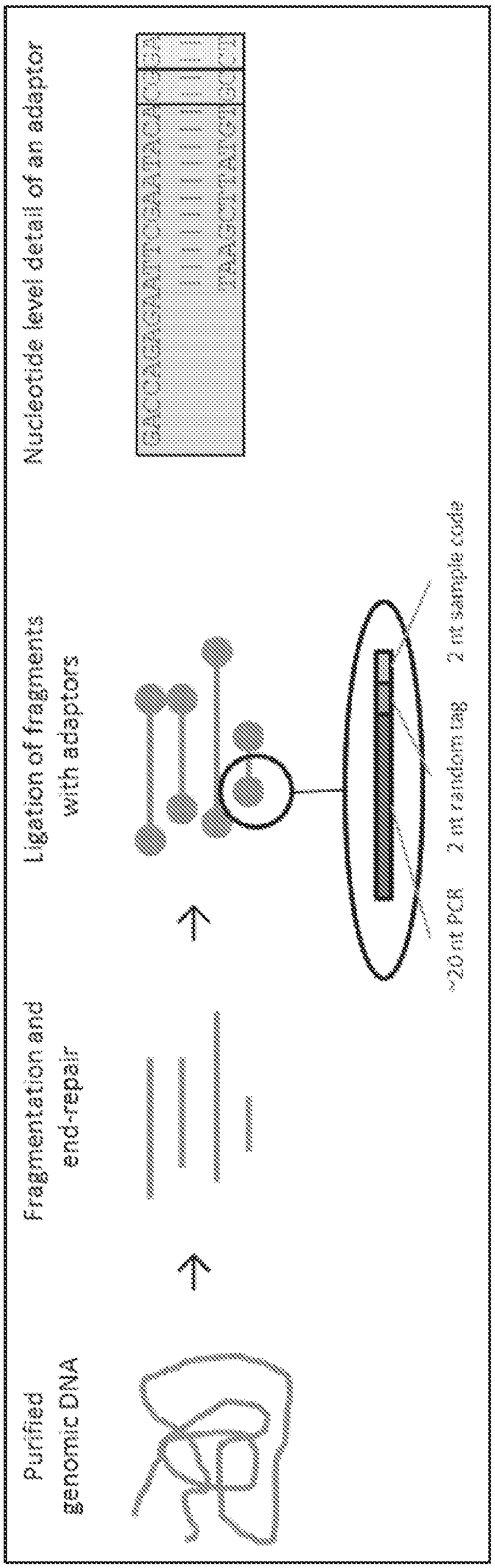

Purified genomic DNA
Fragmentation and end-repair
Ligation of fragments with adaptors
Nucleotide level detail of adaptor
~20 nt PCR
2 nt random tag
2 nt sample code

FIG. 1

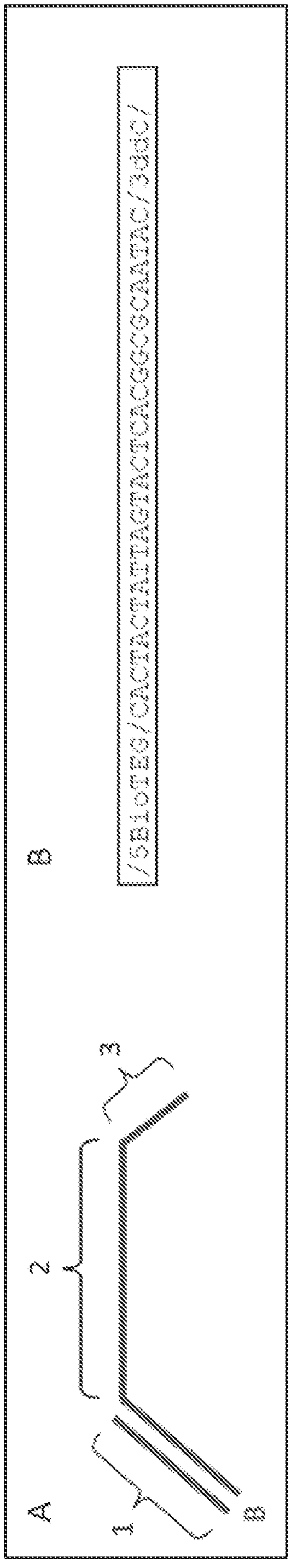

5' GACTTACTTATTAGTTACTCAGCGGCGAAAAG 3'
3' CTGAATGAATAATCAATGAGTCGCCGCTTTTC 5'
B
1
2
3
A

FIG. 2

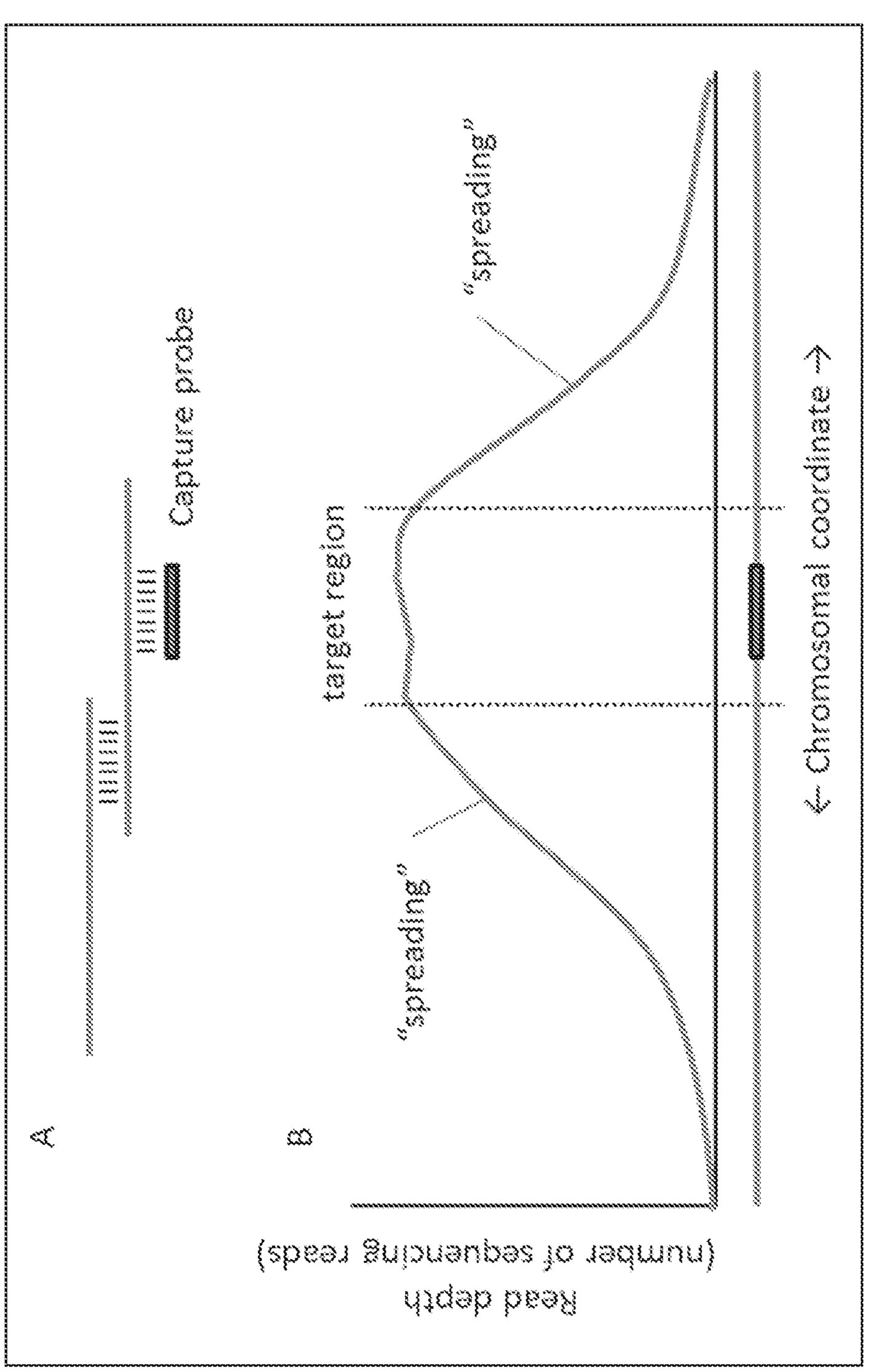

A
Capture probe
target region
"spreading"
"spreading"
← Chromosomal coordinate →
B
"spreading"
"spreading"
Read depth
(number of sequencing reads)

FIG. 3

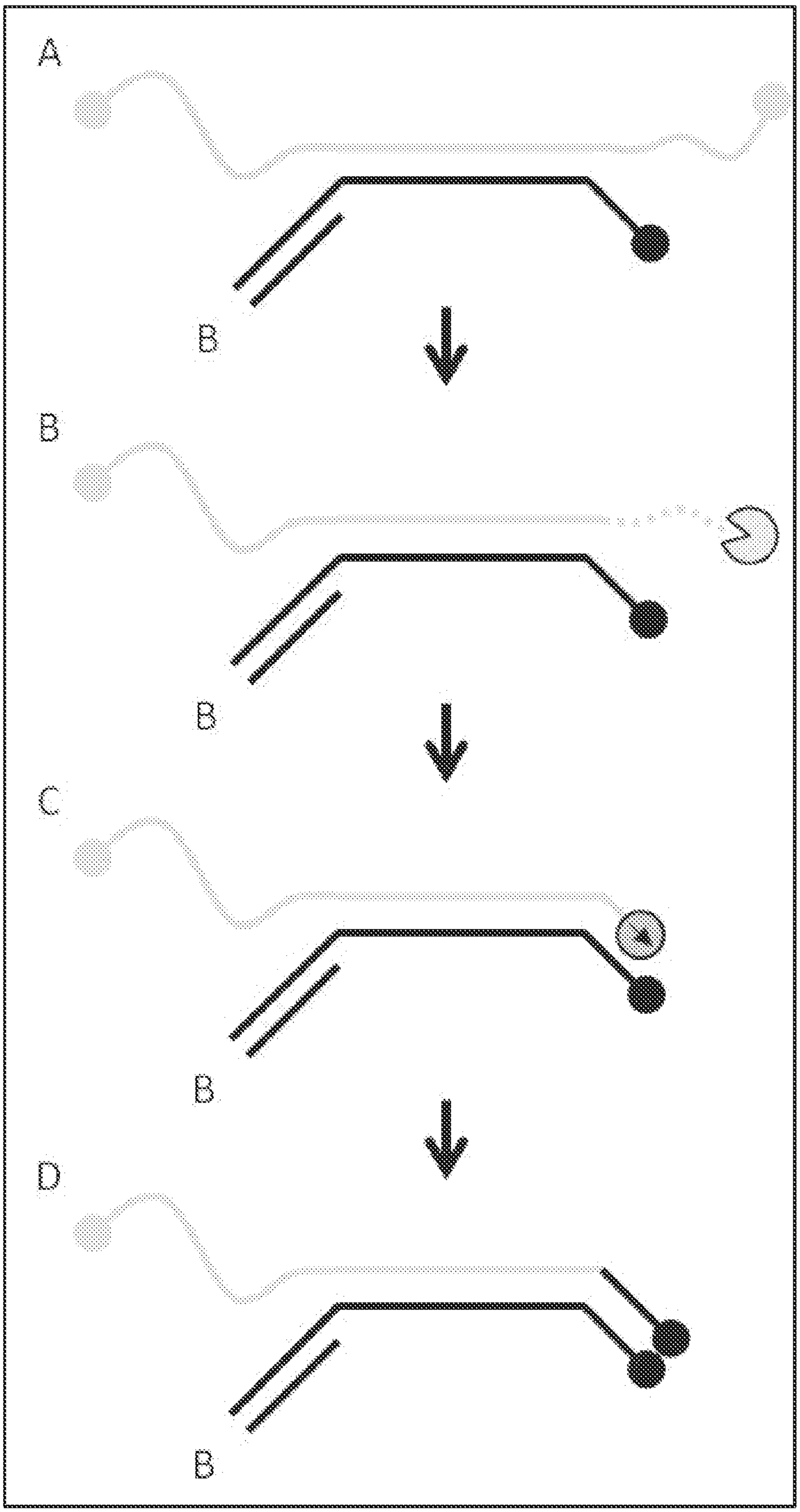


FIG. 4

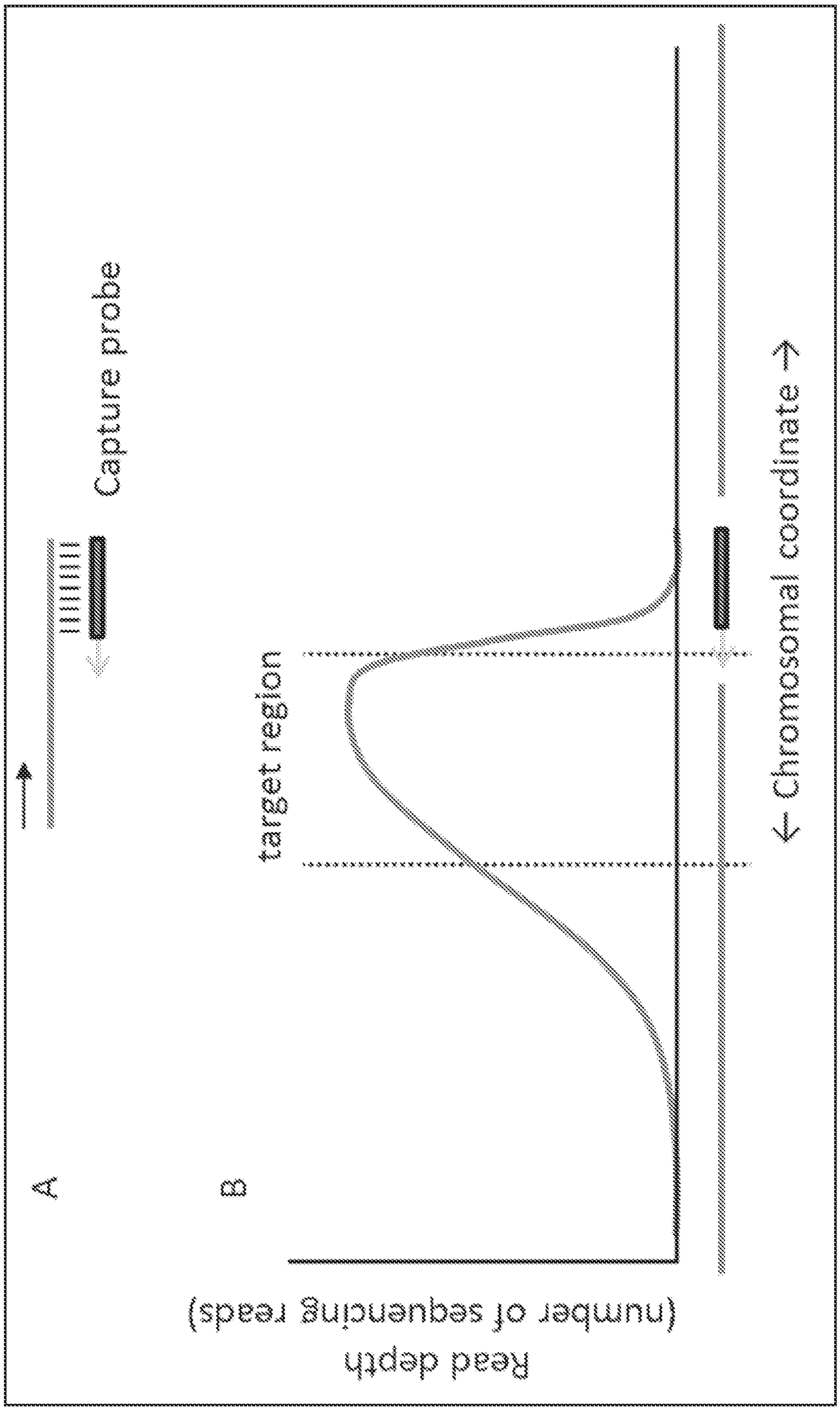

Capture probe
target region
chromosomal coordinate →
Read depth
(number of sequencing reads)

FIG. 5

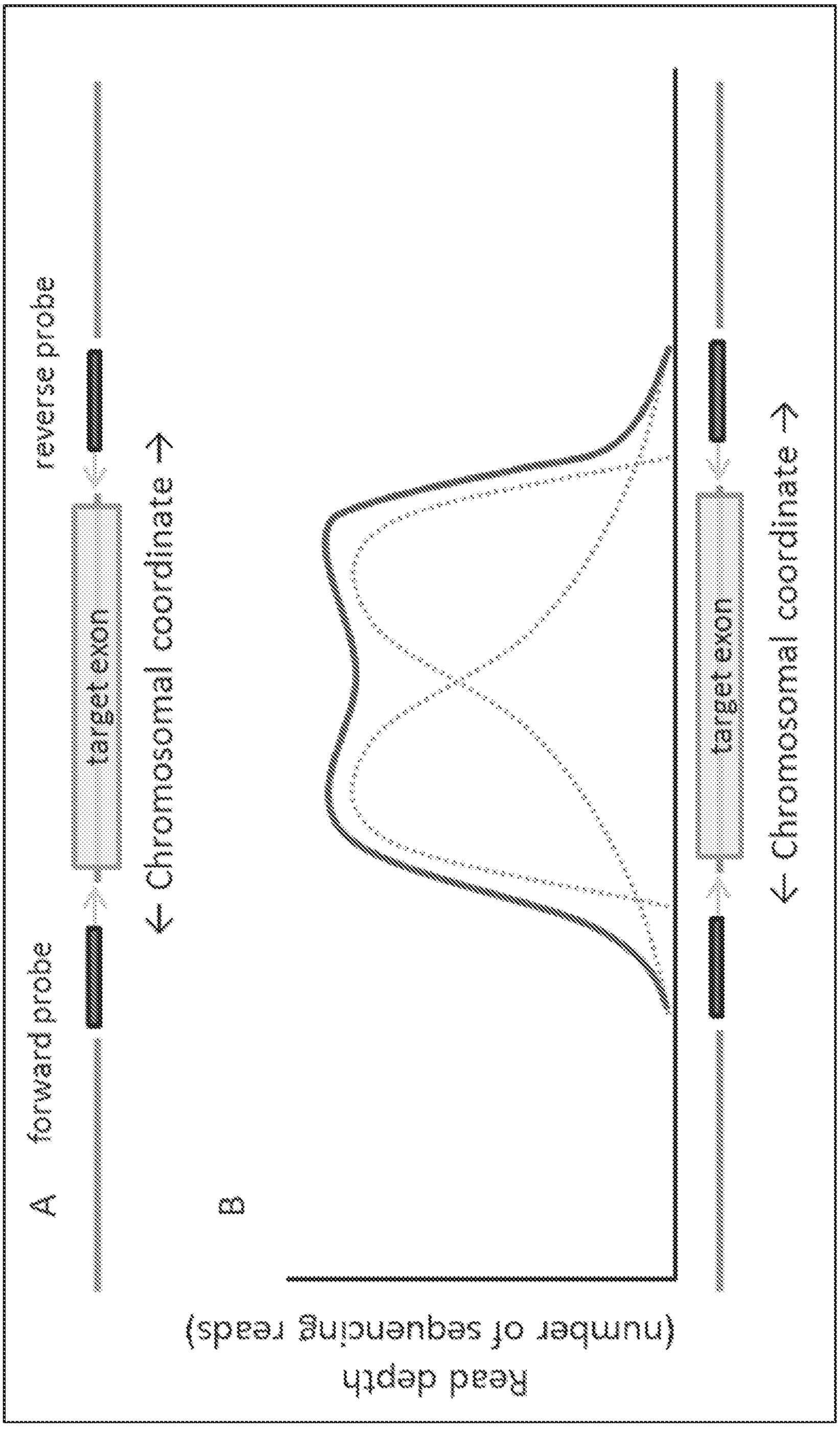

A forward probe
reverse probe
target exon
← Chromosomal coordinate →
Read depth
(number of sequencing reads)
B
target exon
← Chromosomal coordinate →

FIG. 6

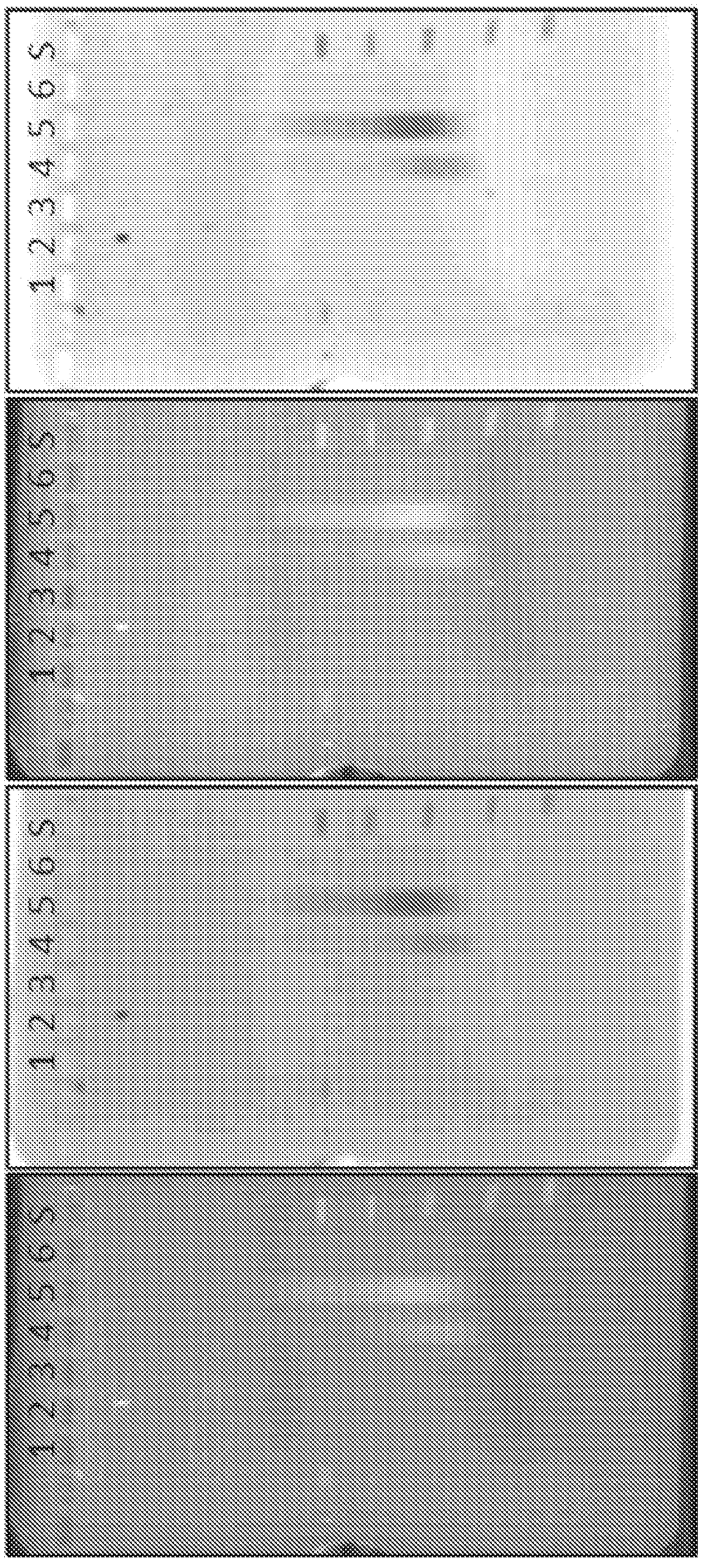


FIG. 7

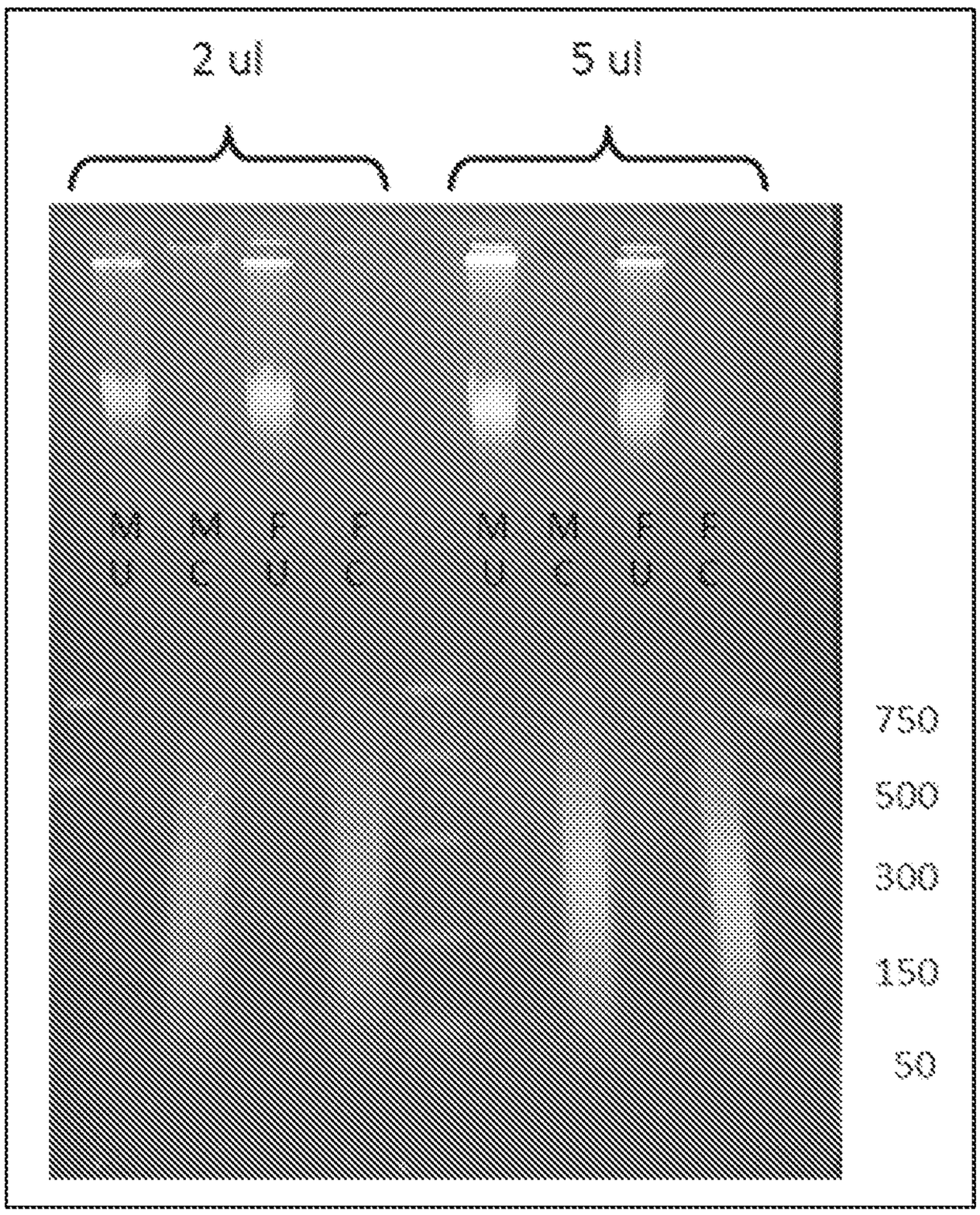


FIG. 8

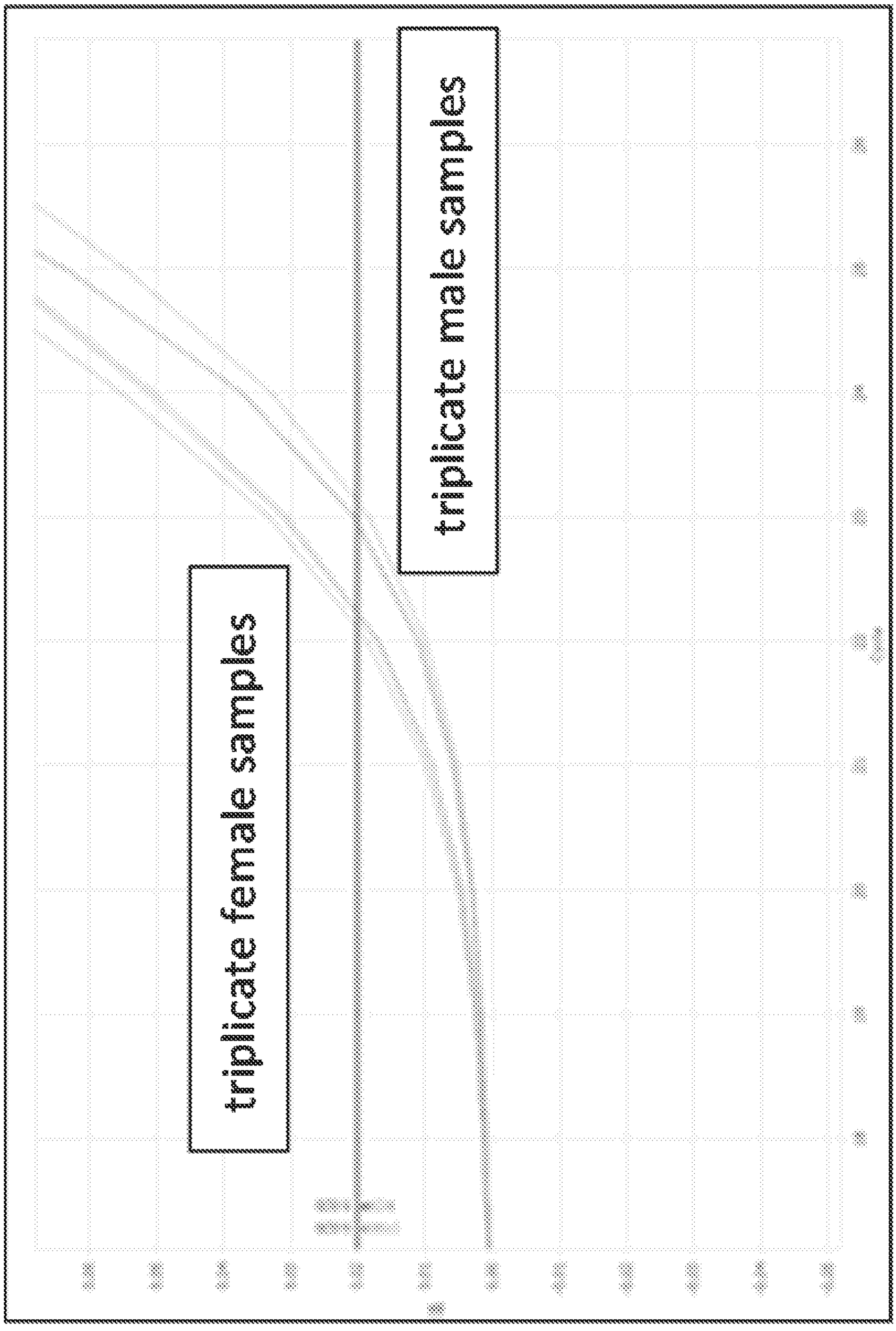


FIG. 9

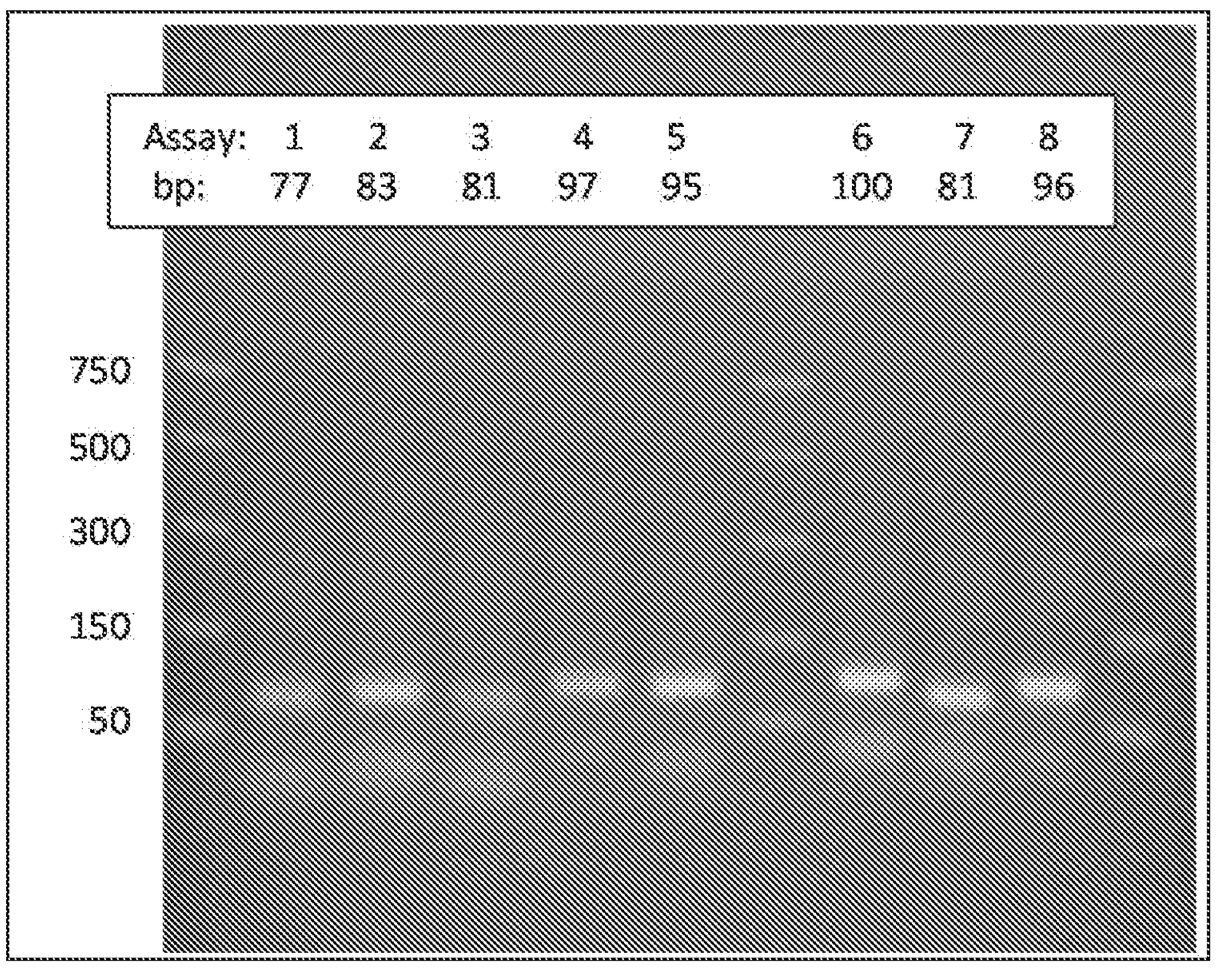


FIG. 10

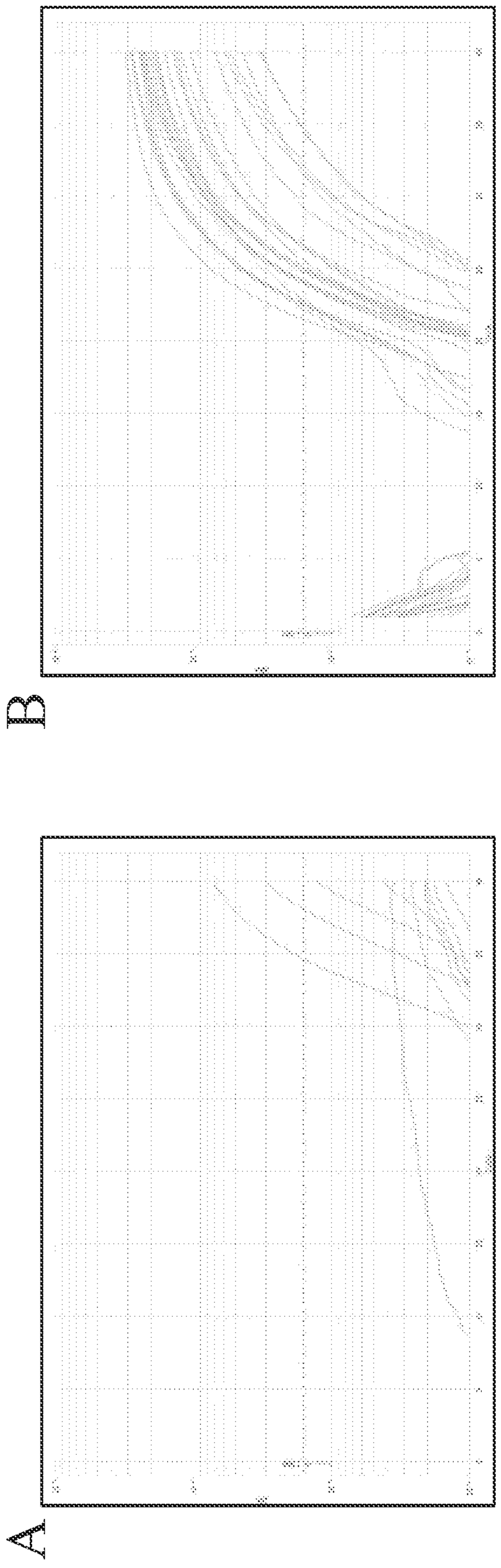


FIG. 11

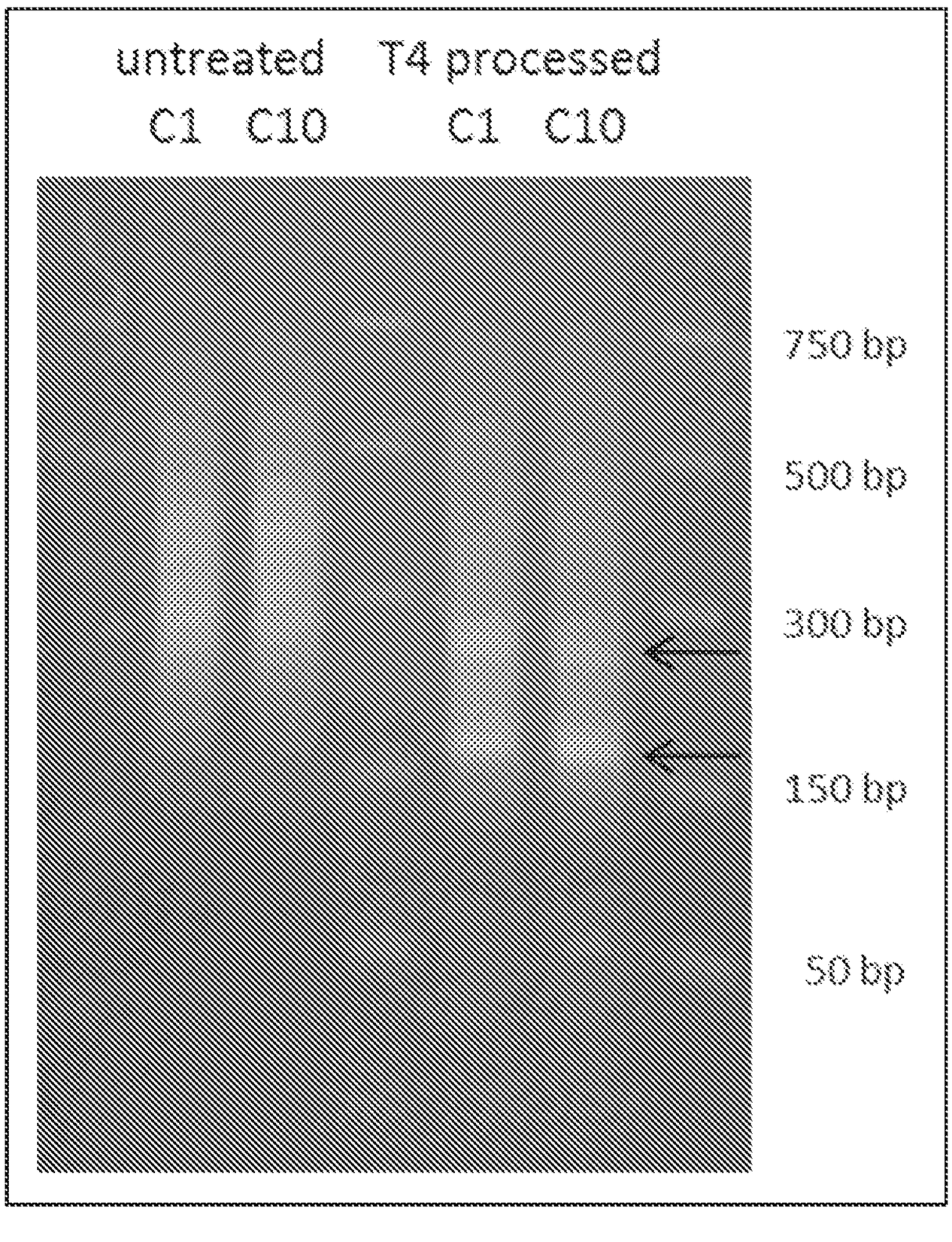

untreated
T4 processed
C1
C10
C1
C10
750 bp
500 bp
300 bp
150 bp
50 bp

FIG. 12

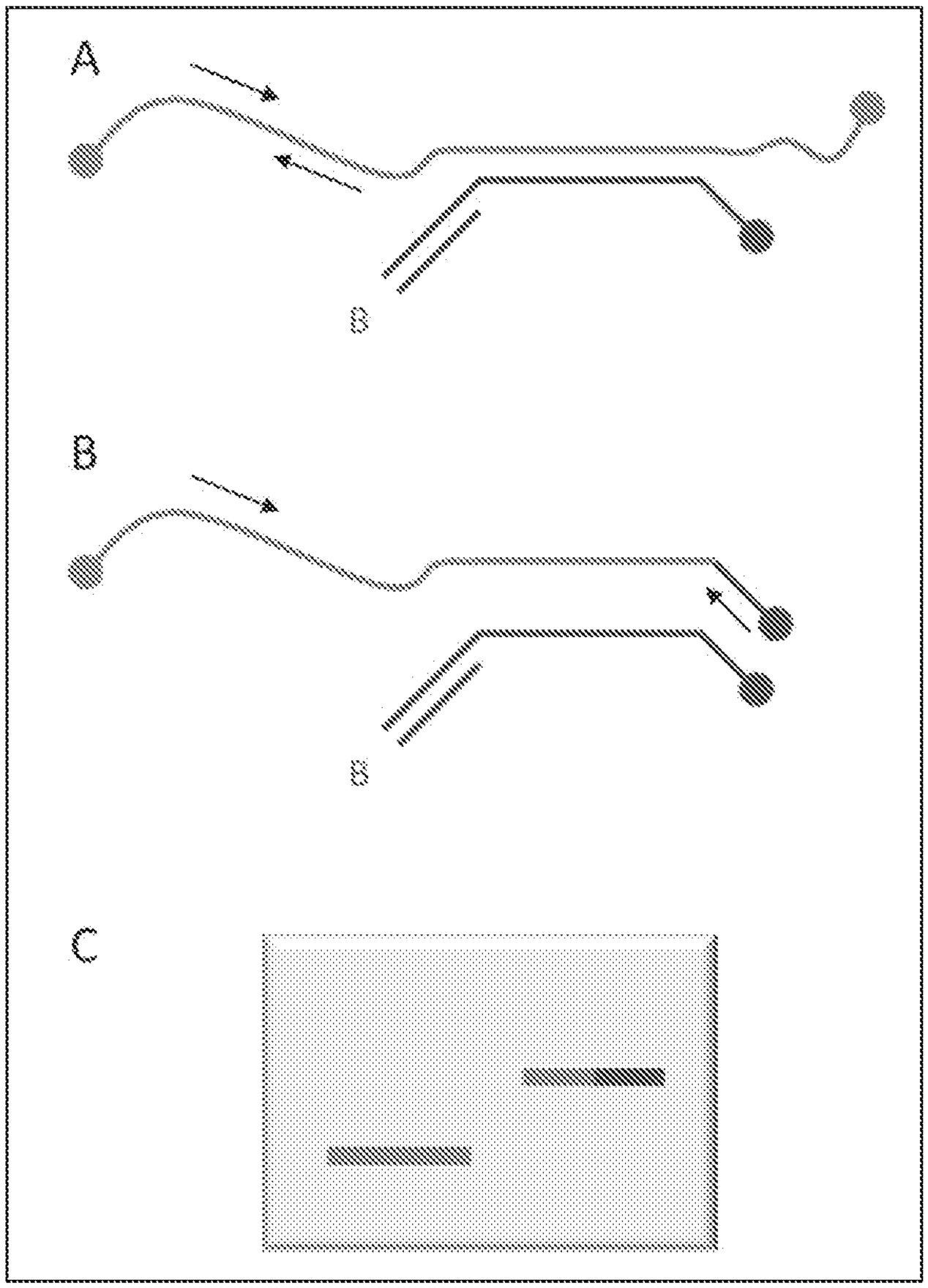


FIG. 13

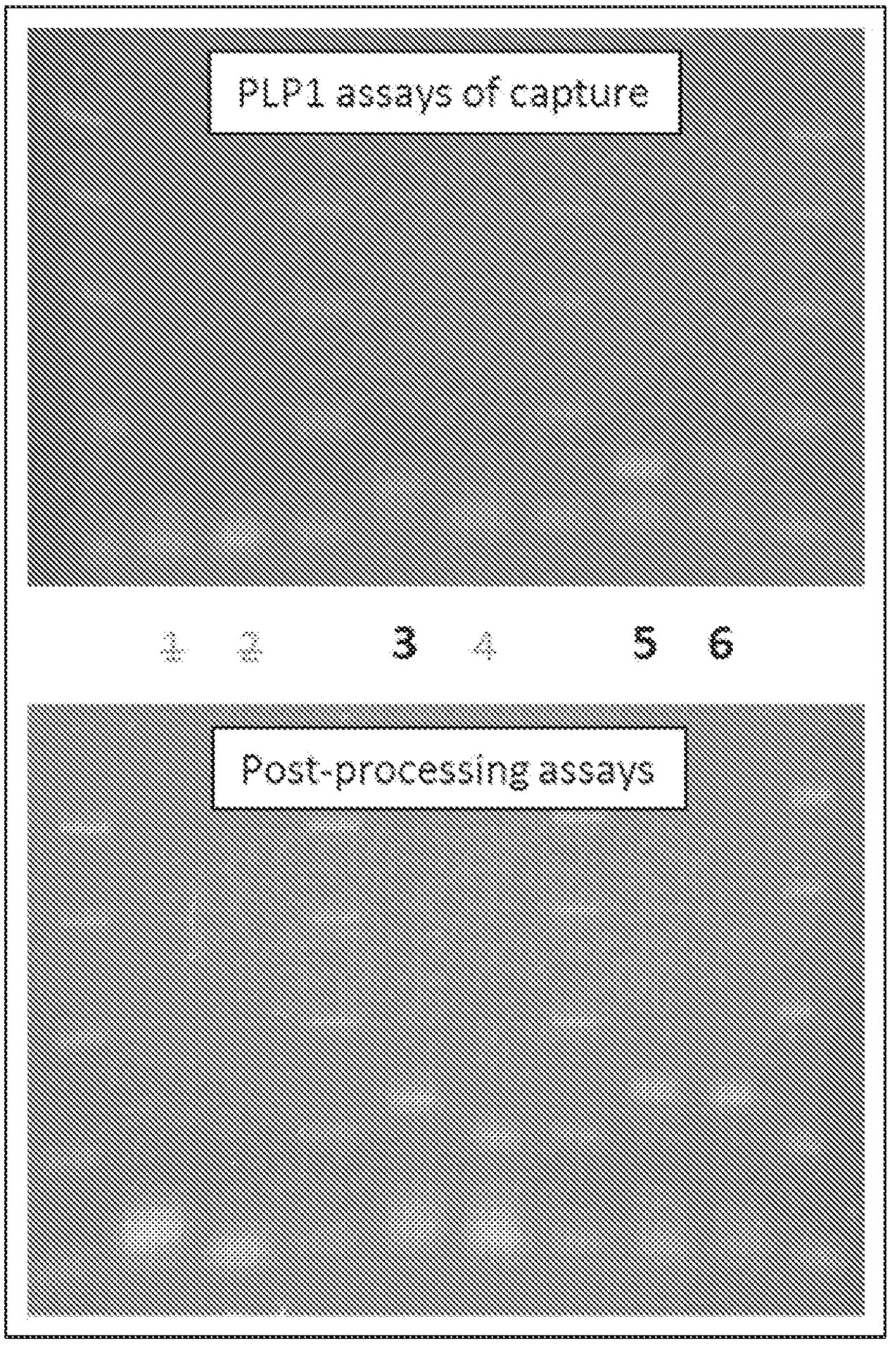


FIG. 14

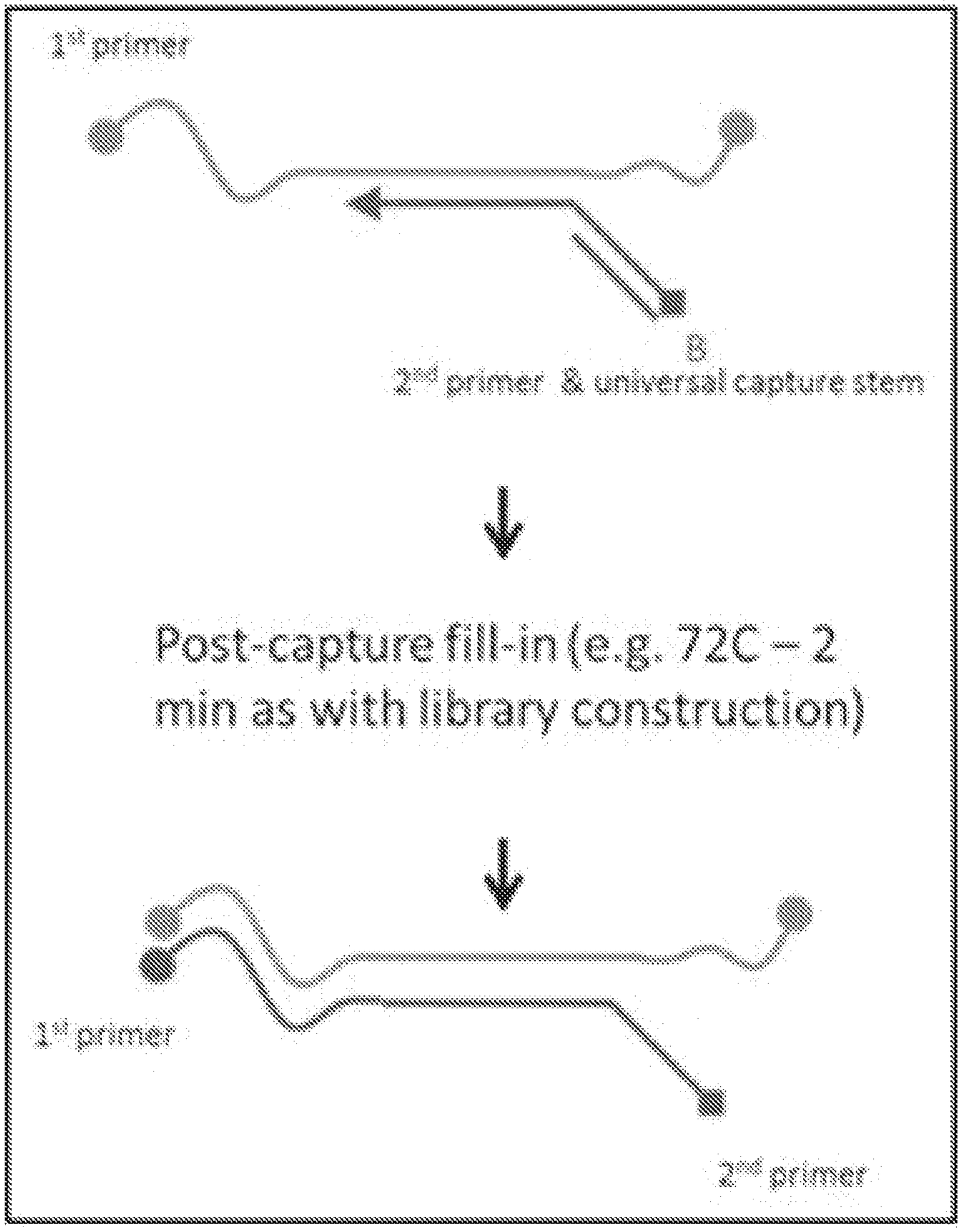

1st primer
B
2nd primer & universal capture stem
Post-capture fill-in (e.g. 72C – 2 min as with library construction)
1st primer
2nd primer

FIG. 15

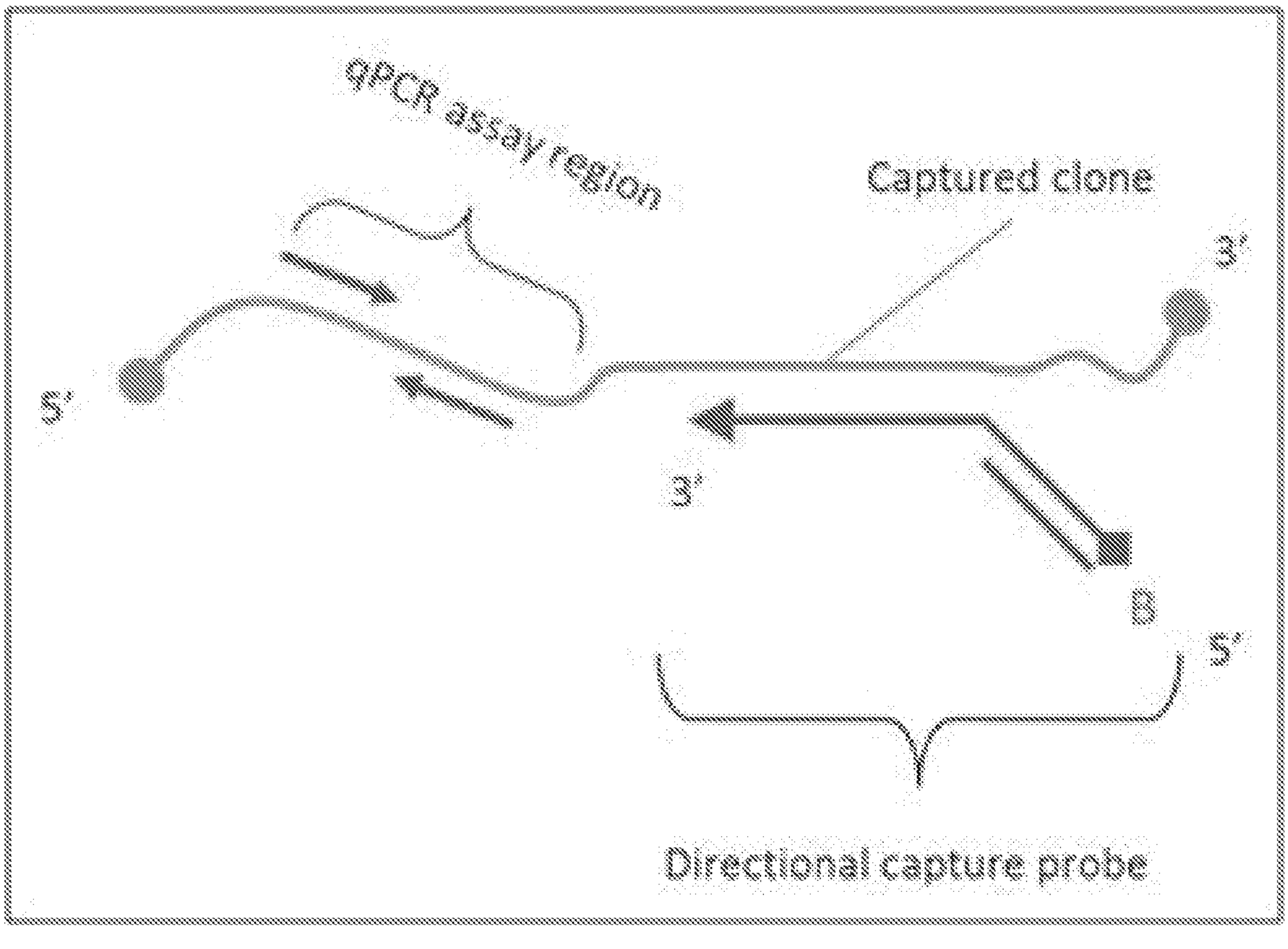

PCR assay region
Captured clone
5'
3'
3'
B
5'
Directional capture probe

FIG. 16

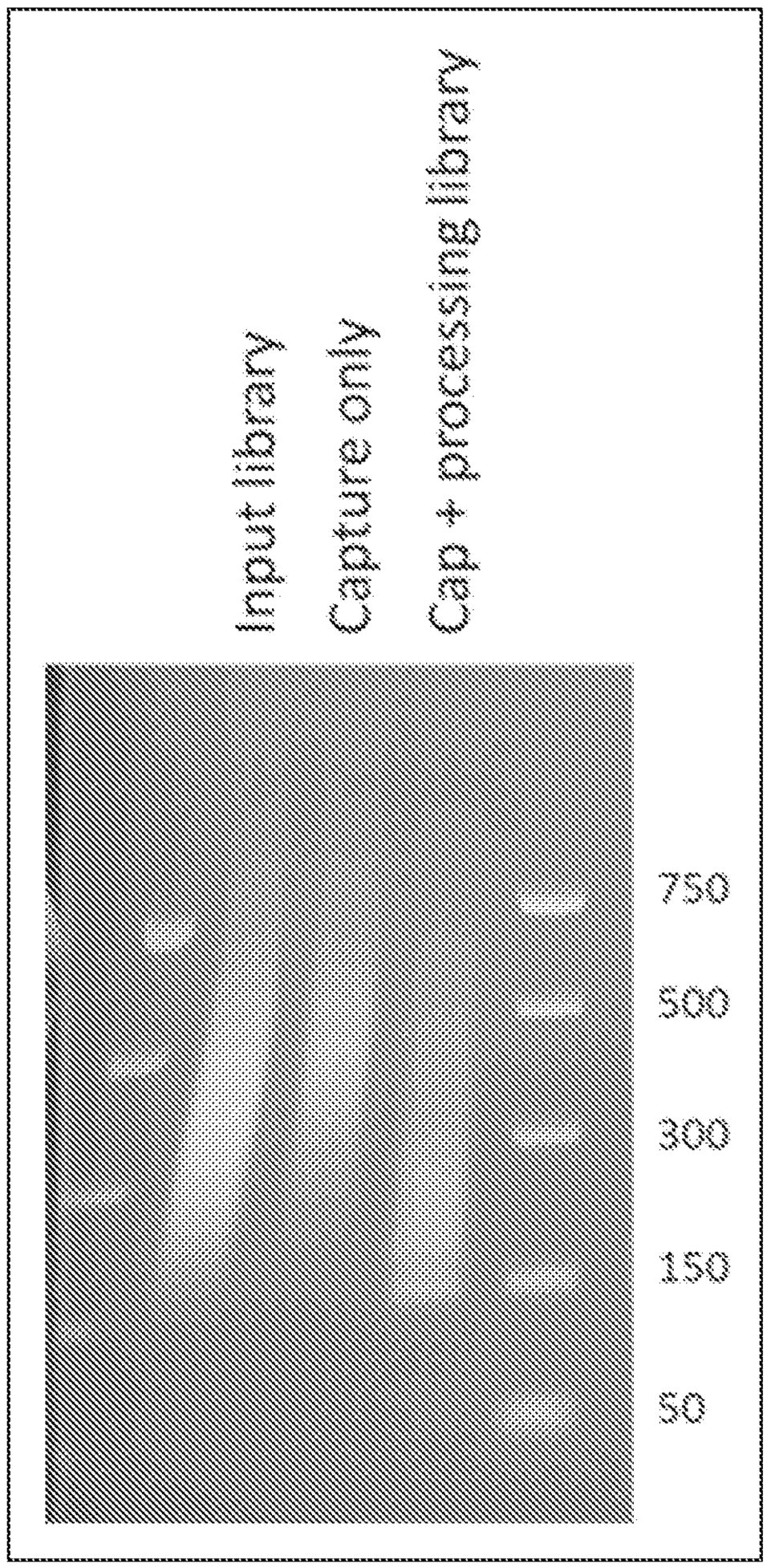
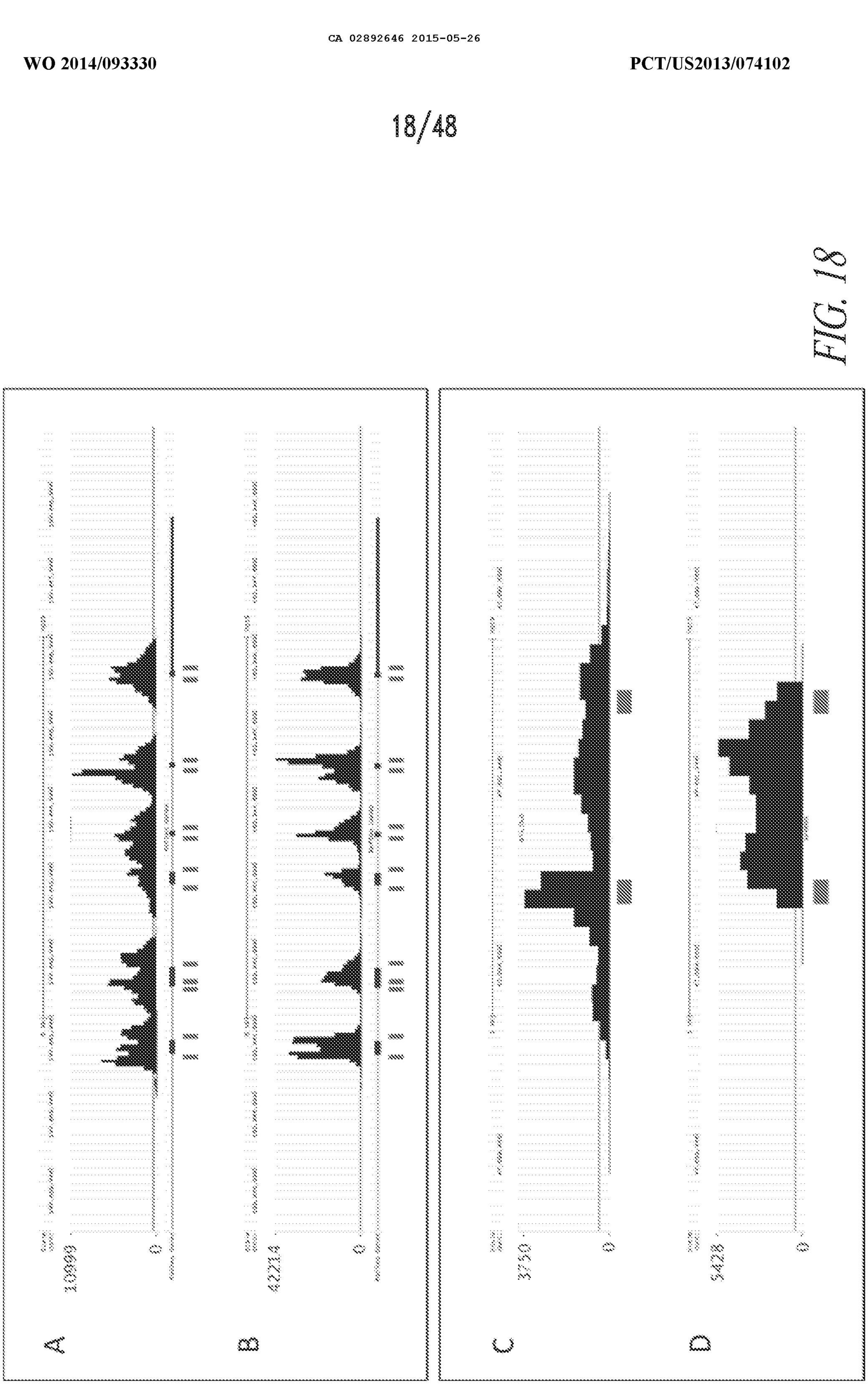

Input library
Capture only
Cap + processing library
750
500
300
150
50

FIG. 17

FIG. 18



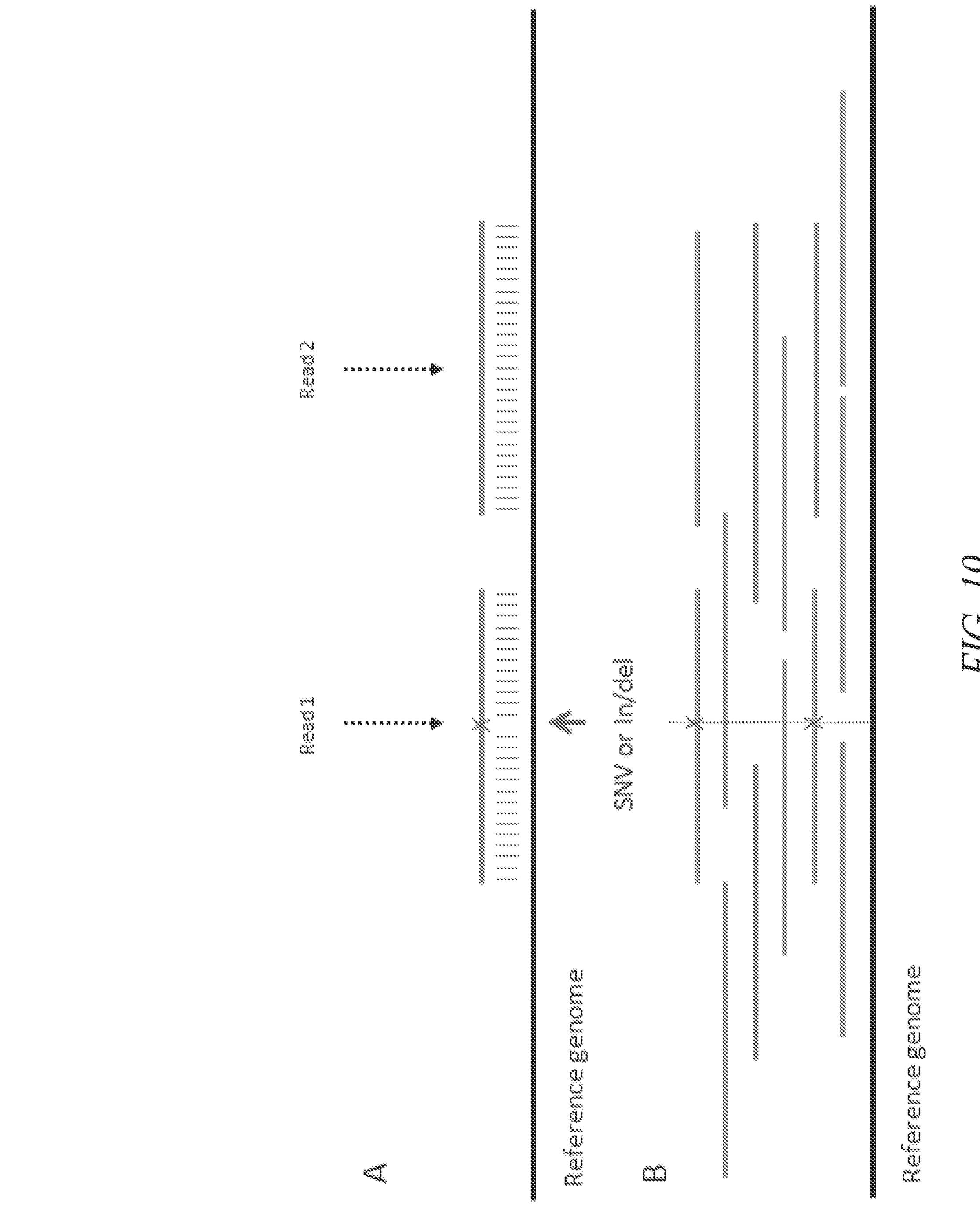
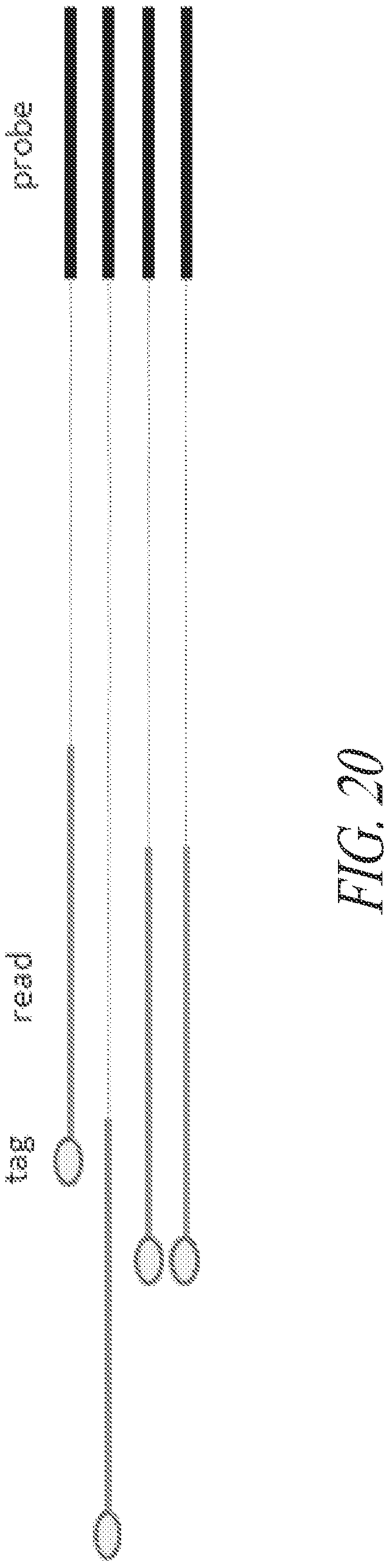

A
Reference genome
Read 1
Read 2
SNV or In/del
B
Reference genome

FIG. 19


probe
test
pear
FIG. 20

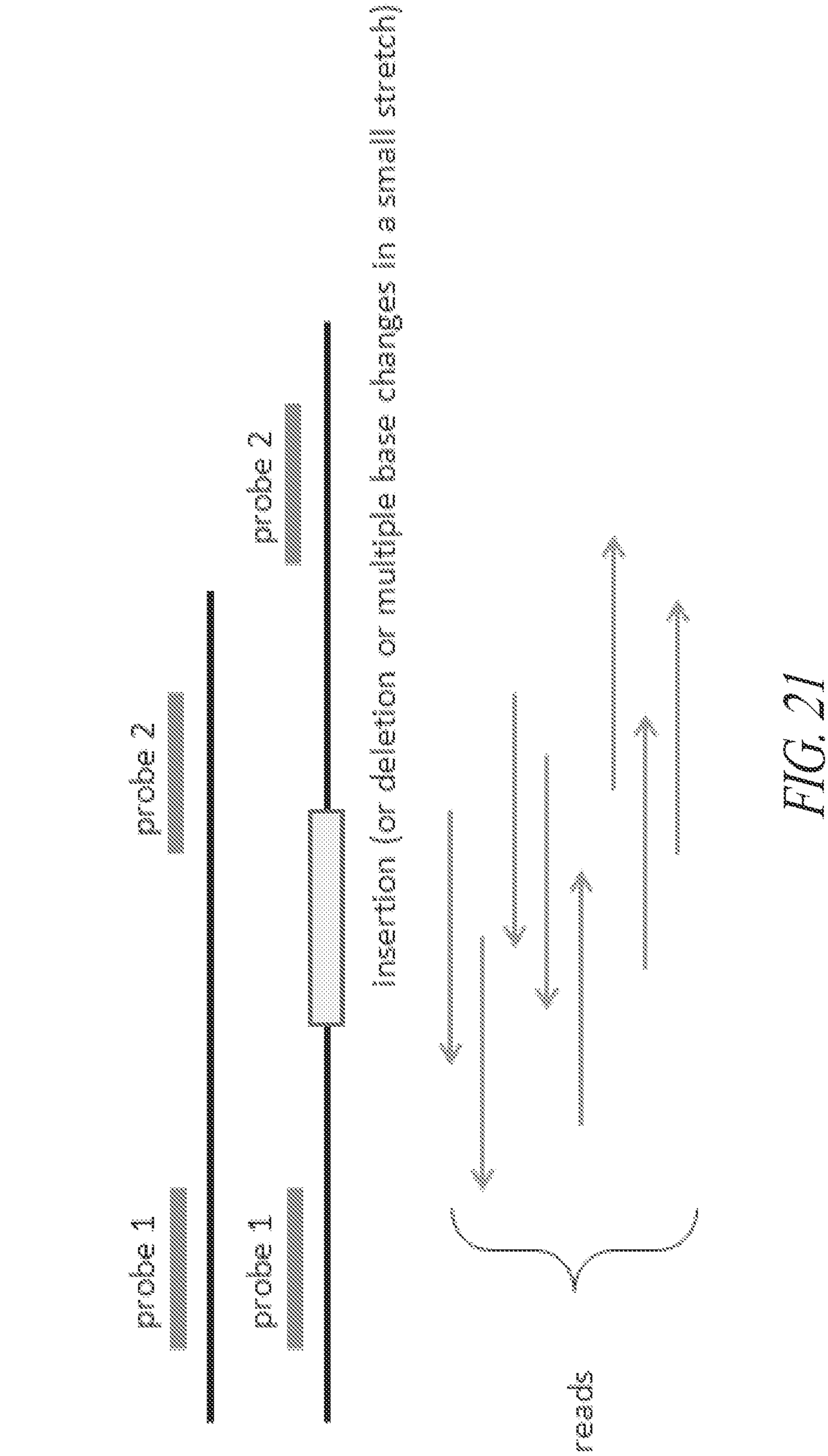

stretch
multiple base changes in small
insertion (or deletion or
probe 2
probe 2
probe 1
probe 1
reads

FIG. 21

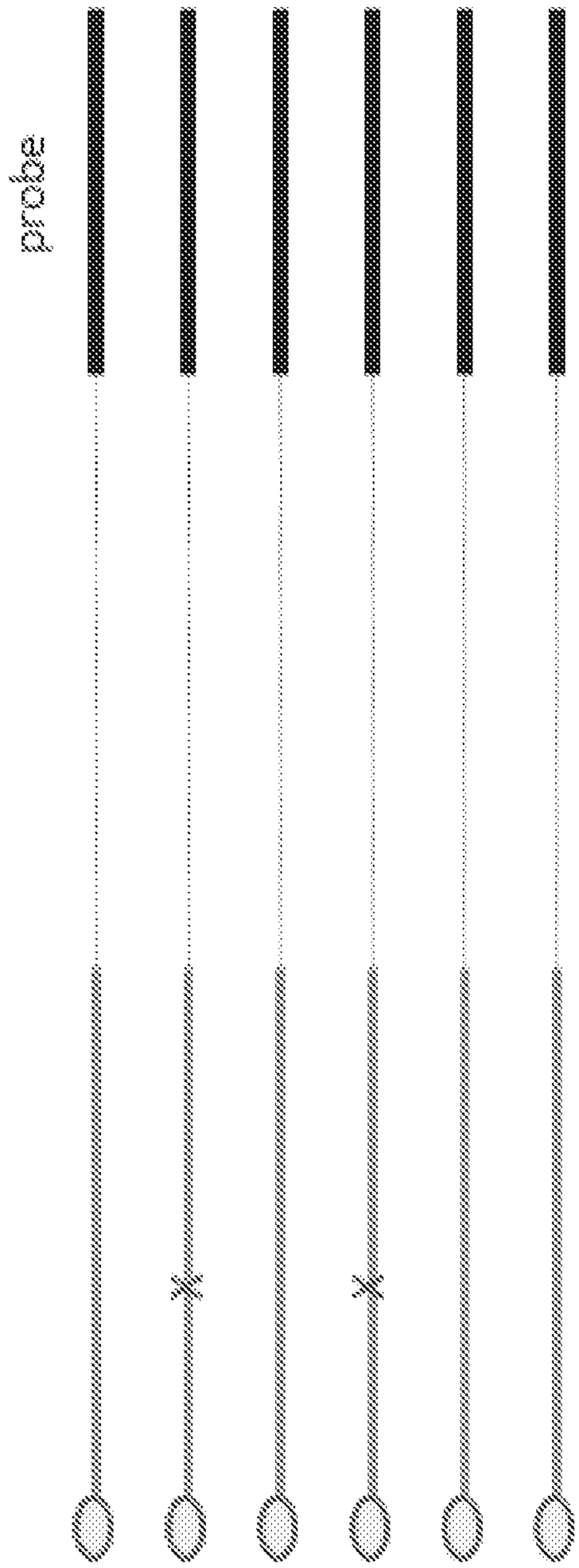

probe
same sequences, strict
sequences, relaxed (e.g., ≥1 mismatches)
encoded reporter
adapter - universal
adapter - target

FIG. 27

FIG. 23

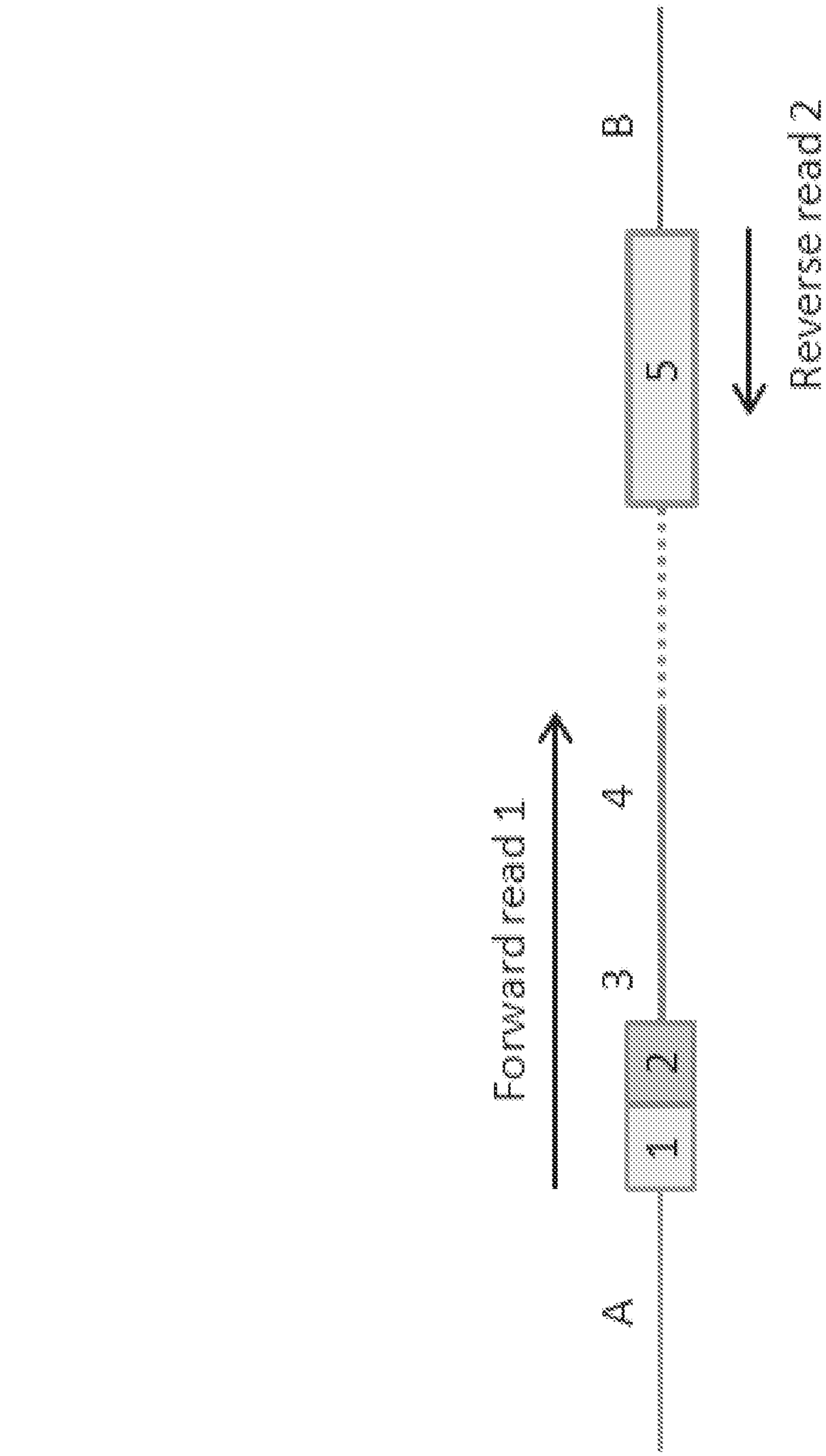


FIG. 4H

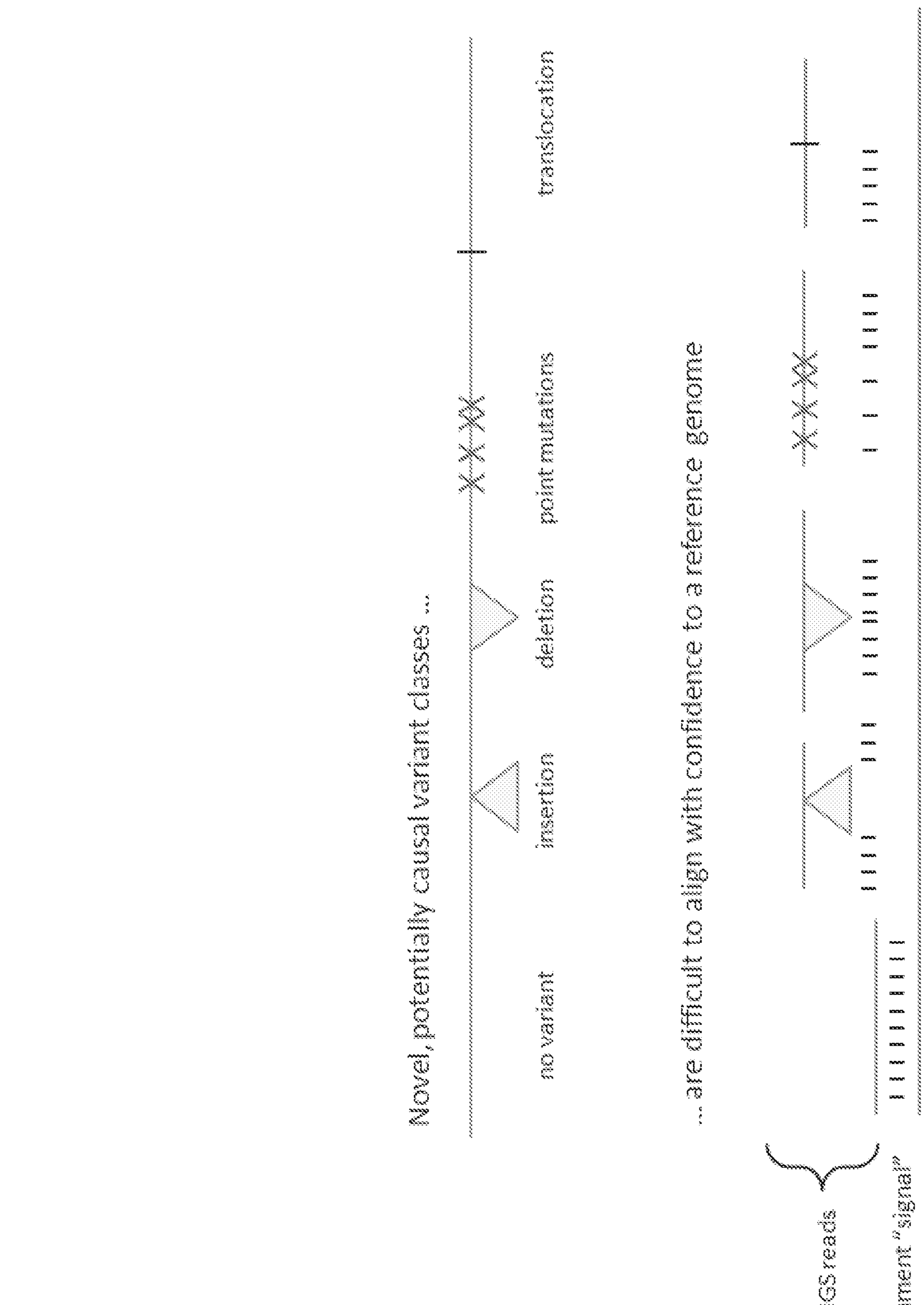

translocation
point mutations
reference genome
... are difficult to align with confidence to a
Novel, potentially causal variant classes ...
no variant
insertion
deletion
NGS reads
alignment "signal"

FIG. 25

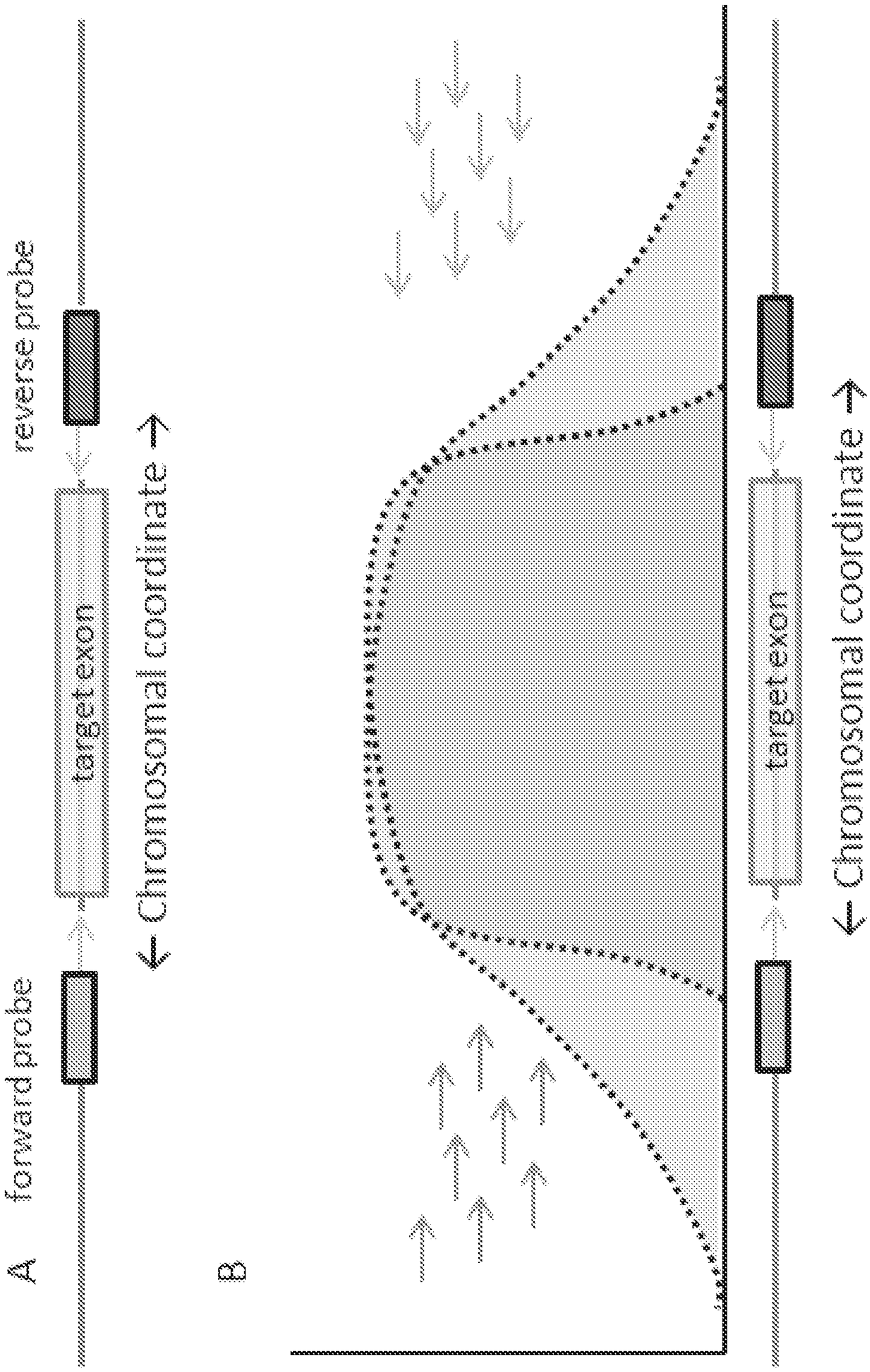

reverse probe
target exon
← Chromosomal coordinate →
forward probe
target exon
← Chromosomal coordinate →
Read depth
(number of sequencing reads)
8
4

FIG. 26

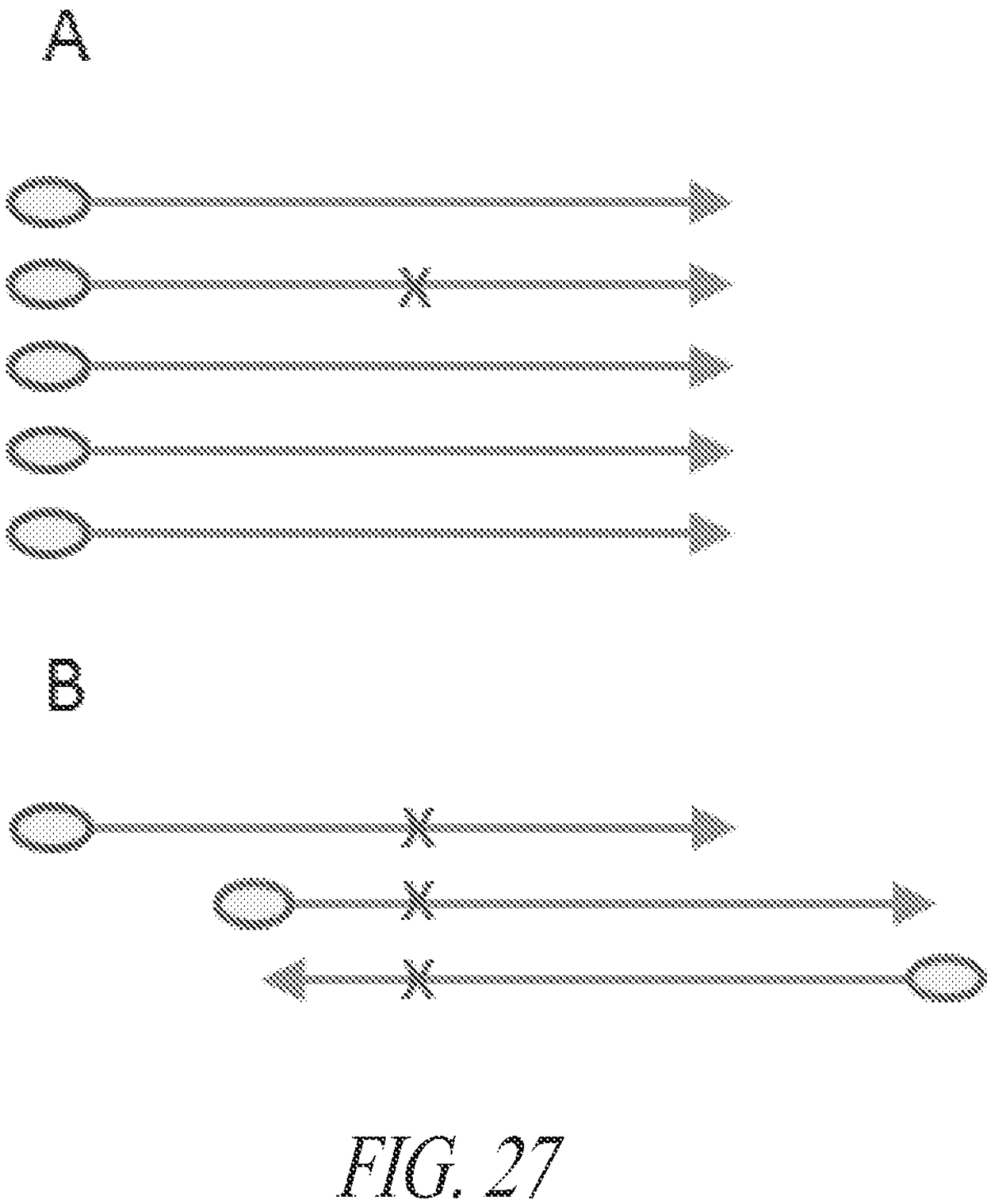
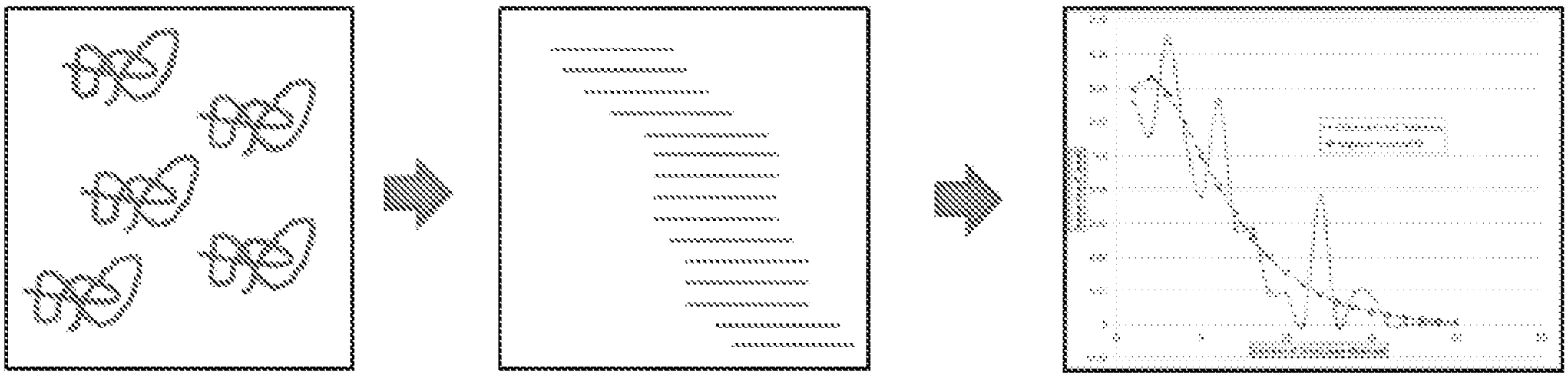
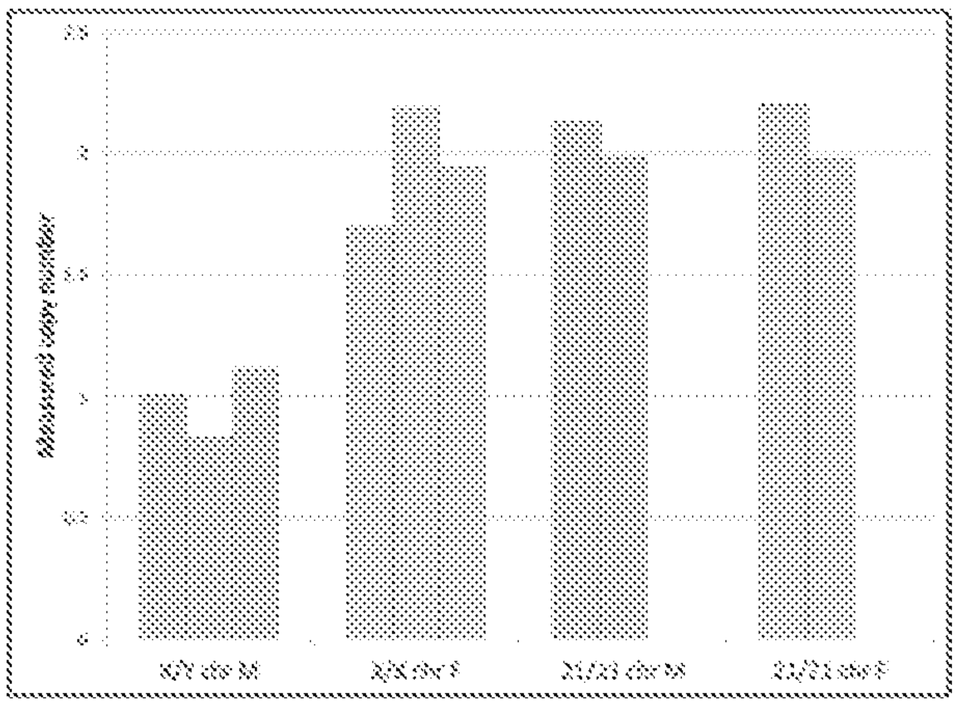


FIG. 27





Representative copy determination at three probes for the X-chromosomal gene PLP1 in a male and a female sample. For comparison, two probes for the autosomal chromosome 21 gene TNFRSF14 gene are also shown.

FIG. 28

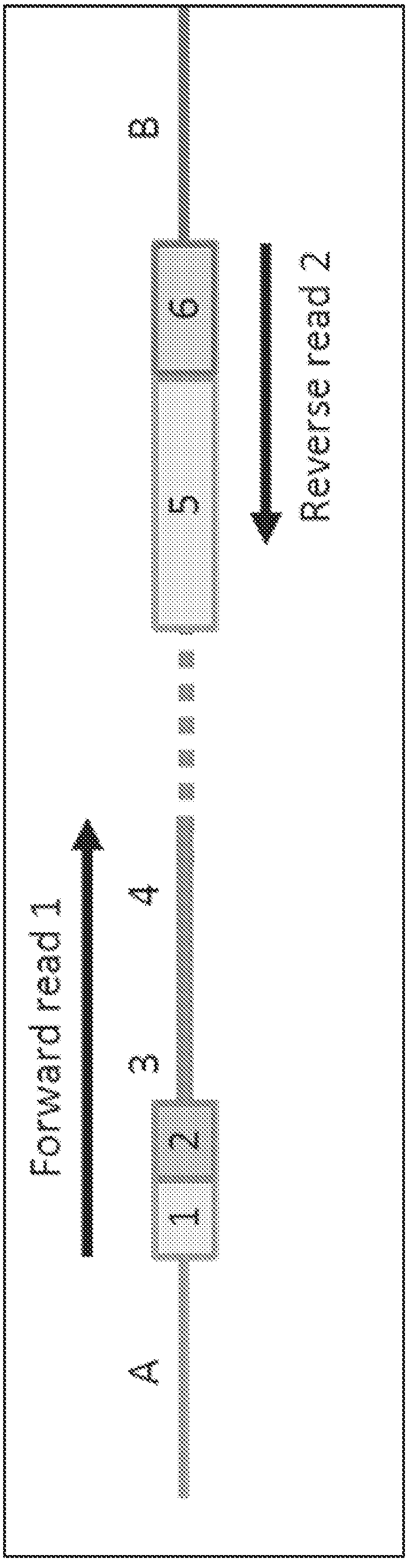


FIG. 29

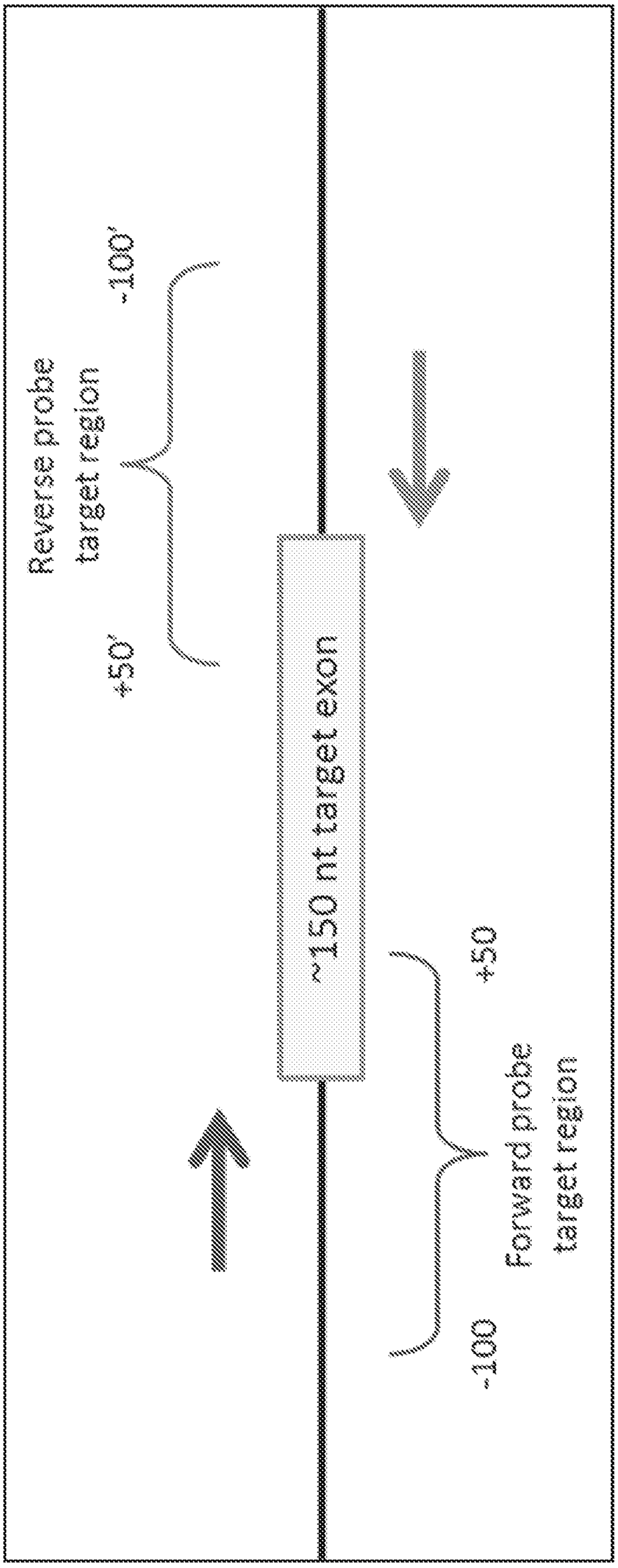

Reverse probe
target region
+50
-100
~150 nt target exon
Forward probe
target region
+50
-100

FIG. 30

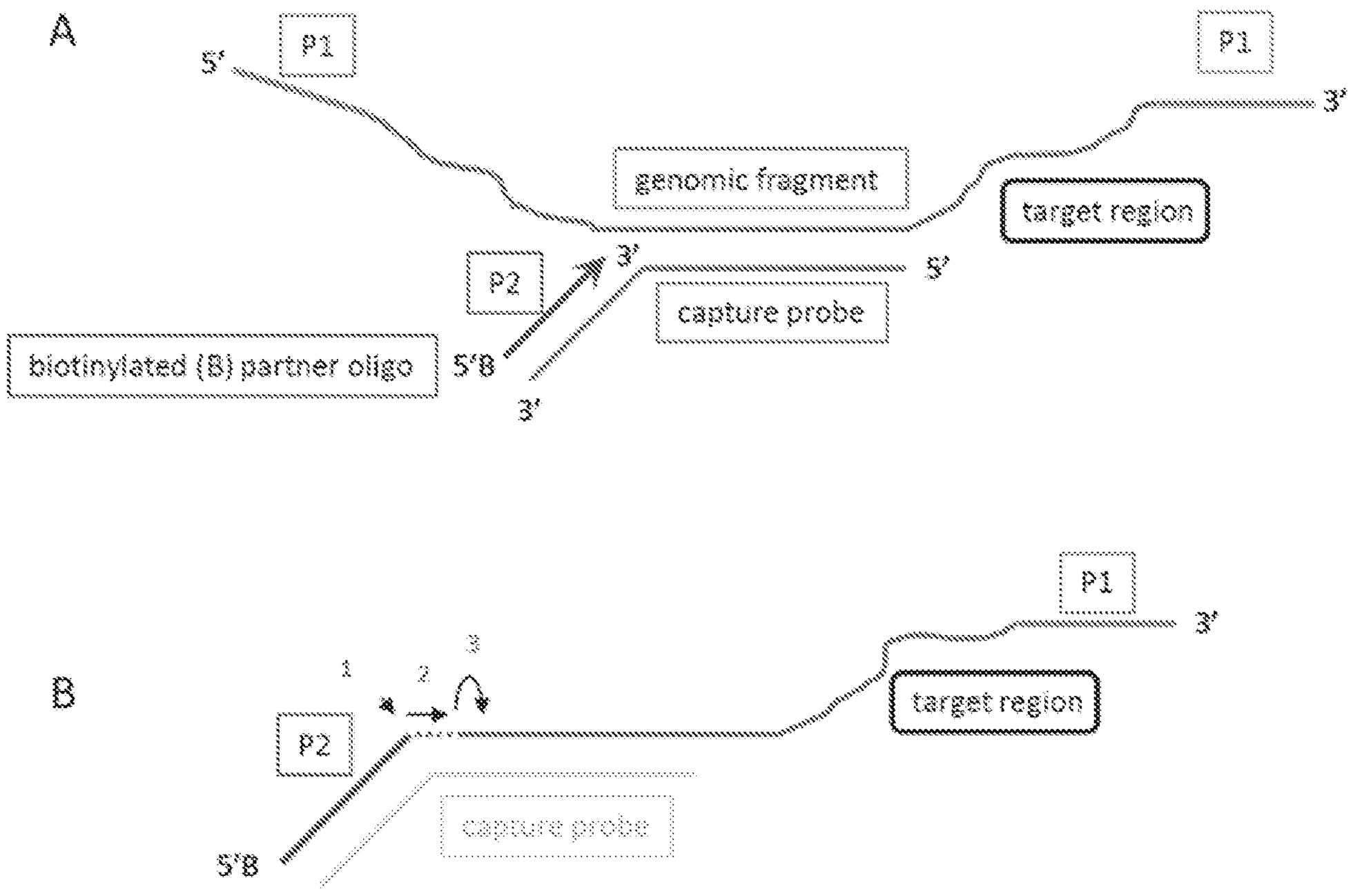

A
5'
P1
P1
3'
genomic fragment
target region
P2
3'
5'
capture probe
biotinylated (B) partner oligo
5'B
3'
B
1
2
3
P1
3'
target region
P2
5'B
capture probe

FIG. 31

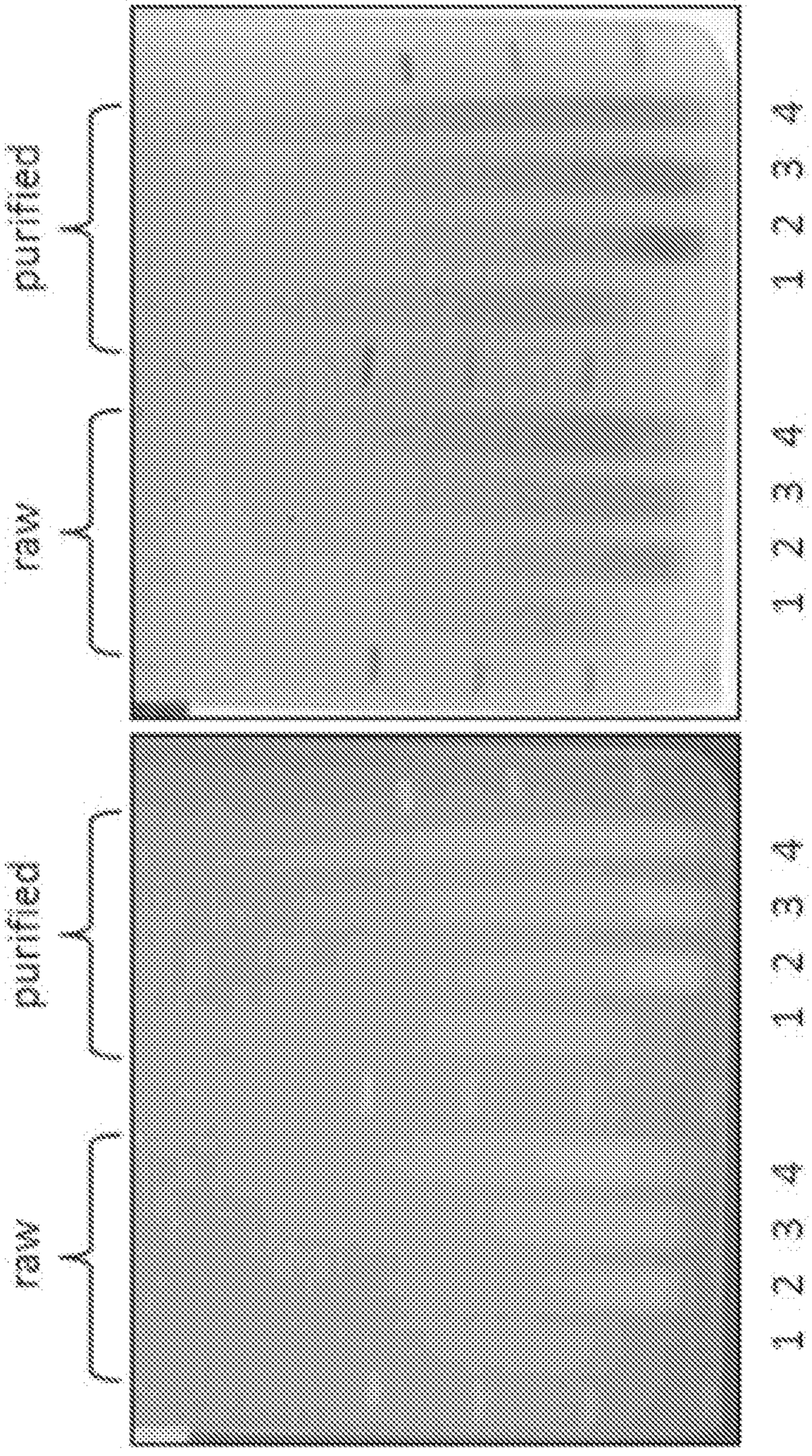


FIG. 32

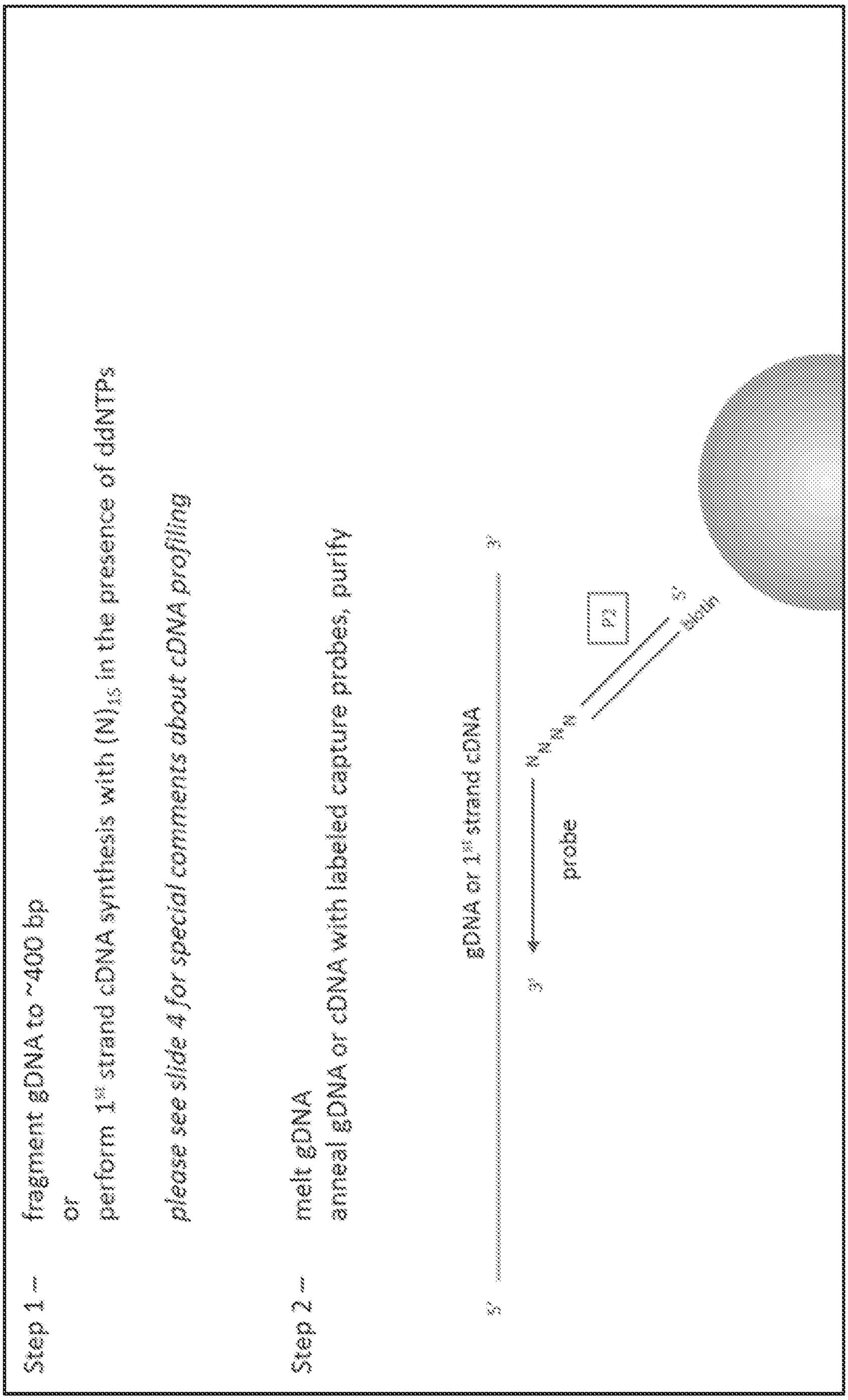

Step 1 – fragment gDNA to ~400 bp
or
perform 1st strand cDNA synthesis with (N)15 in the presence of ddNTPs
please see slide 4 for special comments about cDNA profiling
Step 2 – melt gDNA
anneal gDNA or 1st strand cDNA with labeled capture probes, purify
gDNA or 1st strand cDNA
probe
#2
5'
3'
3'
5'

FIG. 33

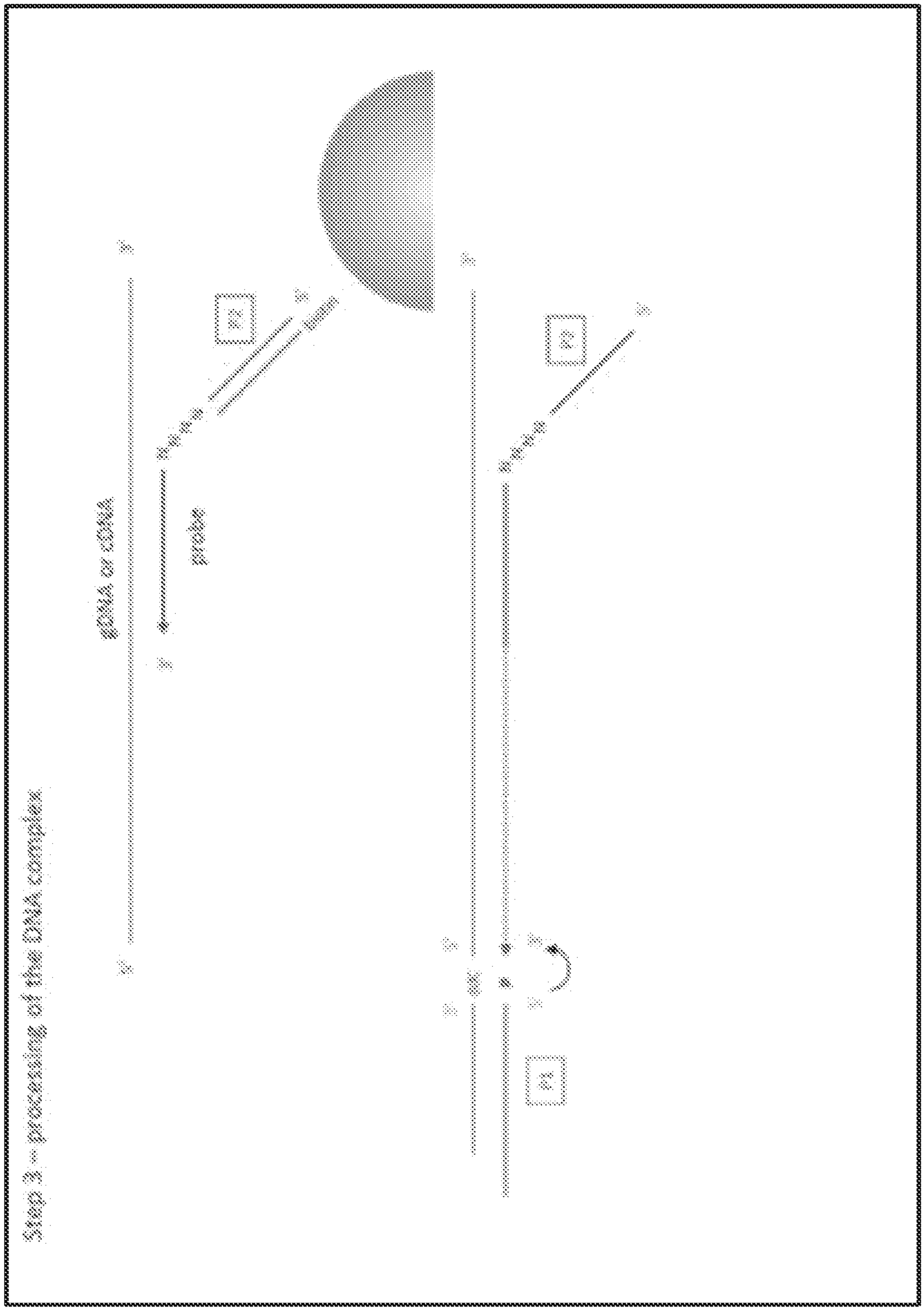


FIG. 34

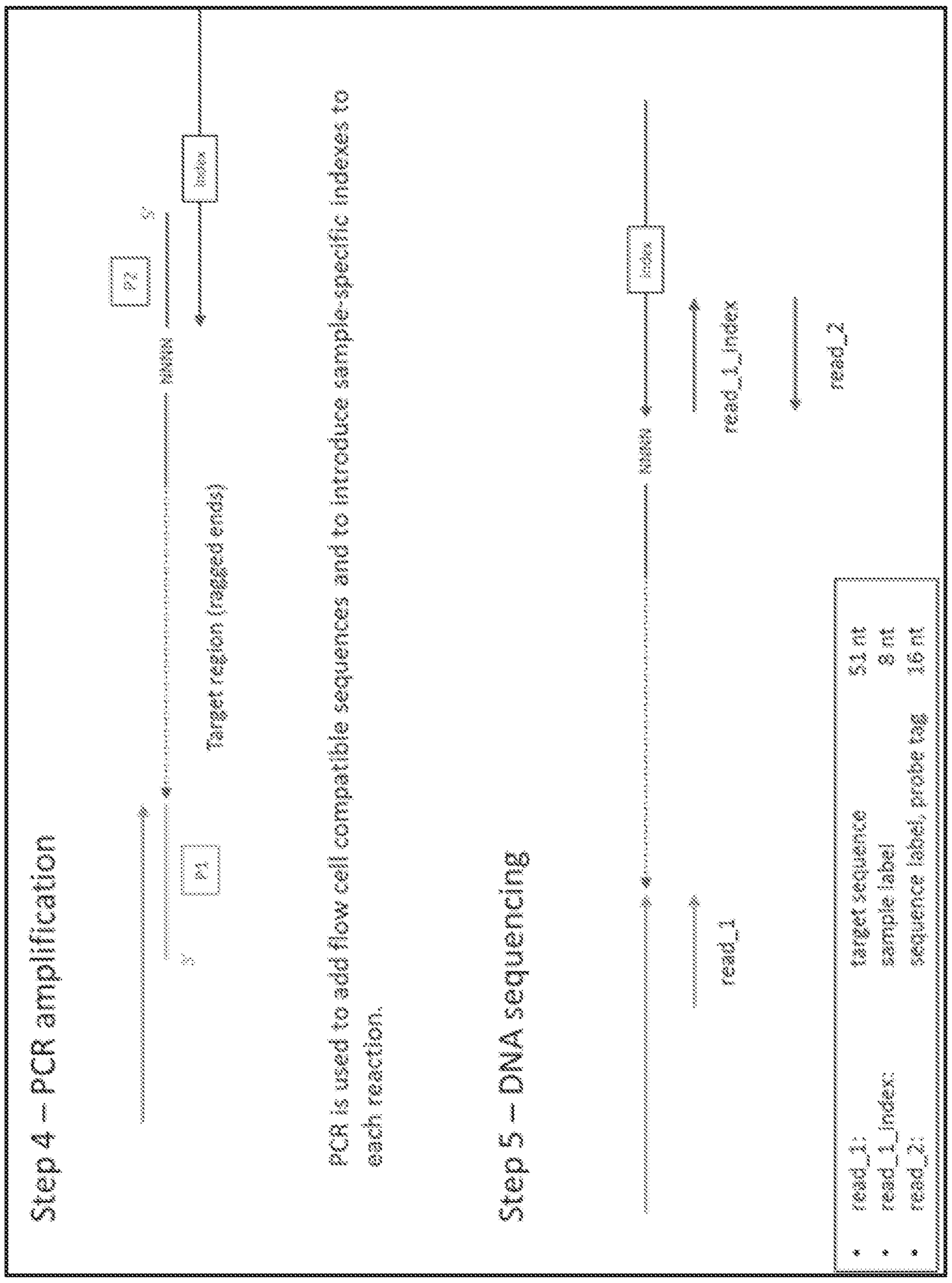

Step 4 – PCR amplification
PCR is used to add flow cell compatible sequences and to introduce sample-specific indexes to each reaction.
Step 5 – DNA sequencing
read_1
read_1_index
read_2
Index
Target region (tagged end)
P5
P7
Index
read_1
read_1_index
read_2
target sequence 51 nt
sample label 8 nt
sequence label, probe tag 16 nt
* read_1:
* read_1_index:
* read_2:

FIG. 35

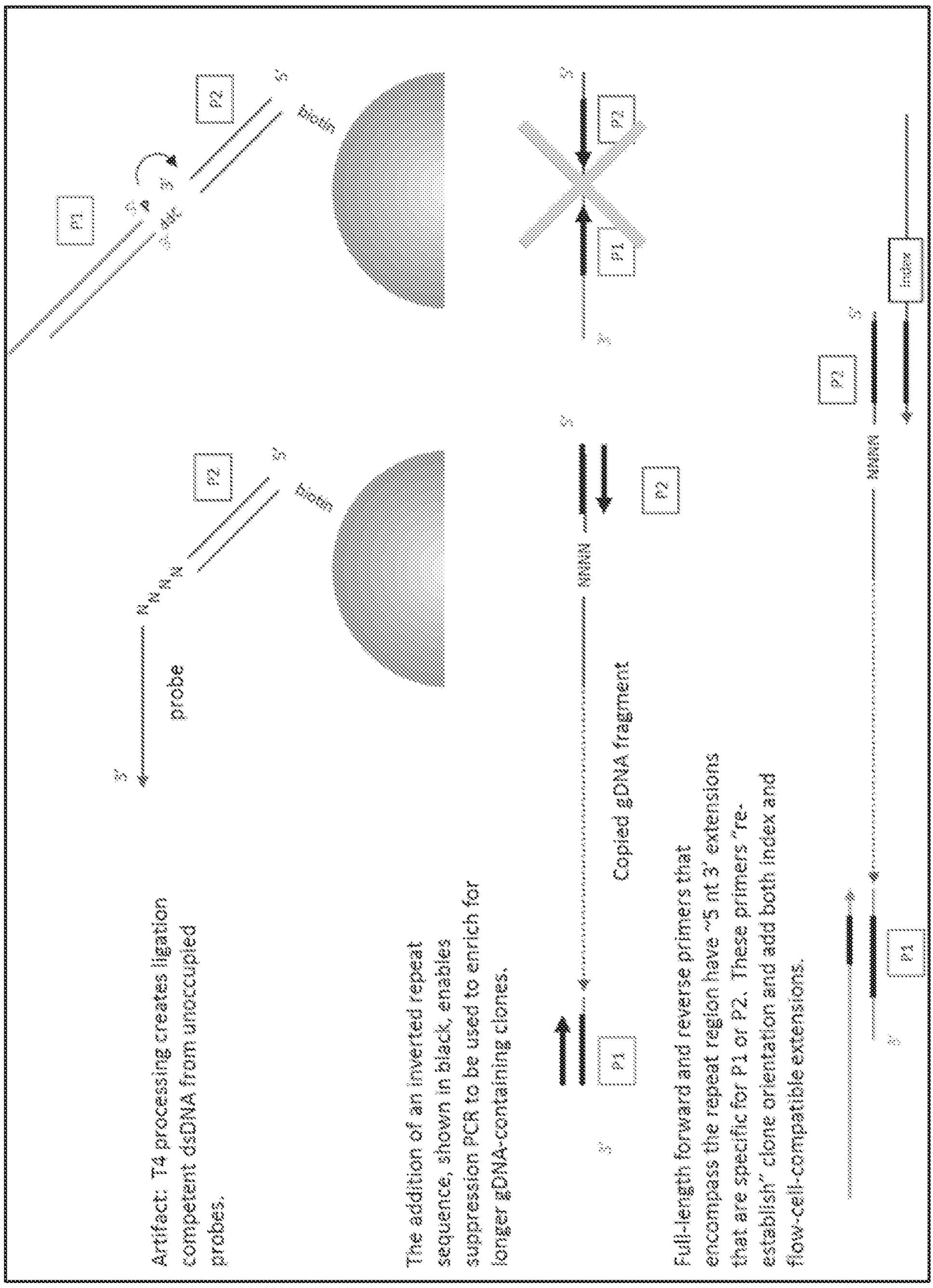

Artifact: T4 processing creates ligation competent dsDNA from uncoupled probes.
5'
3'
probe
The addition of an inverted repeat sequence, shown in black, enables suppression PCR to be used to enrich for longer gDNA-containing clones.
P1
Copied gDNA fragment
P2
Full-length forward and reverse primers that encompass the repeat region have ~5 nt 3' extensions that are specific for P1 or P2. These primers "re-establish" clone orientation and add both index and flow-cell-compatible extensions.
P1
P2
Index
P1
P2

FIG. 36

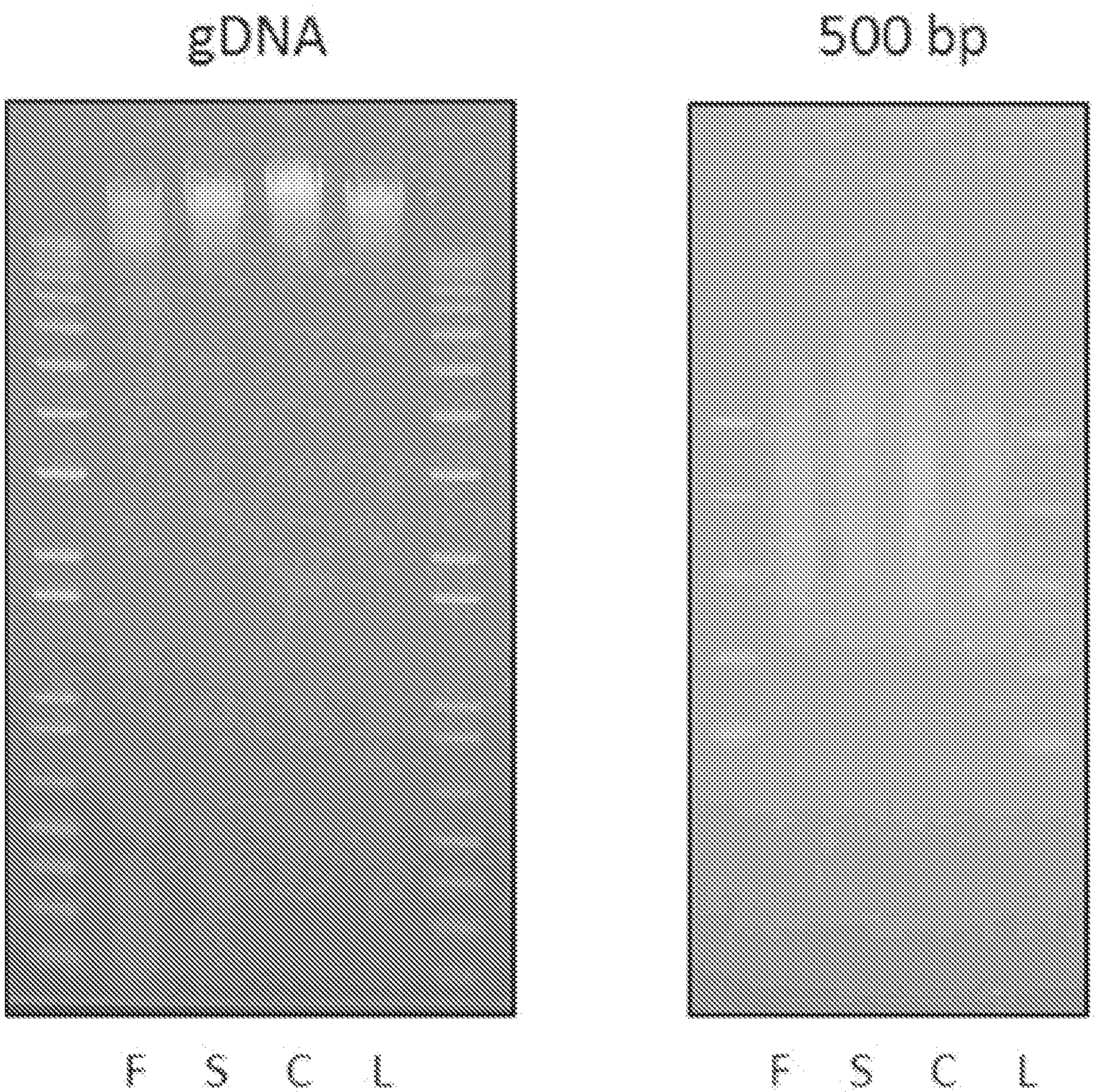


FIG. 37

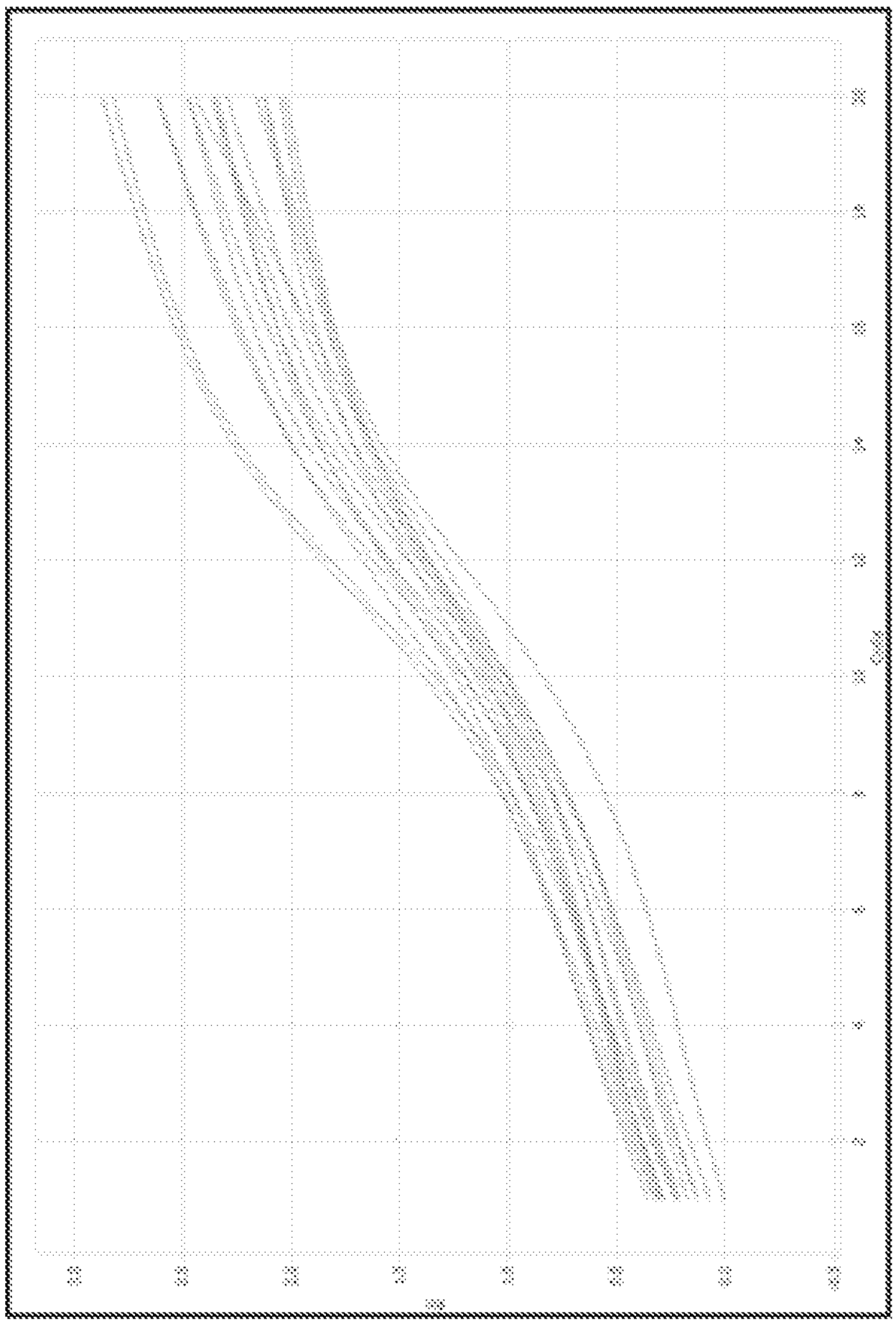


FIG. 38

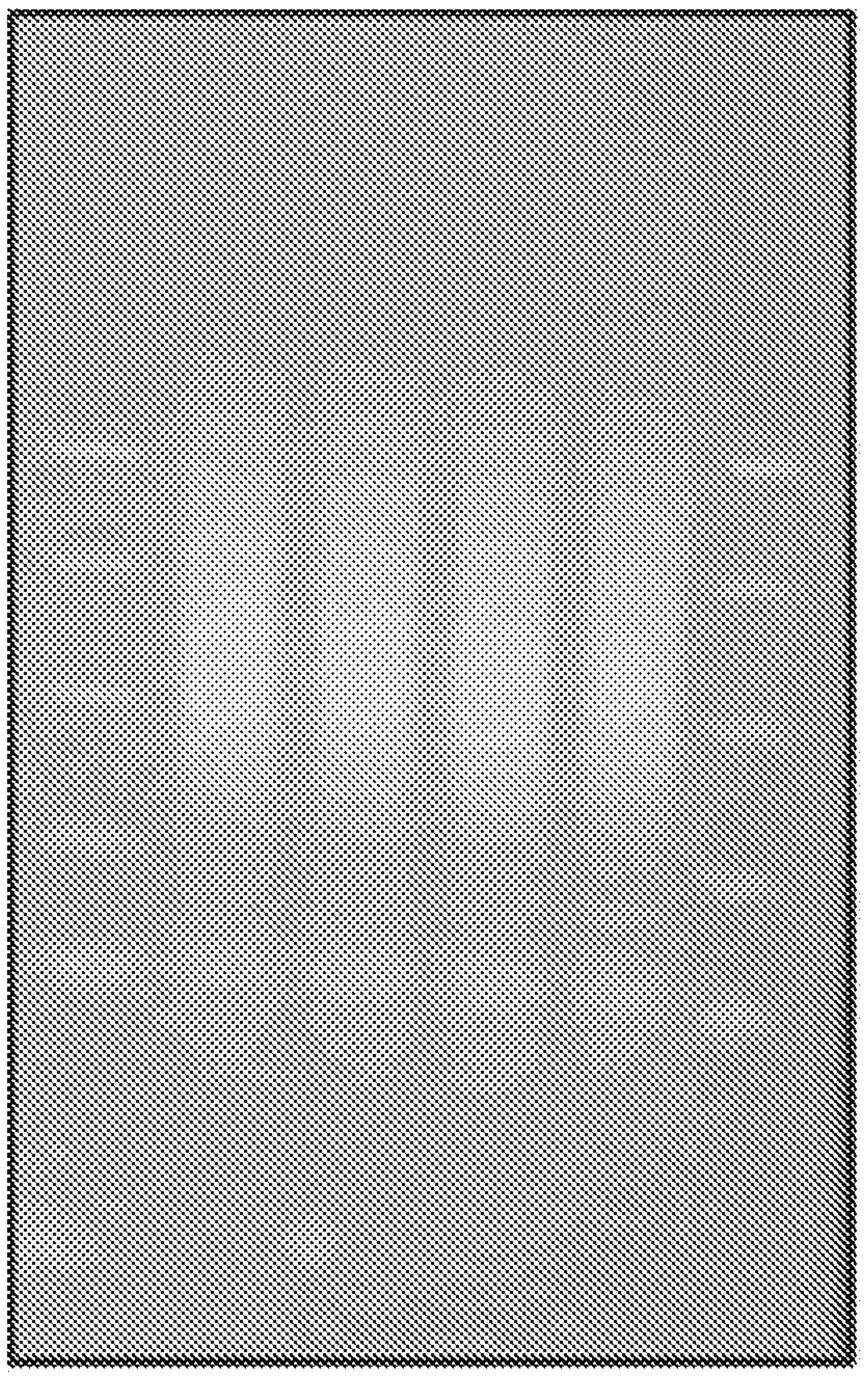


FIG. 39

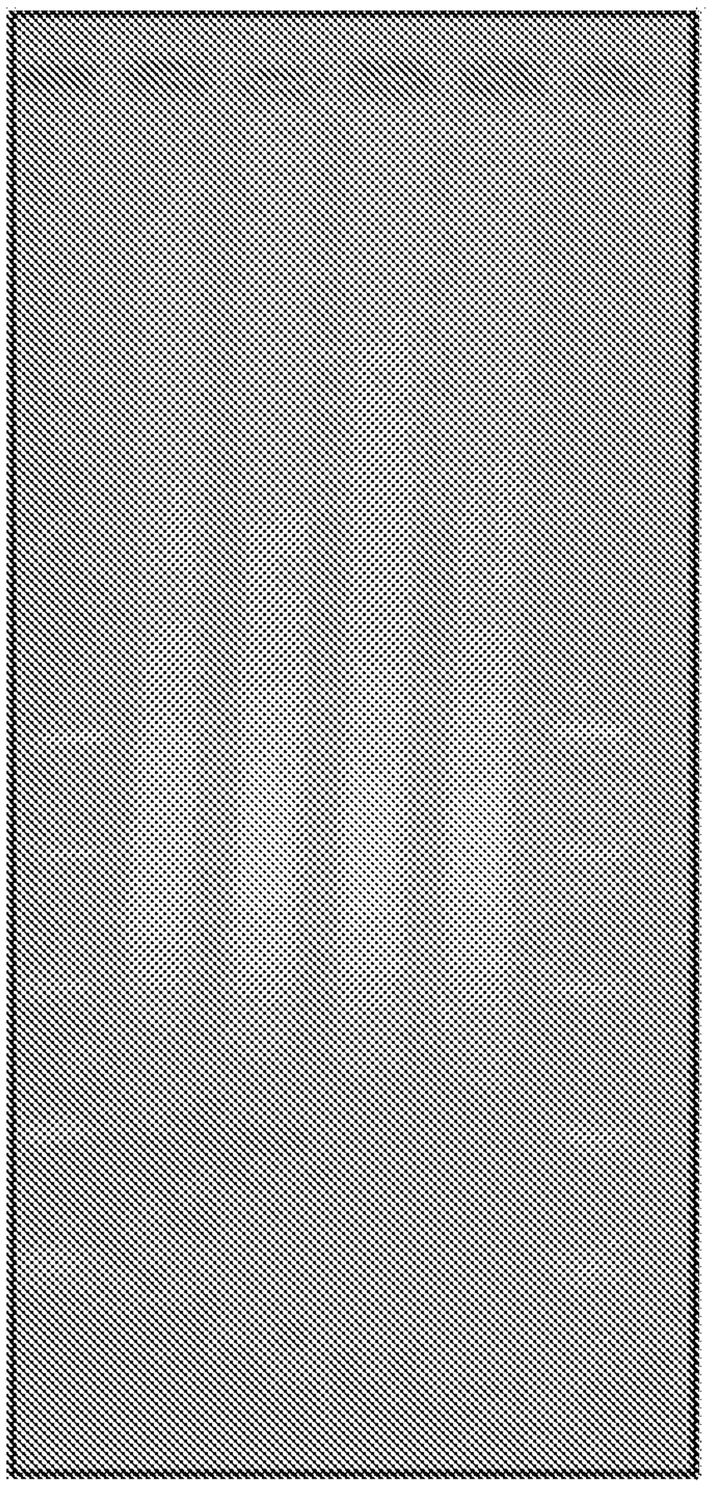


FIG. 40

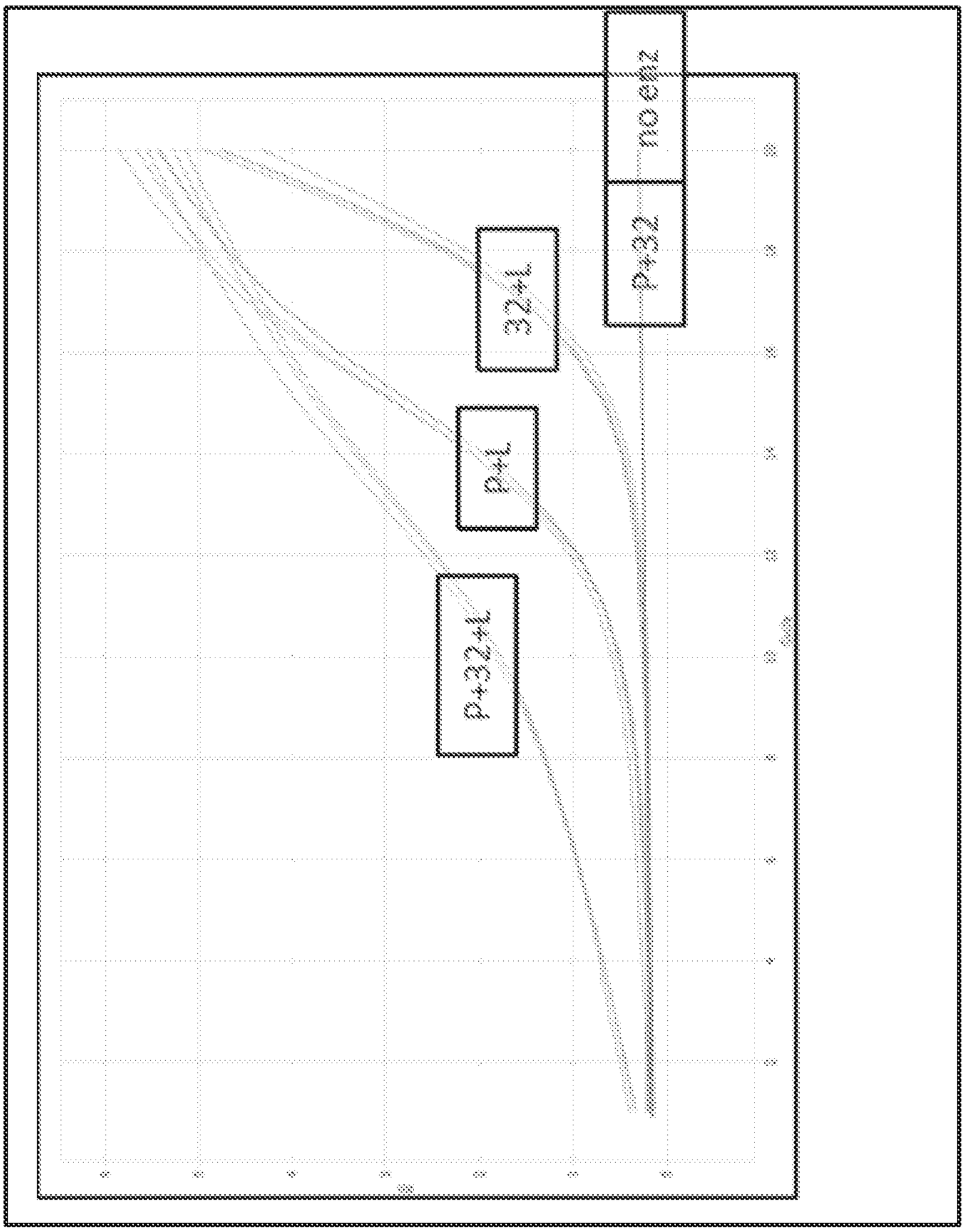


FIG. 41

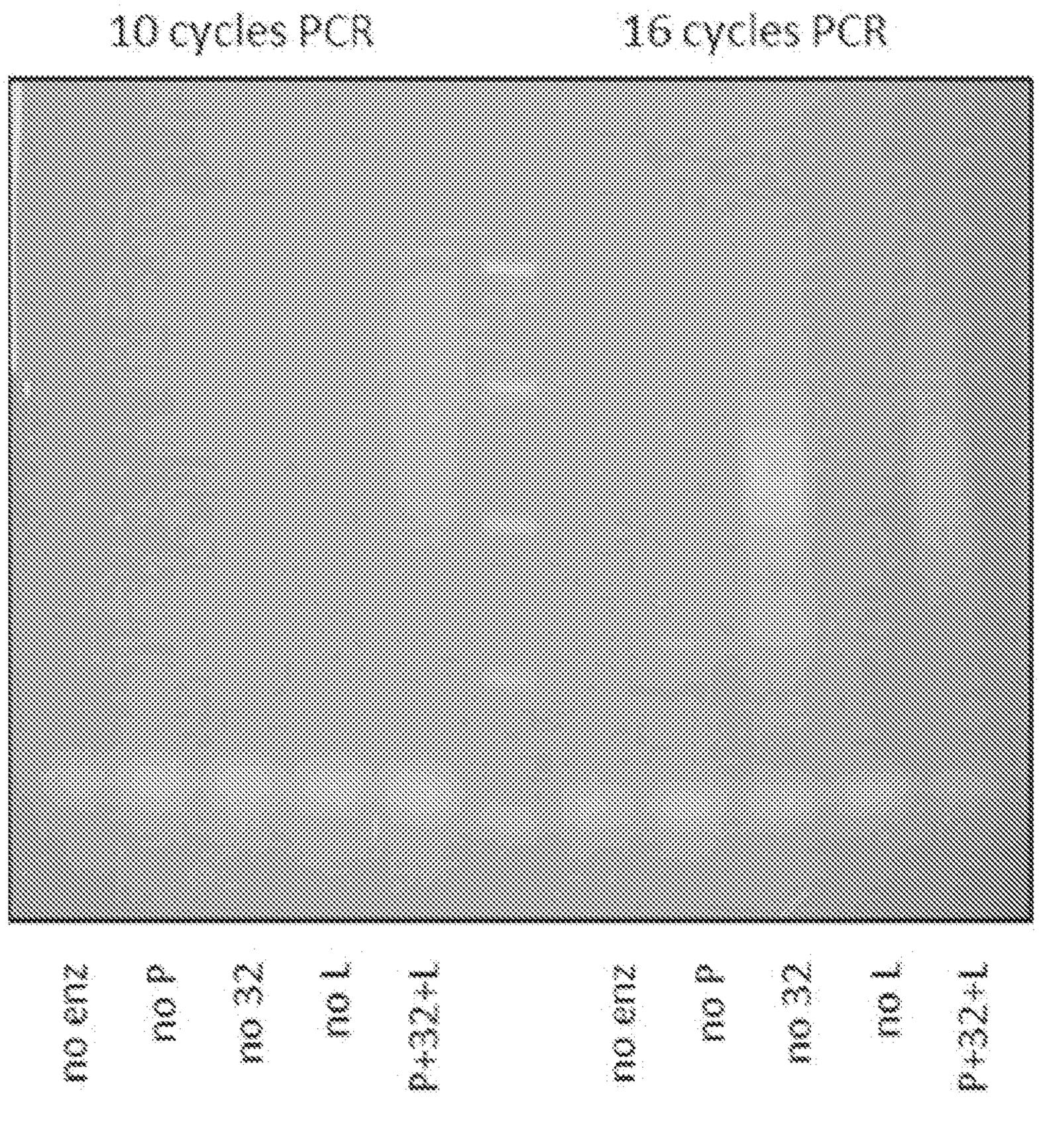

10 cycles PCR
16 cycles PCR
no enz
no p
no 32
no 1
p+32+1
no enz
no p
no 32
no 1
p+32+1

FIG. 42

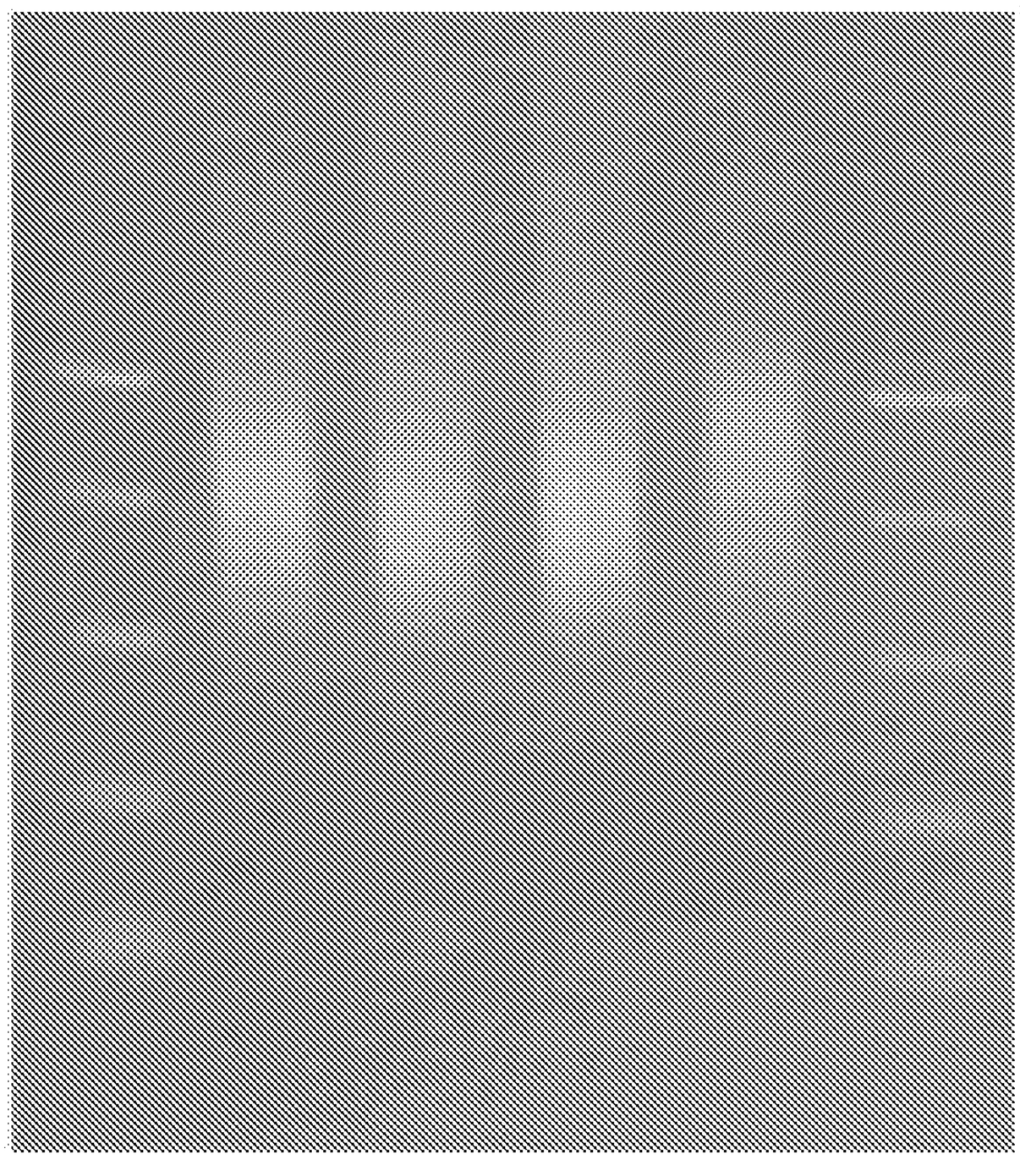


FIG. 43

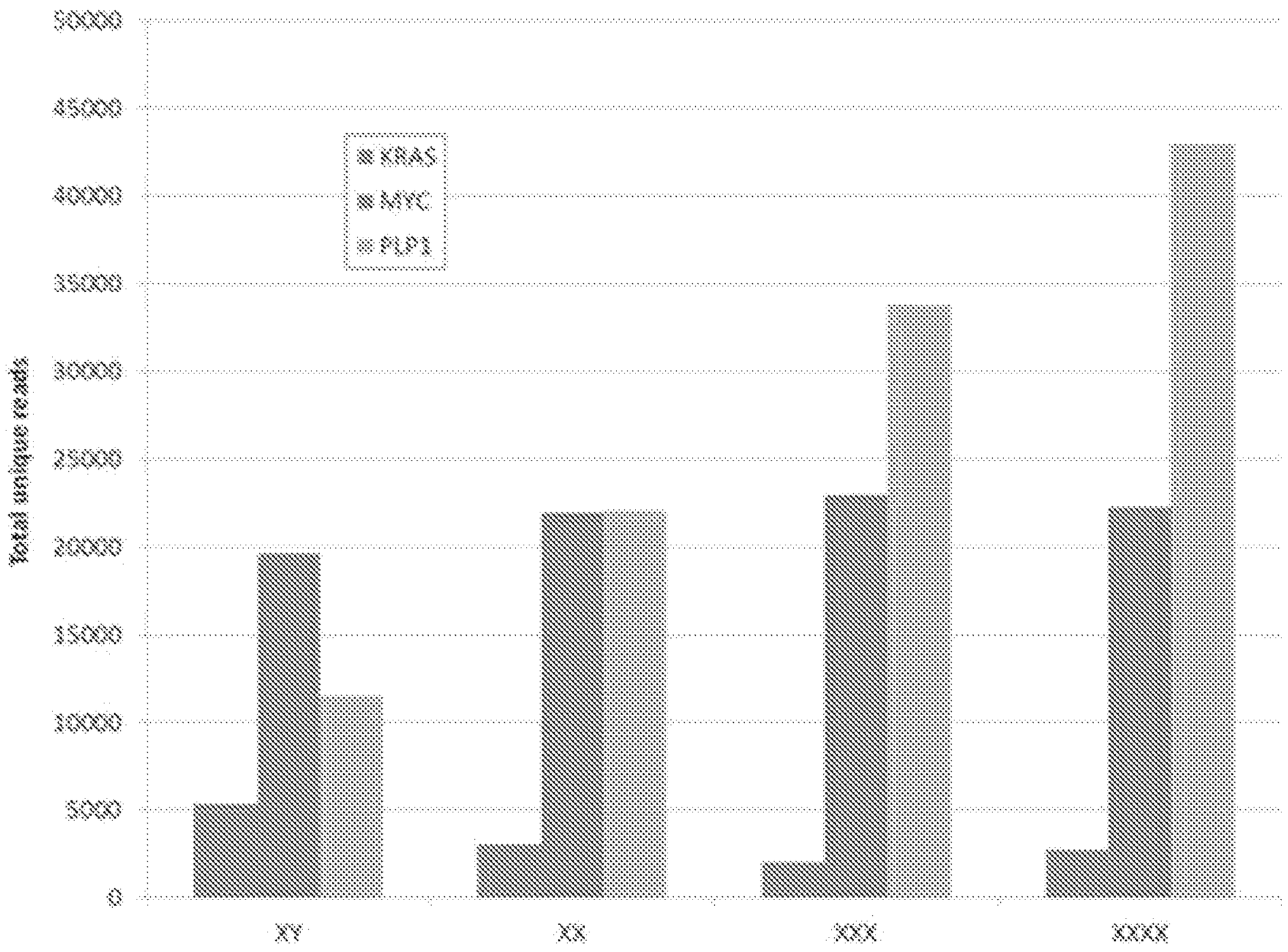

50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Total unique reads
KRAS
MYC
PLP1
XY
XX
XXX
XXXX

FIG. 44



FIG. 45

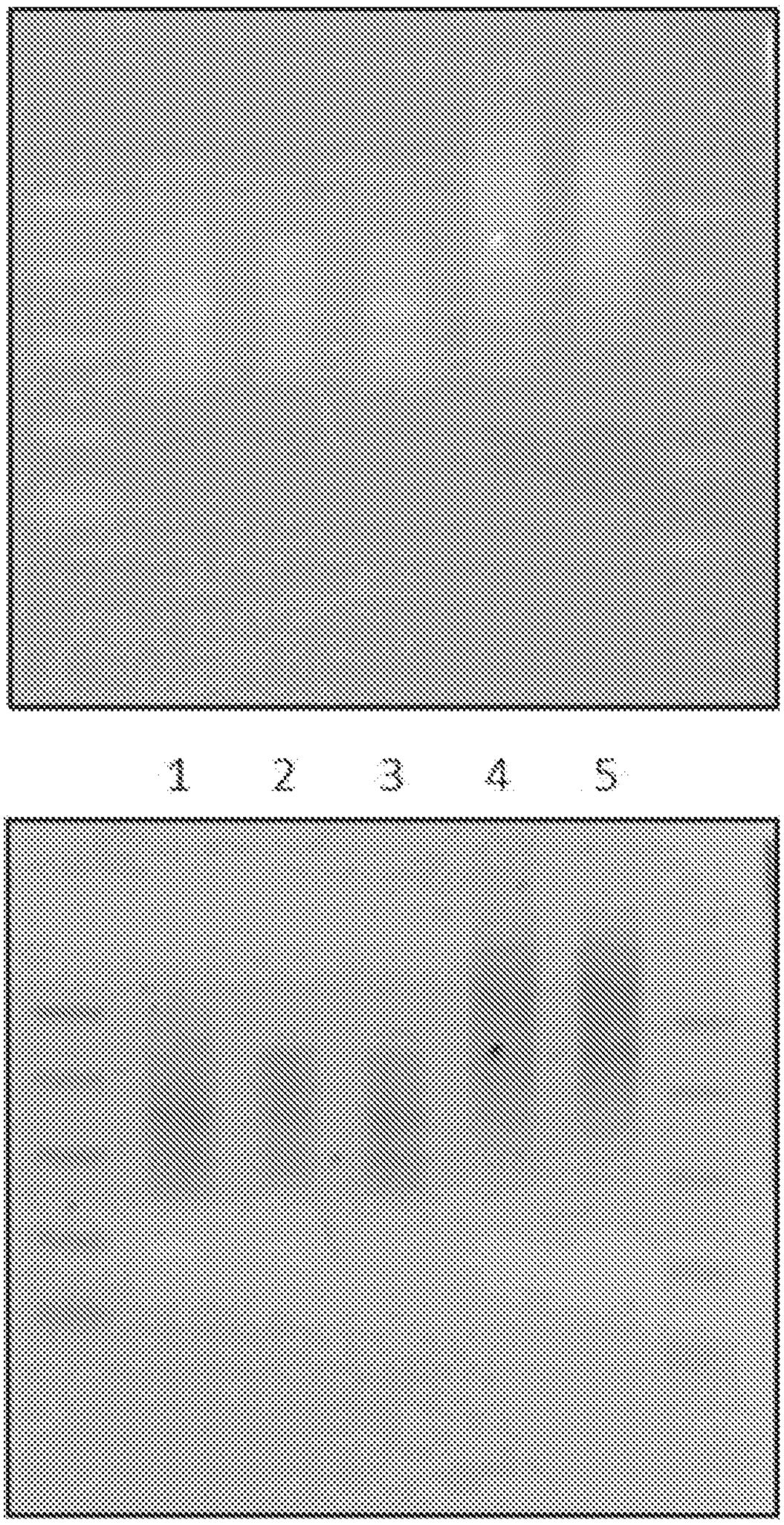


Figure 1. DNA gel of RNA-seq samples.

1. Post capture RNA-seq C+P
2. Pippin prep lane 4
3. Pippin prep lane 5
4. ACA2-20 dT library
5. ACA2_FLFP + exome CAC3_FLRP dT library

FIG. 46

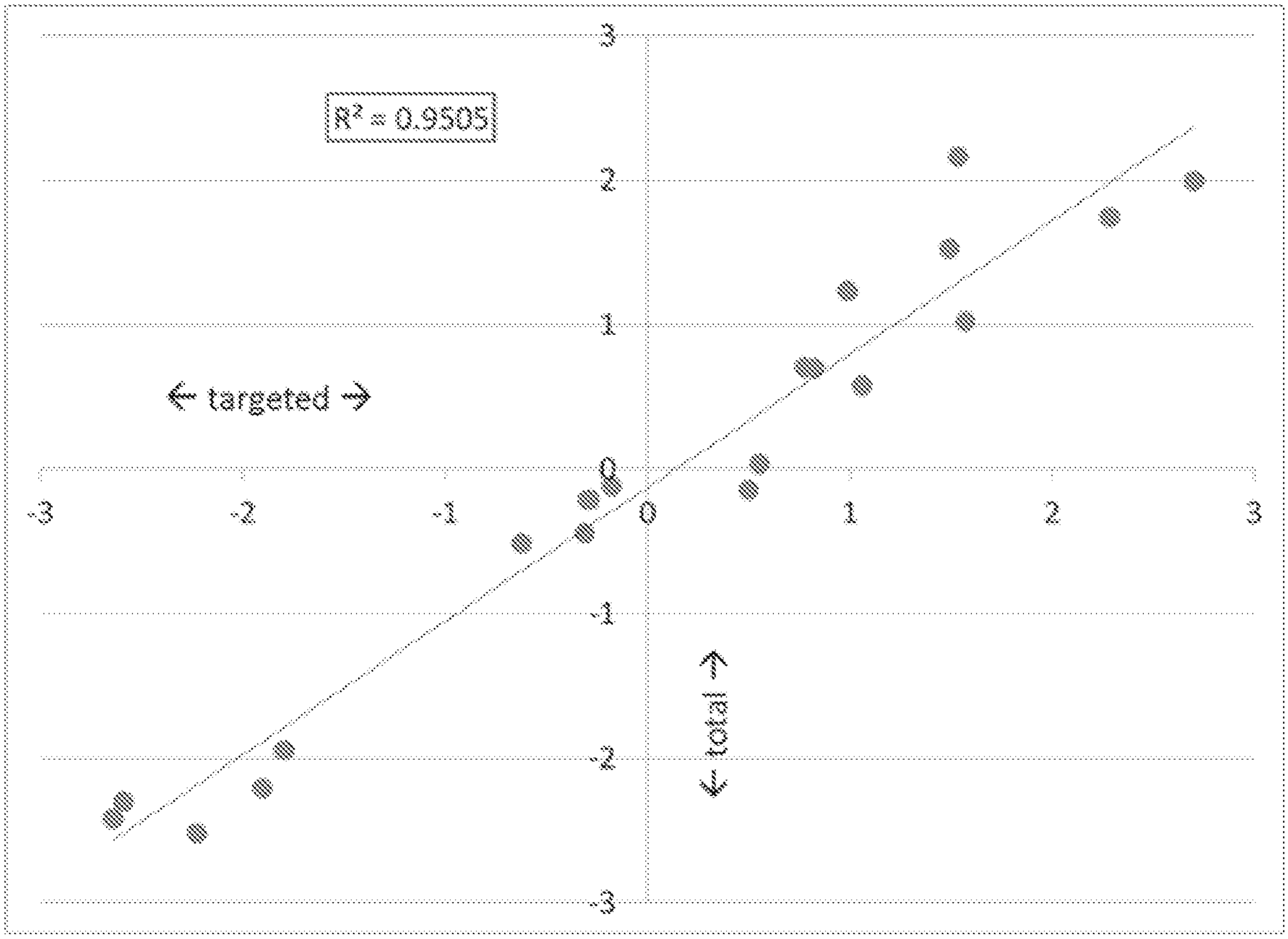


FIG. 47

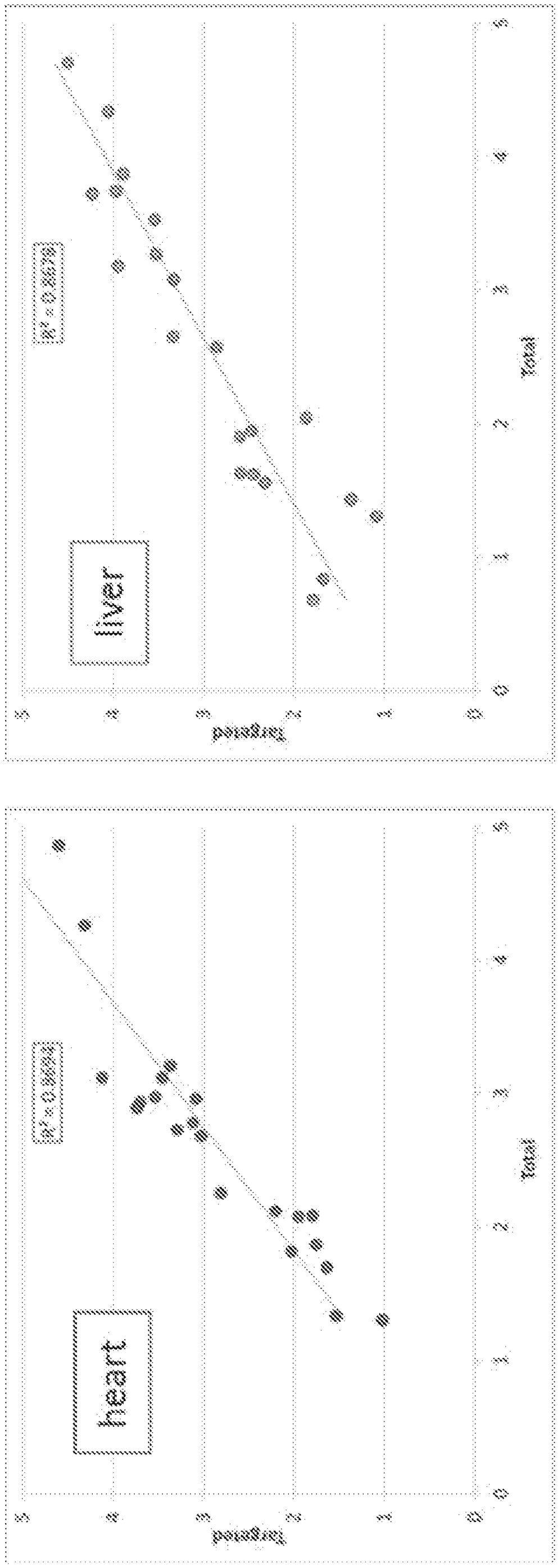
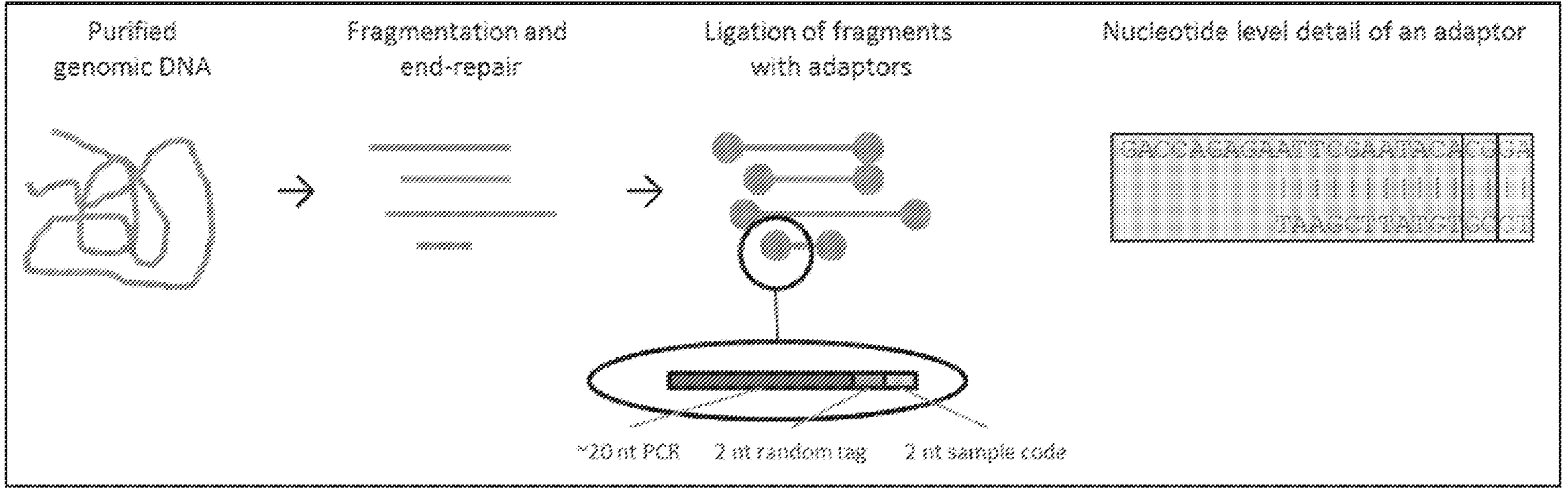

liver
R² = 0.8878
Predicted
Total
heart
R² = 0.8884
Predicted
Total

FIG. 48

FIG. 1


Purified genomic DNA
Fragmentation and end-repair
Ligation of fragments with adaptors
Nucleotide level detail of an adaptor
~20 nt PCR
2 nt random tag
2 nt sample code