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(54) **Title:** APTAMER DETECTION TECHNIQUES

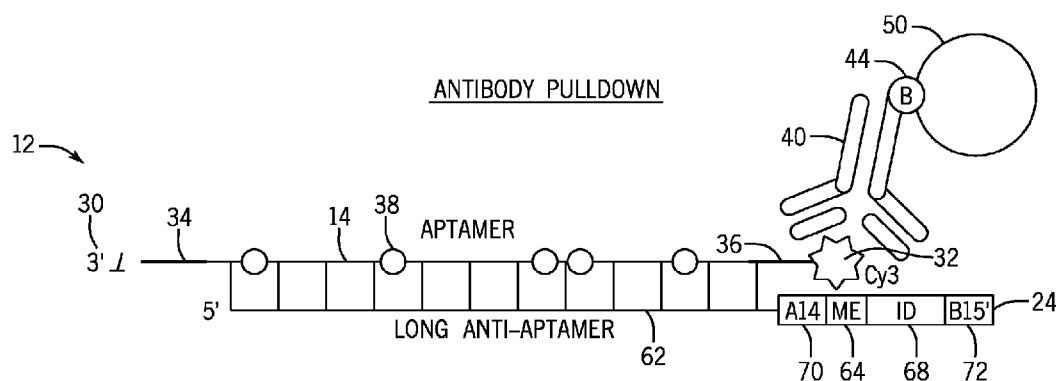


FIG. 1

(57) **Abstract:** Aptamer detection techniques are provided that facilitate sequencing library preparation. In embodiments, reporter probes complementary to aptamers may be used to generate amplification products to form a sequencing library. In embodiments, the aptamer may be used as part of a library preparation.

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- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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APTAMER DETECTION TECHNIQUES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to and the benefit of U.S. Provisional Application No. 63/535,976 filed August 31, 2023, the disclosure of which is hereby incorporated by reference in its entirety herein.

REFERENCE TO ELECTRONIC SEQUENCE LISTING

[0002] The application contains a Sequence Listing which has been submitted electronically in .XML format and is hereby incorporated by reference in its entirety. Said .XML copy, created on August 21, 2024, is named "ILUM_0137PCT.xml" and is 27,646 bytes in size. The sequence listing contained in this .XML file is part of the specification and is hereby incorporated by reference herein in its entirety.

BACKGROUND

[0003] The disclosed technology relates generally to aptamer detection and/or identification techniques. In particular, the technology disclosed relates to nucleic acid sequencing library preparation for direct or indirect aptamer detection in conjunction with an aptamer-based assay.

[0004] The subject matter discussed in this section should not be assumed to be prior art merely as a result of its mention in this section. Similarly, a problem mentioned in this section or associated with the subject matter provided as background should not be assumed to have been previously recognized in the prior art. The subject matter in this section merely represents different approaches, which in and of themselves can also correspond to implementations of the claimed technology.

[0005] Protein expression patterns help define a cell's identity and state. RNA transcripts are often used as a surrogate for protein expression, but the relationship between abundance of

proteins and mRNA is not one-to-one. There are differences caused by regulation of posttranscriptional, translational and protein degradation. Therefore, direct nucleic acid sequencing of RNA transcripts may not provide an accurate estimation of protein expression.

[0006] Aptamers are nucleic acids that bind to molecular targets, such as proteins, with high affinity and specificity. Advancements in aptamer selection and design include Systematic Evolution of Ligands by EXponential enrichment (SELEX). In SELEX, high affinity nucleic acids for different analytes of interest can be isolated from a combinatorial library, permitting high throughput characterization of aptamer-target binding and multiplexed assays for analytes in a complex biological sample. Upon aptamer binding to an analyte target, the binding event can be detected to characterize the presence and concentration of various analytes in the biological sample. However, direct detection of the aptamer molecule is challenging.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] These and other features, aspects, and advantages of the disclosed embodiments will become better understood when the following detailed description is read with reference to the accompanying drawings in which like characters represent like parts throughout the drawings, wherein:

[0008] FIG. 1 is a schematic illustration of an example bi-molecular complex for aptamer detection, according to an embodiment;

[0009] FIG. 2 is a schematic illustration of an example bi-molecular complex for aptamer detection, according to an embodiment;

[0010] FIG. 3 is a schematic illustration of an example aptamer modification used for aptamer detection, according to an embodiment;

[0011] FIG. 4 is a schematic illustration of an example aptamer modification used for aptamer detection, according to an embodiment;

[0012] FIG. 5 shows example click chemistry techniques for the aptamer modification of FIG. 4, according to an embodiment;

[0013] FIG. 6 is a schematic illustration of an example aptamer modification used for aptamer detection, according to an embodiment;

[0014] FIG. 7 shows an example reporter probe labeling technique for aptamer detection, according to an embodiment;

[0015] FIG. 8 shows an example reporter probe labeling technique for aptamer detection, according to an embodiment;

[0016] FIG. 9 shows an example reporter probe adapter incorporation technique, according to an embodiment;

[0017] FIG. 10 shows an example reporter probe adapter incorporation technique, according to an embodiment;

[0018] FIG. 11 shows an example reporter probe adapter incorporation technique, according to an embodiment;

[0019] FIG. 12 shows example aptamer modification technique, according to an embodiment;

[0020] FIG. 13 shows example aptamer modification technique, according to an embodiment;

[0021] FIG. 14 shows an example reporter probe extension technique using an oligo-modified nucleotide analogue, according to an embodiment;

[0022] FIG. 15 shows an example restriction enzyme lock technique using an oligo-modified nucleotide analogue, according to an embodiment;

[0023] FIG. 16 shows an example concatenation technique, according to an embodiment;

[0024] FIG. 17 shows an example concatenation technique, according to an embodiment;

[0025] FIG. 18 shows an example concatenation technique, according to an embodiment;

[0026] FIG. 19 shows an example shows an example concatenation technique, according to an embodiment;

[0027] FIG. 20 shows an example splint ligation technique, according to an embodiment;

[0028] FIG. 21 shows an example splint ligation technique, according to an embodiment;

[0029] FIG. 22 shows an example splint ligation technique, according to an embodiment;

[0030] FIG. 23 shows an example aptamer library preparation technique, according to an embodiment;

[0031] FIG. 24 shows an example aptamer library preparation technique, according to an embodiment;

[0032] FIG. 25 shows an example aptamer library preparation technique, according to an embodiment;

[0033] FIG. 26 shows an example reporter padlock probe technique, according to an embodiment;

[0034] FIG. 27 shows an example padlock extension pullout, according to an embodiment;

[0035] FIG. 28 shows an example reporter probe ligation technique, according to an embodiment;

[0036] FIG. 29 shows a protein-binding sensor technique, according to an embodiment;

[0037] FIG. 30 shows a protein-binding sensor technique, according to an embodiment;

[0038] FIG. 31 shows a protein-binding sensor technique, according to an embodiment;

[0039] FIG. 32 shows a protein-binding sensor technique, according to an embodiment;

[0040] FIG. 33 shows a dynamic range compression technique, according to an embodiment;

[0041] FIG. 34 is a block diagram of a sequencing device configured to acquire sequencing data, according to an embodiment.

DETAILED DESCRIPTION

[0042] The following discussion is presented to enable any person skilled in the art to make and use the technology disclosed, and is provided in the context of a particular application and its requirements. Various modifications to the disclosed implementations will be readily apparent to those skilled in the art, and the general principles defined herein may be applied to other implementations and applications without departing from the spirit and scope of the technology disclosed. Thus, the technology disclosed is not intended to be limited to the implementations shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein.

[0043] Aptamers are short single stranded nucleic acid molecules (ssDNA or ssRNA) that can bind to their specific target molecules with high affinity. Accordingly, aptamers can be used for multiomic applications, such as proteome characterization of a sample in a high-throughput manner. Aptamer-based assays of samples may yield, at certain stages, aptamer-analyte complexes. Detection of the nucleic acid aptamer from these complexes facilitates detection of analytes in the sample. Therefore, it would be beneficial to streamline aptamer detection using techniques for purification and/or downstream characterization. However, incorporating these techniques into an aptamer detection workflow can be complex. Modification of the aptamer itself to incorporate a purification tag may compromise the binding strength of the aptamer for its analyte. In another example, aptamers may include modified nucleic acids that may interfere with downstream processing steps, such as amplification or ligation.

[0044] Certain aptamer detection workflows may use a tri-molecular assay in which two different probes hybridize to different parts of an aptamer. One of the probes can be a reporter probe that is characterized to indirectly detect the aptamer. The other probe can carry an

affinity tag that in turn is used to pull down the tri-molecular complex of the aptamer and the two probes as part of the detection workflow, permitting separation of the bound reporter probe from unbound probes. In a tri-molecular assay, an excess of the biotinylated probe is used, which can be costly. In addition, different aptamers have different melting temperature (T_m) characteristics. With a relatively low T_m aptamer, there may be insufficient nucleotide length to achieve a sufficient melting temperature relationship between one or both of the probes and the aptamer to avoid nonspecific binding at the reaction temperatures. Further, the two-probe arrangement results in less hybridization availability per probe, and therefore lower T_m s for each probe, because the aptamer is divided between two different probe binding sites. The disclosed embodiments provide improvements to a tri-molecular assay by reducing excess of costly ingredients and/or nonspecific binding. Certain embodiments of the disclosure may relate to modifications of the aptamer for detection workflows whereby the modifications do not significantly impact aptamer-analyte binding.

[0045] It should be understood that the disclosed embodiments may be extended to multiple aptamers in a multiplexed aptamer-based assay in parallel. Further, assay eluate of an aptamer-based assay may include multiple copies of an individual aptamer, which is dependent on the concentration of the target molecule of the aptamer in the assessed sample. The aptamer is a single-stranded nucleic acid having a fixed or substantially fixed nucleic acid sequence. Thus, copies or multiples of the individual aptamer may all share a conserved sequence. Different aptamers, referred to generally as aptamers 14 (see FIG. 1), may have different nucleic acid sequences relative to one another, which facilitates different target specificity for respective different aptamers.

[0046] FIG. 1 shows an example bi-molecular complex 12 that may be used for aptamer detection in which an aptamer 14 is used to pull down a complex including the aptamer 14 and an associated reporter probe 24. Rather than using two different bound probes, as in a tri-molecular assay, only a reporter probe 24 is used. Thus, the reporter probe 24 may be longer, which permits reaction temperatures at which nonspecific binding is reduced.

[0047] The aptamer 14 is an example aptamer that includes an 3' inverted dT end 30 and a 5' label 32, illustrated as a Cy3 label (cyanine3). The aptamer may, in embodiments, include terminal or flanking sequences 34,36 that may not directly contribute to analyte binding. Certain aptamers 14 may include one or more modified nucleotides 38 that form part of the binding sequence.

[0048] As part of the detection workflow, the aptamer 14 is contacted with the reporter probe 24 under conditions that permit hybridization to form the complex 12. The reporter probe 24 hybridizes to the aptamer 14 via a complementary region 62, e.g., an aptamer binding region. The complementary region 62 is unique to each individual aptamer 14 and is based on and complementary to the aptamer sequence such that the complementary region 62 hybridizes to the nucleotide sequence of the aptamer 14. In embodiments, the complementary region 62 is complementary to at least a portion of the aptamer sequence, which may or may not include the flanking sequences 34,36. In embodiments, the complementary region 62 is complementary to at least 20 nucleotides of the aptamer sequence. The length of the complementary region 62 may be selected to achieve a certain minimum T_m . In embodiments, the T_m is at least 42-80°C. In embodiments, the complementary region 62 is 10-120 nucleotides in length. In embodiments, the complementary region 62 is 20-60 nucleotides in length.

[0049] The hybridized aptamer-reporter probe complex 12 can be separated from unbound reporter probes via an antibody pulldown using an anti-label antibody 40, which has binding specificity for the 5' label 32, e.g., an anti-Cy3 antibody. The antibody 40 includes an affinity tag 44, such as a biotin 50, which can be captured by beads 50 having an affinity tag binder, such as streptavidin. In an embodiment, the beads 50 are magnetic beads that can be separated from the reaction solution via magnetic properties of the beads 50. Separation using the beads results in a separated portion that includes the bound bi-molecular complex 12. After separation, the reporter probe 24 can be used to characterize the aptamer 14, e.g., via sequencing. Because the aptamer 14 is targeted by the antibody 40, unbound or uncomplexed reporter probes 24 that are not associated with a tagged aptamer 14 will not be captured by the

beads 50. Thus, the sequences or other detection modality of the reporter probes 24 will only reflect those reporter probes 24 that are bound to aptamers 14, which will in turn reflect the composition of aptamers 14 bound to analytes in the assay.

[0050] The reporter probe 24 may include sequences that are associated with a particular aptamer 14 such that the reporter probe 24 is uniquely identifying for its bound aptamer 14. The reporter probe 24 includes a nonhybridizing region 64 that extends away from the complementary region 62 and that does not hybridize to the aptamer 14. Thus, the sequence of the nonhybridizing region 64 can be selected to avoid substantial complementarity with a sequence of the aptamer 14 or the sequences of other aptamers 14 of the aptamer-based assay. The nonhybridizing region 64 can be used for detection as a proxy for the aptamer 14. Accordingly, the nonhybridizing region 64 can include a bar code or identification sequence 68 that is unique to the individual aptamer 14. Thus, different aptamers 14 are associated with respective different identification sequences 68 that are all different from one another and are uniquely identifying. In an embodiment, uniquely identifying sequences are uniquely identifying while accounting for barcode errors (e.g., a 1-2 nucleotide sequence error) during sequencing. Further, the identification sequence 68 may be designed such that the identification sequence 68 is different from the aptamer sequence. In an embodiment, the identification sequence may be 10-50 nucleotides in length.

[0051] To facilitate detection, the nonhybridizing region 64 can include a first primer region 70 and a second primer region 72 that flank the identification sequence 68 such that amplification of the nonhybridizing region 64 using primers to generate amplification products as generally discussed herein, will amplify the identification sequence 68 to permit detection of the aptamer 14. In an embodiment, the amplification is part of preparation of a sequencing library for sequencing.

[0052] Because the nonhybridizing region 64 is single-stranded, the first primer region 70 can represent a primer binding site that is a reverse complement of a first primer, while the second primer region 72 can correspond to the sequence of a second primer that binds to an amplified strand generated from the first primer. The first primer region 70 and a second primer region

72 may be universal primer regions or may be part of universal adapter sequences as discussed herein.

[0053] FIG. 2 shows an embodiment in which the aptamer 14 is modified by adding a biotin 80 to the 5' end 32. The modification may occur as a workflow step within the assay. That is, during previous steps in which the aptamer-analyte complex is formed, the 5' end 32 may have lost a biotin via cleavage as part of a capture and separation step. In the illustrated example, the 5' end 32 is re-biotinylated. The biotin 80 may be linked to a nucleotide added to the 5' end 32 using ligase (e.g., circ ligase, RNA ligases) in a template-independent manner to tag the 5' end 32.

[0054] The tagged aptamer 14 can be contacted with reporter probes 24 to form the bi-molecular complex 12. The reporter probes 24 may be as generally discussed with respect to FIG. 1, and may include the complementary region 62 which is complementary to the aptamer 14, and/or may be complementary to the flanking sequences 34, 36. The reporter probe 24 may also include the nonhybridizing region 64 having the first primer region 70 and the second primer region 72 that flank the identification sequence 68. Thus, when the bi-molecular complex 12 is formed, the complex 12 can be separated from unbound reporter probes 24 via magnetic beads 50 as discussed with respect to FIG. 1 for characterization via the identification sequence 68. The primer sequences 70, 72 may be used for library preparation to generate amplification products and/or add adapter sequences.

[0055] FIG. 3 shows an aptamer modification example in which the aptamer 14 may be structurally modified to include a disulfide group at the 5' end 32. During the workflow, at the stage of aptamer detection (e.g., prior to or concurrently with contact with the reporter probe 24), the aptamer 14 can be contacted with a reducing agent, such as dithiothreitol (DTT). The reduced sulfhydryl at the 5' end 32 is capable of reactive with thiol-reactive compounds, such as maleimide. In an embodiment, beads 50 carrying maleimide can be used to pull out the bi-molecular complex 12 to separate the bound reporter probe 24 for downstream characterization steps.

[0056] FIG. 4 shows an aptamer modification example in which the aptamer 14 may be structurally modified to include a modified nucleotide 90 at the 5' end 32. During the workflow, at the stage of aptamer detection (e.g., prior to or concurrently with contact with the reporter probe 24), the modified nucleotide can be biotinylated or can be directly linked to a capture bead 50 via click chemistry, and the bead, either bound directly or coupled via affinity reaction with biotin, can be used pull out the bi-molecular complex 12 to separate the bound reporter probe 24 for downstream characterization steps. FIG. 5 is an example of modifications using click chemistry that may be used in conjunction with FIG. 4.

[0057] FIG. 6 shows an aptamer modification example in which one or both of the 3' end 30 or the 5' end 32 of the aptamer 14 can include reactive ends that are biotinylated via splint ligation. FIG. 6 shows an example 3' biotinylated splint 102 and ligated oligonucleotide 100 or a biotinylated 5' splint 110 and ligated oligonucleotide 106. Once biotinylated, the aptamer 14, as part of the bi-molecular complex 12 with the reporter probe 24, can be separated using streptavidin-carrying beads 50.

[0058] FIGS. 7-8 shows examples of a bi-molecular complex 12 in which the reporter probe 24 may be provided untagged and, via template-dependent polymerase extension, biotinylated nucleotides are incorporated at the 3' end of the reporter probe. Thus, the reporter probe 24 at the start of the workflow includes a complementary region 62 that does not hybridize to the entire aptamer 14. Instead, a portion of the aptamer 14 including at least 4-10 nucleotides is left available to serve as a template for extension. Once extended, the reporter probe 24 includes biotin affinity tags that can be captured by streptavidin-linked beads 50. FIG. 7 shows a reporter probe 24 that is relatively longer than a truncated reporter probe 24 of FIG. 8. The truncated reporter probe 24 may be relatively less expensive to manufacture due to shorter lengths having higher manufacturing yields. Additional adapter sequences may be added by subsequent amplification steps.

[0059] Certain disclosed embodiments include amplification techniques, ligation techniques, and/or sequencing techniques and corresponding arrangements that can be used to conform the generated amplification products into inputs for sequencing library preparation or, in

embodiments, into a sequencing library that can be sequenced to generate sequence data of the amplification products. Accordingly, the disclosed embodiments may, in embodiments, provide an advantage of incorporating one or more sequencing library preparation steps into the detection of the aptamer 14. Further, the disclosed embodiments may permit certain steps of sequencing library preparation to be omitted or combined, thus increasing detection efficiency. In embodiments, the disclosed embodiments are also directed to sequencing techniques that permit generation of sequence data from sequence reads of the amplification products.

[0060] FIG. 9 shows an example technique for incorporating adapters onto ends of the reporter probe 24. In one workflow path, the reporter probe 24 includes a 5' adapter sequence 120 carrying an affinity tag, e.g., a biotin. In another workflow path, the 5' adapter sequence 120 may not carry the affinity tag. After formation of the bi-molecular complex 12, the 3' adapter can be added by contacting the bi-molecular complex 12 with degenerate adapters 130 including a degenerate portion 134 and an adapter portion 136. Those degenerate adapters 130 having the complementary degenerate portion 134 to the aptamer 14 will bind the aptamer 14 such that the aptamer 14 acts a splint for splint ligation. Excess probes, e.g., excess reporter probes or degenerate adapter 130, can be digested using a single-stranded exonuclease. The reporter probes 24 having the 5' and 3' adapters can be subjected to additional amplification reactions to add indexes or further adapter sequences. The amplification steps select for 3' adapter incorporation, because no amplification products will be generated for reporter probes 24 without ligated 3' adapters, because the 3' adapters provide a priming site. Thus, reporter probes 24 that are not complexed to aptamers 14 will not have the aptamer 14 serve as a ligation splint for incorporation of the 3' adapter and, therefore, will not generate amplification products that rely on the 3' adapter being present for priming.

[0061] In FIG. 10, the workflows of FIG. 9 can be implemented to add the 3' adapter via splint ligation. However, the reporter probe 24, rather than including a contiguous complementary region 62 (see FIG. 1), includes a discontinuous complementary region 150 with internal hairpins 152. Thus, the reporter probe 24 may be lengthened when in the single-stranded

configuration, and the sequence of the reporter probe 24 will not correspond directly as being a complement to the aptamer 14. As discussed with respect to FIG. 9, the reporter probe 25 may carry the affinity-tagged 5' adapter and the 3' adapter can be added via ligation, and additional adapter or indexing sequences can be added via amplification.

[0062] FIG. 11 shows an example of the reporter probe 24 being a primer for extension using the aptamer 14 as a template. The reporter probe 24 carries a 5' adapter 200. In embodiments, the reporter probe extended strand 202 may be A-tailed. For example, taq polymerase may leave an A overhang 204. Using the A-tail 204, a double-stranded oligonucleotide 210 carrying the 3' adapter 212 can be ligated to the 3' end of the extended strand. The extended strand, carrying both 5' and 3' adapters, can be amplified to additional adapter or indexing sequences.

[0063] FIG. 12 shows an example aptamer end-modification technique that removes protected and/or labeled aptamer ends 30, 32 to permit the aptamer to, after modification, directly enter single-stranded library preparation workflows without use of the reporter probe 24. The ends 30, 32 may be removed via fragmentation of the aptamer 14, e.g., via Adaptive Focused Acoustics (AFA) energy (Covaris) or ssDNA endonuclease. The results of such modification may be fragmented ssDNA 216 of variable sizes. However, size selection of fragments and/or resulting sequence data may compensate for lost information due to fragmentation. FIG. 13 shows an end-removal technique that uses oligonucleotides 220, 222 that are complementary to flanking sequences 34, 36 to create a partially double-stranded aptamer 14. The oligonucleotides may be relatively short, such that most or almost all of the aptamer 14 remains after digestion. In an embodiment each of the oligonucleotides 220, 222 may be between 5-25 nucleotides in length. The double-stranded ends can be digested using a double-stranded exonuclease such as Lambda or T7 to yield a modified truncated aptamer 14. The fragmented or truncated aptamers 14 may be processed into a sequencing library using single-stranded library preparation techniques to incorporate a sequencing adapter onto one or both aptamer ends. Techniques for single-stranded library preparation may be as disclosed in U.S. Patent Publication No. 20220348906, entitled "Methods and compositions for analyzing nucleic

acid”, which is hereby incorporated by reference in its entirety. In addition or alternatively, single-stranded library preparation may employ techniques as in the xGen™ ssDNA & Low-Input DNA Library Preparation Kit.

[0064] FIG. 14 shows a schematic illustration of a relatively shorter reporter probe 24 that hybridizes, via the complementary region 62, to a partial sequence of the aptamer 14. In the illustrated example, the complementary region 62 is 25 nucleotides. However, longer or shorter complementary regions 62 are contemplated. The reporter probe 24 includes a nonhybridizing region at the 5' end having an adapter sequence 250, shown by way of example as ME' B15'. Thus, the reporter probe 24 carries the 5' adapter. The reporter probe 3' end can be extended via polymerase incorporation of an oligo-modified nucleotide analogue 252. An oligo-modified nucleotide analogue may include a modified nucleotide 254 that includes, e.g., at a 1' position of the ribose, an oligonucleotide adapter 260. The oligonucleotide adapter 260 may have a reactive 3' end. After incorporation of the modified nucleotide 254, the 3' end of the oligonucleotide adapter 260 serves as the extension point. Thus, in an embodiment, the 3' position of the modified nucleotide may be blocked to prevent extension directly from the modified nucleotide. After incorporation, the reporter probe 24 is extended with the 3' adapter sequence 262 and the index sequence 264 of the oligonucleotide adapter 260 to yield an extended reporter probe 280. In an embodiment, the index sequence 264 may provide a sample-specific index. Oligo-modified nucleotide analogues may be as disclosed in WO2022251510 (PCT/US2022/031150), which is incorporated by reference in its entirety herein.

[0065] The use of the oligo-modified nucleotide analogue may permit a shorter complementary region 62 to be used, which may be less expensive to manufacture. Further, the oligo-modified nucleotide analogue is only added when the reporter probe 24 is bound to the aptamer 14. Thus, the presence of amplified products using the 3' adapter sequence 262 and the 5' adapter sequence 250 as priming sites is a result of binding. Amplified products are generated by a polymerase that adds across a scar or lesion at the site of the modified nucleotide 254. Thus, a lower fidelity polymerase may more efficiently extend across the

lesion. However, to maintain sufficient information and fidelity for aptamer identification, a polymerase blend including both high and low fidelity polymerases may be provided.

[0066] FIG. 15 is a schematic illustration of a reporter probe 24 that includes a restriction enzyme lock that, when in a configuration bound to the aptamer 14 is in an inoperable or open configuration but, when no corresponding aptamer 14 is present, is assembled in a closed configuration that permits digestion.

[0067] As illustrated, when bound to the aptamer 14, the reporter probe 24 is hybridized across the complementary region 62. This holds complementary portions 300, 302 of a restriction site in a physically separate state. If no corresponding aptamer 14 is present, the complementary portions 300, 302 anneal to create an assembled restriction site 306, such that a specific restriction enzyme is capable of cutting at the restriction site 306. In the illustrated example, contacting the reaction mixture with the restriction enzyme after permitting reporter probe hybridization will cut at the restriction site 306 to separate adapters 70, 72. Thus, at downstream amplification steps, no amplification across cut fragments is possible. However, the bound reporter probes 24 can be amplified across the adapters 70, 72 to generate amplification products that include the identification sequence 68. One complementary portion 302 of the restriction site 306 and the adapters 70, 72 and the identification sequence 68 are present on the nonhybridizing region 64. The other complementary portion 300 may extend from the other end of the reporter probe 24 in a second nonhybridizing region. In an embodiment, the reporter probe 24 is provided as a circular probe such that the nonhybridizing region 64 is a loop.

[0068] To avoid cross-binding between complementary portions 300, 302 bound to different reporter probes 24, the pairing may extend into the identification sequence 68. Further, methylated blockers can be provided. To avoid any existing cut sites within the aptamer sequence when bound to the reporter probe 24, the complementary region 62 may be methylated to block cutting. Cut fragments can be digested using an exonuclease. The restriction enzyme may be heat-deactivated after cutting.

[0069] FIG. 16 is a schematic illustration of a technique for concatenating or assembling different reporter probes 24 into a single fragment. As disclosed herein, the reporter probes 24 may be sequenced as part of an aptamer detection workflow. A sequence of the identification sequence 68 can be correlated to its uniquely identified aptamer 14. Because a typical sequencing read length can be 50-300bases, depending on the sequencing technique, sequencing reporter probes 24 having total lengths under 100 bases may not fully take advantage of available sequencing read length and, therefore, may be less efficient. Further, some sequencing techniques may operate more efficiently with longer fragments. An advantage of the illustrated approach is that, rather than having separate sequence reads for a large number of short reporter probes 24, identification sequences 68 of different reporter probes 24 can be assembled together into a single fragment that can be sequenced, thus achieving the same information using fewer sequenced fragments. The order and particular assembly of the identification sequences 68 may be generally random, because the presence of the identification sequence 68 within a multi-sequence fragment is sufficiently identifying for its aptamer 14.

[0070] The illustrated example provides a four unit assembled fragment using three different linkers. However, other lengths and unit combinations are also contemplated, such as 5-15 units. In addition, while the illustrated example is a Gibson one-step assembly, two or more step assemblies are also contemplated. Further, while the illustrated embodiment relates to reporter probe assembly, in certain embodiments, the assembly may use double-stranded fragments including the aptamer sequence.

[0071] As illustrated, at a first step of the workflow, aptamer-reporter probe complexes 12 are separated from unbound reporter probes 24, e.g., using affinity tag binder-carrying beads 50 or by other separation techniques discussed herein. The separated complexes are subjected to cleavage conditions that cleave the nonhybridizing region 64 from the complementary region 62, yielding single-stranded cleaved nonhybridizing regions 64 representing the pool of reporter probes 24 that formed complexes. For example, a U-cleavage site 320 may be present within the reporter probes 24. In other embodiments, the complementary region 62 is removed

via exonuclease digestion of double-stranded nucleic acids. In an embodiment, the aptamer 14 may be polymerase-extended to yield a fully-double stranded fragment with the nonhybridizing region 64 used for downstream one-step assembly steps.

[0072] The reporter probes 24 are designed such that, within the population of reporter probes, the sequences flanking the identification sequences 68 are selected from a set of linkers. In the illustrated example, a first identification sequence 68a is flanked by an adapter sequence 72 and a first linker 330, a second identification sequence 68b is flanked by the first linker 330 and a second linker 332, a third identification sequence 68c is flanked by the second linker 332 and a third linker 334, and a fourth identification sequence 68d is flanked by the third linker 334 and an adapter sequence 70. Each linker may be distinguishable from the other linkers in the set and the adapters 70, 72. In this manner, the total unit number of the assembled fragment is four units. However, using more or fewer linkers can change the total unit number. The reporter probes 24 are designed such that appropriate subsets of the probes 24 have the appropriate linkers for assembly, and dummy linkers may be provided for dynamic range compression as discussed herein. In addition, multiple unique linker sets may be used within a sample to create different assemblies. While the illustrated example is shown with reporter probes 24, in embodiments, the aptamer 14 may carry the adapter sequences 70, 72 and linkers.

[0073] At a next step in the workflow, primers specific for and/or complementary to the adapter sequences 70, 72 and linkers are contacted with the single-stranded nonhybridizing regions 64 to generate double-stranded fragments 340 for use in the Gibson assembly. Any undesired primer binding towards the 5' end of the nonhybridizing regions 64 can be displaced by temperature or polymerase extension. The double-stranded fragments 340 are then contacted with an assembly reaction mixture that may include a 5' to 3' exonuclease, a polymerase, and a ligase. In an embodiment, the enzymes may be T5 exonuclease, Phusion polymerase and Taq ligase. The primers specific for the adapters 70, 72 can contain phosphorothioate-modified 3' ends, which protect the adapters from 3' exonuclease chew back activity during assembly. The assembly reaction mixture, after contact with the double-stranded fragments 340, generates a four unit fragment 344 that includes four different

identification sequences 68a, 68b, 68c, 68d. It should be understood that, in embodiments, the four identification sequences 68 may be different identification sequences 68 or may contain repeated same identification sequences 68, depending on the particular design of the reporter probes 24 and bridging linkers. The four unit fragment 344 can be indexed via amplification to generate an indexed four unit fragment 346 that can be sequenced.

[0074] FIG. 17 is an arrangement in which the identification sequences 68 are concatenated or assembled using splint ligation. The reporter probes 24 may be eluted and, in embodiments, cleaved at cleavage sites 320 to generate single-stranded nonhybridizing regions 64. Complementary splints 350 across the linkers can be used in a splint ligation reaction to assemble a concatenated strand 352. The concatenated strand 352 can be used as a template for amplification to generate a double-stranded fragment 354 with concatenated identification sequences 68a, 68b, 68c, 68d. As discussed, additional amplification steps may be used to index the double-stranded fragment 354.

[0075] FIG. 18 is an arrangement in which the nonhybridizing regions 64 of the reporter probes 24 are designed to be hybridize to one another at their ends in an extension-ligation reaction. At the start of the workflow, the reporter probes 24 are in an arrangement with blocking oligonucleotides 360 complementary to the linkers. This is because, the linkers in the arrangement of FIG. 18 are self-annealing to permit assembly at the extension-ligation step. However, linker self-annealing during aptamer hybridization may interfere with aptamer hybridization. After cleavage to release the nonhybridizing regions 64, the blockers 360 can be removed (e.g., via denaturing and, in embodiments, exonuclease digestion to reduce competition during assembly). Self-binding of the linkers and extension-ligation results in a double-stranded fragment 364, which may be indexed in a one or two step amplification process, as discussed herein.

[0076] FIG. 19 is an aptamer concatenation technique in which single-stranded aptamers 14 are converted to double-stranded aptamer fragments 380 using random primer extension from randomers 382. If the extension is tailed, the double-stranded aptamer fragments 380 are blunted and then ligated to one another in a blunt end ligation to create an assembled double-

stranded fragment 386 with multiple aptamers 14. In an embodiment, the assembled double-stranded fragment 386 can be used as template for a double-stranded library preparation technique. In an embodiment, the assembled double-stranded fragment 386 can be tagged or otherwise indexed with, for example a sample-specific index. The sequencing results can be aligned to an aptamer reference to count the aptamers present.

[0077] The concatenation techniques of FIGS. 16-19 are discussed in the context of aptamers. However, the generation of concatenated double-stranded fragments may be applied to other oligonucleotides. For example, a concatenated double-stranded fragment may be generated from multiple different sequencing indexes, barcodes or UMIs. In another embodiment, the disclosed concatenation techniques may be used for sequencing other short fragments to generate longer fragments for more efficient sequencing.

[0078] FIGS. 20-22 show different techniques for generating an adapterized strand using splint ligation. In FIG. 20, the aptamer includes an extending sequence 400 that carries both a first adapter 401 and a first partial identification sequence 402. The reporter probe 24 carries a second adapter 404 and a second partial identification sequence 405. Ligation of the first partial identification sequence 402 and the second partial identification sequence 405 yields a complete identification sequence 68 flanked by the adapters 401, 404 by ligating the aptamer 14 to the reporter probe 24, and may be accomplished using a split 406. FIG. 21 shows an arrangement in which the aptamer 14 is ligated to the reporter probe 24 using two different splints 410, 412 the yields the complete identification sequence 68 flanked by the adapters 401, 404. FIG. 22 is a tri-molecular assay arrangement in which two different probes 414, 416 are ligated to one another via use of splints 420, 422.

[0079] FIGS. 23-25 show different techniques for aptamer library preparation. In FIG. 23, the aptamer 14 includes adapters 430, 432, which can be used as universal primers to generate double-stranded library fragments 434. Different primer groups may be used for dynamic range compression (e.g. high primer sequences, medium primer sequences, and low primer sequences). In FIG. 24, primers 440, 442 are used that are specific for the flanking sequences 34, 36. The primers 440, 442 carry adapters, such that the generated double-stranded library

fragments 444 are adapterized for the appropriate sequencing workflow and, in embodiments, indexed. In FIG. 25, primers 450, 452 are used that are specific for the aptamer sequence. Because the aptamer sequence may include modified nucleotides, the polymerase may be selected to be tolerant of modifications. The primers 450, 452 carry adapters, such that the generated double-stranded library fragments 454 are adapterized for the appropriate sequencing workflow and, in embodiments, indexed in a subsequent amplification to generate indexed fragments 456.

[0080] FIGS. 26-27 show an example padlock reporter probe arrangement in which the ends of the reporter probe 24 hybridize to the aptamer 14. As shown in FIG. 26, when bound, the reporter probe 24 is sealed with ligase and circularized. When unbound, the reporter probe 24 is linear. Exonucleases targeting linear DNA can be used to remove unbound reporter probes 24. The circularized reporter probe 24 can be amplified to yield double-stranded library fragments 460. FIG. 27 shows an example padlock probe pulldown

[0081] FIG. 28 show an example split reporter probe arrangement in which a first probe 470 bound to the aptamer 14 carries a first adapter and a second probe 472 bound to the aptamer 14 carries a second adapter. Binding of the probes 470, 472 permits the aptamer 14 to act as the ligation splint to generate a ligated reporter probe 476. Amplification using the adapter sequences generates double-stranded library fragments 480 are adapterized for the appropriate sequencing workflow and, in embodiments, indexed in a subsequent amplification to generate indexed fragments. In certain embodiments, the ligated reporter probe 476 can carry an affinity tag for separation using affinity tag binders linked to beads 50.

[0082] FIGS. 29-32 relate to sensor-based sequencing techniques. In an embodiment, the sensor may be a bi-stable polynucleotide sensor as set forth in WO/2019/059961, the disclosure of which is incorporated by reference herein in its entirety. As illustrated, the aptamers 14 target two epitopes of a protein. Upon binding the analyte (protein) the origami structure changes shape as shown. The Sensor ID may be a barcode that may be surrounded by PCR primers, for amplification and sequencing for detection to serve as a reporter. The sensing in the illustrated embodiment may include converting the conformational change in

the sensor, upon protein binding, to a release or creation of a DNA barcode reporter molecule that can be quantitated by sequencing. Essentially the biotin group is obscured from binding by the conformational change. Thus, protein-bound sensors do not bind to a solid support and can be selected from free sensors in this way.

[0083] The bottom of FIG. 29 shows addition or formation of a pullout handle by proximity. The oligonucleotides have complementary regions that would not be high enough T_m to force closure of the sensor structure (weak-lock, as in the link WO/2019/059961). In the presence of the protein, the complementary regions can now hybridize due to being in proximity. As shown, a polymerase can be used to extend from the complementary region and incorporate biotinylated NTPs for subsequent selection on a solid support (e.g. beads). The complementary regions could also create a dsDNA recognition site for a targeting protein with a pullout handle (i.e. dCas9 or Tn5)

[0084] In the embodiments of FIG. 30 indicated as generating the sensor ID by proximity, the sensor ID is generated in the presence of the protein, e.g., via protein binding. By extending the reporter through complementary regions, the reporter can obtain both PCR primers for amplification (blue and red). In the presence of the protein some dsDNA is created as a substrate for Tn5 tagmentation, which would add the red adaptor and make the reporter active. In the presence of the protein, the ends of the single stranded 'halves' of the reporter are in close enough proximity to be ligated by a ssDNA ligase (e.g. circ ligase or RNA ligases).

[0085] In the embodiment shown in the right portion of FIG. 30, the weak complementary regions hybridize in the presence of the protein, and a polymerase extends eluting a bound oligonucleotide sensor. The same concept could be applied to cleave off a reporter with creation of a double stranded DNA restriction endonuclease site. i.e. if the reporter was a ssDNA oligo that was 3' or 5' of the cut site, it could be eluted off after cleavage.

[0086] FIG. 31 shows the cut site concept is continued, but rather than the sensor conformational change driving the elution, the protein binding directly retains the cut sensor-

reporter, whereas free reporters would be eluted. The final step would deliberately denature or remove the protein to elute only reporter that had bound a protein

[0087] FIG. 32 relates to an embodiment in which a clustering element is added to a detection readout. Essentially the sensor ID displacement concept in FIG. 32 is provided, but the protein detection step is carried out on an NGS flow cell. A protein binding event would displace a reporter, which would be captured by a surface primer, which could be clustered and sequenced to identify the sensor and the bound protein.

[0088] FIG. 33 shows a dynamic range compression embodiment. One dynamic range compression technique is using split-dilution. Essentially, high abundant proteins are diluted into a separate assay, whereas low abundant proteins are not diluted as much. In the illustrated example, aptamers that do not contain a photo-cleavage group can be mixed, at a known ratio, with aptamers that contain the active cleavable group. The photocleavage step elutes the bound aptamer-protein complexes before re-catching. By removing the cleavage group for some aptamers, the bound complexes will remain bound to the first beads and the signal will be 'tuned' down by the degree of the 'dummy' or non-photo-cleavage group aptamers. This method could remove the need for dilution groups altogether and streamline the tip, plates and magnetic steps of the assay. Thus, aptamer modification can be used to generate dynamic range compression.

[0089] It should be understood that the disclosed reporter probe and/or aptamer arrangements are by way of example, and any of the disclosed arrangements may be used in conjunction with disclosed techniques. Further, the illustrated adapter sequences are shown by way of example, and other adapters may be incorporated at the 5' and/or 3' end depending on the desired sequencing workflow.

[0090] The reporter probes 24 and/or the aptamers 14 may include adapter sequences. In an embodiment, the nonhybridizing region 64 or regions of the reporter probe 24 can include a minimum sequence of just the primer regions 70, 72 flanking the identification sequence to

introduce an adapter sequence, such as examples of sequences, or their complements, for primer 1 and primer 2 used in Illumina® sequencing preparations, A14, B15, during amplification. In other embodiments, universal capture primer sequences and/or sample index sequences can be incorporated into oligonucleotides generated from the reporter probes 24 or aptamers 14, such as via amplification and/or ligation and extension. Certain arrangements that include indexes may incorporate a custom or bridged primer during sequencing to accommodate the different indexes. Other embodiments may include custom options for sequencing libraries using single reads from surface P5 for example, or for adding dark sequencing by synthesis cycles where common sequences exist in adapter regions.

[0091] The adapter sequences A14-ME, ME, B15-ME, ME', A14, B15, and ME are provided below:

[0092] A14-ME: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' (SEQ ID NO: 1)

[0093] B15-ME: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3' (SEQ ID NO: 2)

[0094] ME': 5'-phos-CTGTCTCTTATACACATCT-3' (SEQ ID NO: 3)

[0095] A14: 5'-TCGTCGGCAGCGTC-3' (SEQ ID NO: 4)

[0096] B15: 5'-GTCTCGTGGGCTCGG-3' (SEQ ID NO: 5)

[0097] ME: AGATGTGTATAAGAGACAG (SEQ ID NO.: 6)

[0098] The primer region or primer binding region can include a region having the sequence of a universal Illumina® capture primer or a region specifically hybridizing with a universal Illumina® capture primer. Universal Illumina® capture primers include, e.g., P5 5'-AATGATACGGCGACCACCGA-3' ((SEQ ID NO: 7)) or P7 (5'-CAAGCAGAAGACGGCATACGA-3' (SEQ ID NO: 8)), or fragments thereof. A region specifically hybridizing with a universal Illumina® capture primer can include, e.g., the

reverse complement sequence of the Illumina® capture primer P5 ("anti-P5": 5'-TCGGTGGTCGCCGTATCATT-3' (SEQ ID NO: 9) or P7 ("anti-P7": 5'-TCGTATGCCGTCTTCTGCTTG-3' (SEQ ID NO:10)), or fragments thereof.

[0099] A conserved primer region can additionally or alternatively include a region having the sequence of an Illumina® sequencing primer, or fragment thereof, or a region specifically hybridizing with an Illumina® sequencing primer, or fragment thereof. Illumina® sequencing primers include, e.g., SBS3 (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' (SEQ ID NO: 11)) or SBS8 (5'-CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3' (SEQ ID NO: 12)). A region specifically hybridizing with an Illumina® sequencing primer, or fragment thereof, can include, e.g., the reverse complement sequence of the Illumina® sequencing primer SBS3 ("anti-SBS3": 5'-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-3' (SEQ ID NO: 13)) or SBS8("anti-SBS8":

5'-AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCG-3' (SEQ ID NO: 14)), or fragments thereof. The incorporation of sequencing primer sequences in the reporter probes may be either directly or via subsequent amplification, ligation, or other sequencing library preparation steps.

[00100] In an embodiment, the disclosed amplification products for an aptamer-based assay may include amplification products of different aptamers 14 that differ from one another based on different identification sequences 68 but that have conserved or universal primer regions 70, 72. In this manner, a single primer set can be used to amplify reporter probes 24 that have variable identification sequences 68. Provided herein are library preparation kits that include primers that are capable of generating the amplification products from the reporter probes 24 or modified aptamers 14 to generate sequencing libraries.

[00101] In an embodiment, the sequencing may use Illumina® NGS primers. The following primers are shown by way of example.

Read 1 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3' (SEQ ID NO: 15)

Read 2 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG (SEQ ID NO: 16)

Paired End Read 1 5' ACACTCTTTCCTACACGACGCTCTTCCGATCT (SEQ ID NO: 17)

Paired End Read 2 5' CGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCT (SEQ ID NO: 18)

Index 1 Read 5' CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG (SEQ ID NO: 19)

Index 2 Read 5' AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC (SEQ ID NO: 20)

It should be understood that the index read primers may be designed to include the particular index sequence associated with a particular sample in an aptamer-based assay. Thus, the index primers may have a nucleotide region, shown as i5 or i7, that varies in sequence between different samples of a multiplexed sample. Other samples in the run can be prepared with primers that include their respective indexes. Accordingly, certain sequence reads may be obtained with universal primers while other sequence reads are obtained with primers or a mix of primers that are specific to indexes of one or more samples in a multiplexed reaction.

[00102] In an embodiment, unique molecular identifiers (UMIs) may be incorporated onto the reporter probes 24, e.g., via ligation. UMIs are short sequences used to uniquely tag each molecule in a sample library to provide error correction and reduce sequencing bias.

[00103] Adapters for sequencing or other assays may be added one or more ligation and/or PCR steps. In embodiments, adapters may be added in stages using step-out amplification. Relatively longer reporters may include integral adapters, but may be more expensive, less pure, and/or, if too long, less feasible to synthesize due to lower yields. Accordingly, in certain embodiments, adapter incorporation via direct or indirect ligation steps may be used to modify a relatively shorter reporter probe 24 that participates in aptamer binding but that does not

include the adapter sequences (e.g., index sequences, primer binding sequences, functional sequences). The disclosed adapter ligation techniques may be used in conjunction with the dynamic range compression workflows as provided herein, e.g., using dummy probes or reporters. Further, in certain embodiments, the disclosed adapter ligation techniques as discussed herein may be PCR-free workflows that avoid thermocycling. In an embodiment, a PCR-free workflow provides an advantage of reduced potential amplicon contamination and removing the requirement for separate areas for pre and post PCR working.

[00104] In certain embodiments, the disclosed techniques may be used in conjunction with dynamic range compression techniques, as generally discussed with respect to PCT/US2023/017778, the disclosure of which is incorporated by reference in its entirety. For example, in certain embodiments, high abundance aptamers 14 may be diluted in the detection results by using nonamplifiable dummy reporter probes mixed with amplifiable reporter probes 24. The dummy reporter probes compete for aptamer binding but do not generate any detectable sequence. In an embodiment, the reporter probes 24 may be part of a mixture with dummy reporter probes in ratios selected based on the abundance of the corresponding aptamer 14. The more dummy reporter probes present relative to reporter probes 24, the higher the level of range compression and the greater the attenuation of the abundance. In one example, the dummy reporter probes may be blocked at a 3' end such that the 3' end is nonextendable. Failure to extend at the 3' end may result in failure to incorporate a 3' adapter, such that the dummy reporter probes are nonamplifiable.

[00105] In one example, (see FIG. 15), where restriction enzymes or restriction endonucleases are provided to cut unbound reporter probes 24, different restriction sites may be used for targets of different abundance. Complementary oligonucleotides to a particular site used for high abundance targets may be provided in the reaction mixture to artificially create annealing conditions that permit cutting. Thus, the complementary oligonucleotides may permit cutting even when the reporter probe 24 is bound to the aptamer 14 to cause dynamic range compression. In another example, the complementary oligonucleotides may include a complementary identification sequence that is a full or partial complement to the

identification sequence 68. Thus, the complementary oligonucleotides can be targeted to particular aptamer targets using the complement of the identification sequence 68.

[00106] In another example, dynamic range compression may be achieved by providing a mix of ligate-able ends and nonligate-able ends (see FIGS. 21-23). The nonligate-able end will result in ligation failure and, therefore, a ligated fragment containing both adapter ends will not be generated. Thus, amplification across the adapter ends will not yield any amplification products.

[00107] FIG. 34 is a schematic diagram of a sequencing device 500 that may be used in conjunction with the disclosed embodiments for acquiring sequencing data of identification sequences and/or index sequences as generally discussed herein. The sequencing device 500 may be implemented according to any sequencing technique, such as those incorporating sequencing-by-synthesis methods described in U.S. Patent Publication Nos. 2007/0166705; 2006/0188901; 2006/0240439; 2006/0281109; 2005/0100900; U.S. Pat. No. 7,057,026; WO 05/065814; WO 06/064199; WO 07/010,251, the disclosures of which are incorporated herein by reference in their entireties. Alternatively, sequencing by ligation techniques may be used in the sequencing device 500. Such techniques use DNA ligase to incorporate oligonucleotides and identify the incorporation of such oligonucleotides and are described in U.S. Pat. No. 6,969,488; U.S. Pat. No. 6,172,218; and U.S. Pat. No. 6,306,597; the disclosures of which are incorporated herein by reference in their entireties. Some embodiments can utilize nanopore sequencing, whereby target nucleic acid strands, or nucleotides exonucleolytically removed from target nucleic acids, pass through a nanopore. As the target nucleic acids or nucleotides pass through the nanopore, each type of base can be identified by measuring fluctuations in the electrical conductance of the pore (U.S. Patent No. 7,001,792; Soni & Meller, *Clin. Chem.* 53, 1996–2001 (2007); Healy, *Nanomed.* 2, 459–481 (2007); and Cockcroft, et al. *J. Am. Chem. Soc.* 130, 818–820 (2008), the disclosures of which are incorporated herein by reference in their entireties). Yet other embodiments include detection of a proton released upon incorporation of a nucleotide into an extension product. For example, sequencing based on detection of released protons can use an electrical detector and associated techniques that are

commercially available from Ion Torrent (Guilford, CT, a Life Technologies subsidiary) or sequencing methods and systems described in US 2009/0026082 A1; US 2009/0127589 A1; US 2010/0137143 A1; or US 2010/0282617 A1, each of which is incorporated herein by reference in its entirety. Particular embodiments can utilize methods involving the real-time monitoring of DNA polymerase activity. Nucleotide incorporations can be detected through fluorescence resonance energy transfer (FRET) interactions between a fluorophore-bearing polymerase and γ -phosphate-labeled nucleotides, or with zeromode waveguides as described, for example, in Levene et al. *Science* 299, 682–686 (2003); Lundquist et al. *Opt. Lett.* 33, 1026–1028 (2008); Korlach et al. *Proc. Natl. Acad. Sci. USA* 105, 1176–1181 (2008), the disclosures of which are incorporated herein by reference in their entireties. Other suitable alternative techniques include, for example, fluorescent in situ sequencing (FISSEQ), and Massively Parallel Signature Sequencing (MPSS). In particular embodiments, the sequencing device 500 may be a HiSeq, MiSeq, or HiScanSQ from Illumina (La Jolla, CA). In other embodiment, the sequencing device 500 may be configured to operate using a CMOS sensor with nanowells fabricated over photodiodes such that DNA deposition is aligned one-to-one with each photodiode.

[00108] The sequencing device 500 may be “one-channel” a detection device, in which only two of four nucleotides are labeled and detectable for any given image. For example, thymine may have a permanent fluorescent label, while adenine uses the same fluorescent label in a detachable form. Guanine may be permanently dark, and cytosine may be initially dark but capable of having a label added during the cycle. Accordingly, each cycle may involve an initial image and a second image in which dye is cleaved from any adenines and added to any cytosines such that only thymine and adenine are detectable in the initial image but only thymine and cytosine are detectable in the second image. Any base that is dark through both images is guanine and any base that is detectable through both images is thymine. A base that is detectable in the first image but not the second is adenine, and a base that is not detectable in the first image but detectable in the second image is cytosine. By combining the information from the initial image and the second image, all four bases are able to be discriminated using one channel.

[00109] In the depicted embodiment, the sequencing device 500 includes a separate sample processing device 502 and an associated computer 504. However, as noted, these may be implemented as a single device. Further, the associated computer 504 may be local to or networked or otherwise in communication with the sample processing device 502. In the depicted embodiment, the biological sample may be loaded into the sample processing device 502 on a sample substrate 510, e.g., a flow cell or slide, that is imaged to generate sequence data. For example, reagents that interact with the biological sample fluoresce at particular wavelengths in response to an excitation beam generated by an imager 512 and thereby return radiation for imaging. For instance, the fluorescent components may be generated by fluorescently tagged nucleic acids that hybridize to complementary molecules of the components or to fluorescently tagged nucleotides that are incorporated into an oligonucleotide using a polymerase. As will be appreciated by those skilled in the art, the wavelength at which the dyes of the sample are excited and the wavelength at which they fluoresce will depend upon the absorption and emission spectra of the specific dyes. Such returned radiation may propagate back through the directing optics. This retrobeam may generally be directed toward detection optics of the imager 512.

[00110] The imager detection optics may be based upon any suitable technology, and may be, for example, a charged coupled device (CCD) sensor that generates pixilated image data based upon photons impacting locations in the device. However, it will be understood that any of a variety of other detectors may also be used including, but not limited to, a detector array configured for time delay integration (TDI) operation, a complementary metal oxide semiconductor (CMOS) detector, an avalanche photodiode (APD) detector, a Geiger-mode photon counter, or any other suitable detector. TDI mode detection can be coupled with line scanning as described in U.S. Patent No. 7,329,860, which is incorporated herein by reference. Other useful detectors are described, for example, in the references provided previously herein in the context of various nucleic acid sequencing methodologies.

[00111] The imager 512 may be under processor control, e.g., via a processor 514, and the sample receiving device 502 may also include I/O controls 516, an internal bus 518, non-

volatile memory 520, RAM 522 and any other memory structure such that the memory is capable of storing executable instructions, and other suitable hardware components that may be similar to those described with regard to FIG. 34. Further, the associated computer 504 may also include a processor 524, I/O controls 526, communications circuitry 527, and a memory architecture including RAM 528 and non-volatile memory 530, such that the memory architecture is capable of storing executable instructions 532. The hardware components may be linked by an internal bus, which may also link to the display 534. In embodiments in which the sequencing device 500 is implemented as an all-in-one device, certain redundant hardware elements may be eliminated.

[00112] The processor 514, 524 may be programmed to assign individual sequencing reads to a sample based on the associated index sequence or sequences according to the techniques provided herein. In particular embodiments, based on the image data acquired by the imager 512, the sequencing device 500 may be configured to generate sequencing data that includes base calls for each base of a sequencing read. Further, based on the image data, even for sequencing reads that are performed in series, the individual reads may be linked to the same location via the image data and, therefore, to the same template strand. In this manner, index sequencing reads may be associated with a sequencing read of an insert sequence before being assigned to a sample of origin. The processor 514, 524 may also be programmed to perform downstream analysis on the sequences corresponding to the inserts for a particular sample subsequent to assignment of sequencing reads to the sample.

[00113] In certain embodiments, the I/O controls 516, 526 may be configured to receive user inputs that automatically select sequencing parameters based on the reporter probes 24 and the associated sequence library preparation techniques. For example, in cases where custom primers or dark cycles are incorporated into the sequencing run, the sequencing device can select from preprogrammed operating instructions and/or receive user inputs to cause the sequencing device to operate according to the desired sequence parameters. In an embodiment, the user input may be a selection of a sequence library preparation kit or reading a barcode or identifier of a sequence library preparation kit.

[00114] In embodiments of the disclosed techniques, aptamer detection may be based on a presence of the uniquely identifying identification sequence 68 for an individual aptamer in sequencing data generated by the sequencing device 500. Accordingly, in an embodiment, the sequencing device 500 may perform analysis of sequence reads to identify one or more identification sequences 68 for a panel of aptamers. Based on the identified aptamers, a notification or report of positive aptamer identification may be generated. In an embodiment, the notification is provided on the display 534 or communicated via the communications circuitry 527 to a remote device or a cloud server.

[00115] As used herein, an aptamer may refer to a non-naturally occurring nucleic acid that has specific binding affinity for a target molecule. The binding of the aptamer to the target molecule can result in catalytically changing the target molecule, reacting with the target molecule in a way that modifies or alters the target molecule or the functional activity of the target molecule, covalently attaching to the target molecule (as in a suicide inhibitor), and facilitating the reaction between the target molecule and another molecule. In one embodiment, the target molecule is a three dimensional chemical structure, other than a polynucleotide, that binds to the aptamer through a mechanism which is predominantly independent of Watson/Crick base pairing or triple helix binding. In an embodiment, the aptamer is not a nucleic acid having the known physiological function of being bound by the target molecule.

[00116] Aptamers include nucleic acids that are identified from a candidate mixture of nucleic acids. A specific binding affinity of an aptamer for its target may refer to aptamer binding to its target generally with a much higher degree of affinity than it binds to other, non-target, components in a mixture or sample. Different aptamers may have either the same number or a different number of nucleotides. Aptamers may be DNA or RNA and may be single stranded, double stranded, or contain double stranded regions. The aptamers discussed herein can be used in any diagnostic, imaging, high throughput screening or target validation techniques or procedures or assays for which aptamers, oligonucleotides, antibodies and ligands, without limitation can be used.

[00117] Aptamers as disclosed herein may be used in aptamer-based assays, such as those disclosed in U.S. Pat. Nos. 7,855,054 and 7,964,356 and U.S. Publication Nos. US/2011/0136099 and US/2012/0115752. In one example, a panel of aptamers to different target molecules is provided attached to a solid support. The attachment of the aptamers to the solid support is accomplished by contacting a first solid support with the aptamer/s and allowing the releasable first tag included on the aptamer to associate, either directly or indirectly, with an appropriate first capture agent that is attached to or part of the first solid support. A test sample is then prepared and contacted with the immobilized aptamers that have a specific affinity for their respective target molecules, which may or may not be present in the sample. If the test sample contains the target molecule(s), an aptamer-target affinity complex will form in the mixture with the test sample. In addition to aptamer-target affinity complexes, uncomplexed aptamer will also be attached to the first solid support. The aptamer-target affinity complex and uncomplexed aptamer that has associated with the probe on the solid support is then partitioned from the remainder of the mixture, thereby removing free target and all other uncomplexed matter in the test sample (sample matrix); i.e., components of the mixture not associated with the first solid support. This partitioning step is referred to herein as the Catch-1 partition (see definition below). Following partitioning the aptamer-target affinity complex, along with any uncomplexed aptamer, is released from the first solid support using a method appropriate to the particular releasable first tag being employed.

[00118] In one embodiment, aptamer-target affinity complexes bound to the solid support are treated with an agent that introduces a second tag to the target molecule component of the aptamer-target affinity complexes. In one embodiment, the target is a protein or a peptide, and the target is biotinylated by treating it with NHS-PEO4-biotin. The second tag introduced to the target molecule may be the same as or different from the aptamer capture tag. If the second tag is the same as the first tag, or the aptamer capture tag, free capture sites on the first solid support may be blocked prior to the initiation of this tagging step. In this exemplary embodiment, the first solid support is washed with free biotin prior to the initiation of target tagging. Tagging methods, and in particular, tagging of targets such as peptides and proteins are described in U.S. Pat. No. 7,855,054.

[00119] Partitioning is completed by releasing of uncomplexed aptamers and aptamer-target affinity complexes from the first solid support. In one embodiment, the first releasable tag is a photocleavable moiety that is cleaved by irradiation with a UV lamp under conditions that cleave $\geq 90\%$ of the first releasable tag. In other embodiments, the release is accomplished by the method appropriate for the selected releasable moiety in the first releasable tag. Aptamer-target affinity complexes may be eluted and collected for further use in the assay or may be contacted to another solid support to conduct the remaining steps of the assay.

[00120] In one embodiment, a second partition is performed (referred to herein as the Catch-2 partition, see definition below) to remove free aptamer. As described above, in one embodiment, a second tag used in the Catch-2 partition may be added to the target while the aptamer-target affinity complex is still in contact with the solid support used in the Catch-0 capture. In other embodiments, the second tag may be added to the target at another point in the assay prior to initiation of Catch-2 partitioning. The mixture is contacted with a solid support, the solid support having a capture element (second) adhered to its surface which is capable of binding to the target capture tag (second tag), preferably with high affinity and specificity. In one embodiment, the solid support is magnetic beads (such as DynaBeads MyOne Streptavidin C1) contained within a well of a microtiter plate and the capture element (second capture element) is streptavidin. The magnetic beads provide a convenient method for the separation of partitioned components of the mixture. Aptamer-target affinity complexes contained in the mixture are thereby bound to the solid support through the binding interaction of the target (second) capture tag and the second capture element on the second solid support. The aptamer-target affinity complex is then partitioned from the remainder of the mixture, e.g. by washing the support with buffered solutions, including buffers comprising organic solvents including, but not limited to glycerol.

[00121] Aptamers are then selectively eluted from aptamer-target complexes with buffers comprising chaotropic salts from the group including, but not limited to sodium perchlorate, lithium chloride, sodium chloride and magnesium chloride. Aptamers retained on Catch-2 beads by virtue of aptamer/aptamer interaction are not eluted by this treatment.

[00122] In another embodiment, the aptamer released from the Catch-2 partition is detected and optionally quantified by detection methods as discussed herein, such as via next generation sequencing techniques. For example, via amplification and/or sequencing of probes that bind to the eluted aptamers. In certain embodiments, the detection includes detection results that provide relative and/or estimated absolute concentrations of detected aptamers. The detection results may include a notification or output of a positive or negative detection result or a relative concentration or estimated concentration for a particular aptamer ID or a particular target of the aptamer.

[00123] In certain embodiments of the disclosure, the disclosed probes of the probe set 20 can include one or more conserved regions, such as a conserved primer region, e.g., a first conserved primer region and a second conserved primer region. A conserved region is conserved between at least some other probes of the probe set 20 such that the conserved region has an identical or similar nucleotide sequence as compared between the probes. For example, for a given reporter probe 24, all probes 24 can have a same first conserved primer region and a second conserved primer region. In this manner, primers based on the first conserved primer region and the second conserved primer region can be used to amplify any captured probes 24.

[00124] One or more probes as discussed herein may include an identification sequence that can include one or more nucleotide sequences that can be used to identify one or more specific aptamers. The identification sequence can be an artificial sequence. The identification sequence can comprise at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more consecutive nucleotides. In some embodiments, the identification sequence comprises at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more consecutive nucleotides.

[00125] One or more probes as discussed herein may include an affinity tag. Affinity tags can be useful for a variety of applications, for example the bulk separation of target nucleic acids hybridized to hybridization tags. As used herein, the term “affinity tag” and grammatical equivalents can refer to a component of a multi-component complex, wherein the components of the multi-component complex specifically interact with or bind to each other. For example

an affinity tag can include biotin or poly-His that can bind streptavidin or nickel, respectively. Other examples of multiple-component affinity tag complexes are listed, for example, U.S. Patent Application Pub. No. 2012/0208705, U.S. Patent Application Pub. No. 2012/0208724 and Int. Patent Application Pub. No. WO 2012/061832, each of which is incorporated by reference in its entirety.

[00126] The disclosed embodiments provide a different primers and probes. Probes and/or primers of the disclosed embodiments are designed to be complementary to a target sequence (either the target sequence of the sample or to other probe sequences), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by “substantially complementary” herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions.

[00127] A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5-10° C. lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g. 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g. greater than 50 nucleotides).

[00128] In certain embodiments, probe contacting steps may be run under stringency conditions which allows formation of the hybridization complex only in the presence of target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration, pH, organic solvent concentration, etc. The size of the primer nucleic acid may vary, as will be appreciated by those in the art, in general varying from 5 to 500 nucleotides in length. Primers may be between 10 and 100, between 15 and 50, and from 10 to 35 depending on the use and amplification technique.

[00129] The disclosed techniques are directed to dynamic range compression in one or more applications, such as for analysis of an eluate of an aptamer-based assay. The dynamic range compression may include one or more amplification steps that can be part of sequencing library preparation that may oligonucleotide adapters to reporter probes for downstream sequencing. The adapters may be attached to the target polynucleotide in any other suitable manner. In some embodiments, the adapters are introduced in a multi-step process, such as a two-step process, involving ligation of a portion of the adapter to the target polynucleotide having a universal primer sequence. The second step includes extension, for example by PCR amplification, using primers that include a 3' end having a sequence complementary to the attached universal primer sequence and a 5' end that contains other sequences of an adapter. By way of example, such extension may be performed as described in U.S. Pat. No. 8,053,192, which is hereby incorporated by reference in its entirety. Additional extensions may be performed to provide additional sequences to the 5' end of the resulting previously extended polynucleotide.

[00130] In some embodiments, the adapter may be ligated to the reporter probes. Any suitable adapter may be attached to a target polynucleotide, such as a reporter probe, via any suitable process, such as those discussed herein. The adapter can include a library-specific index tag sequence (e.g., i5, i7). The index tag sequence may be attached to the target polynucleotides from each library before the sample is immobilized for sequencing. The index tag is not itself formed by part of the target polynucleotide, but becomes part of the template

for amplification. The index tag may be a synthetic sequence of nucleotides which is added to the target as part of the template preparation step. Accordingly, a library-specific index tag is a nucleic acid sequence tag which is attached to each of the target molecules of a particular library, the presence of which is indicative of or is used to identify the library from which the target molecules were isolated. Preferably, the index tag sequence is 20 nucleotides or less in length. For example, the index tag sequence may be 1-10 nucleotides or 4-6 nucleotides in length. A four nucleotide index tag gives a possibility of multiplexing 256 samples on the same array, a six base index tag enables 4,096 samples to be processed on the same array. The adapters may contain more than one index tag so that the multiplexing possibilities may be increased.

[00131] The adapters may include any other suitable sequence in addition to the index tag sequence. For example, the adapters may include universal extension primer sequences, which are typically located at the 5' or 3' end of the adapter and the resulting polynucleotide for sequencing. The universal extension primer sequences may hybridize to complementary primers bound to a surface of a solid substrate. The complementary primers include a free 3' end from which a polymerase or other suitable enzyme may add nucleotides to extend the sequence using the hybridized library polynucleotide as a template, resulting in a reverse strand of the library polynucleotide being coupled to the solid surface. Such extension may be part of a sequencing run or cluster amplification.

[00132] In some embodiments, the adapters include one or more universal sequencing primer sequences. The universal sequencing primer sequences may bind to sequencing primers to allow sequencing of an index tag sequence, a target sequence, or an index tag sequence and a target sequence. In some embodiments, the disclosed reporter probes, e.g., reporter probe 24, may include a “sequencing adaptor” or “sequencing adaptor site”, that is to say a region that comprises one or more sites that can hybridize to a primer. In some embodiments, a sequence can include at least a first primer site useful for amplification, sequencing, and the like.

[00133] After adapter incorporation, the disclosed reporter probes may be sequenced. In one example, the sequencing may be via Illumina's sequencing-by-synthesis and reversible terminator-based sequencing chemistry. Illumina's sequencing technology relies on the attachment of fragmented genomic DNA to a planar, optically transparent surface on which oligonucleotide anchors are bound. Template DNA is end-repaired to generate 5'-phosphorylated blunt ends, and the polymerase activity of Klenow fragment is used to add a single A base to the 3' end of the blunt phosphorylated DNA fragments. This addition prepares the DNA fragments for ligation to oligonucleotide adapters, which have an overhang of a single T base at their 3' end to increase ligation efficiency. The adapter oligonucleotides are complementary to the flow-cell anchors. Under limiting-dilution conditions, adapter-modified, single-stranded template DNA is added to the flow cell and immobilized by hybridization to the anchors. Attached DNA fragments are extended and bridge amplified to create an ultra-high density sequencing flow cell with hundreds of millions of clusters, each containing ~1,000 copies of the same template. In one embodiment, the randomly fragmented genomic DNA is amplified using PCR before it is subjected to cluster amplification. Alternatively, an amplification-free genomic library preparation is used, and the randomly fragmented genomic DNA is enriched using the cluster amplification alone. The templates are sequenced using a robust four-color DNA sequencing-by-synthesis technology that employs reversible terminators with removable fluorescent dyes. High-sensitivity fluorescence detection is achieved using laser excitation and total internal reflection optics. Sequence are aligned against a truth table or stored correlations between aptamer identity and identification sequences using specially developed data analysis pipeline software.

[00134] This written description uses examples to enable any person skilled in the art to practice the disclosed embodiments, including making and using any devices or systems and performing any incorporated methods. The patentable scope is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal languages of the claims.

CLAIMS

What is claimed is:

1. A method of aptamer detection, comprising:
contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and
detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting an individual aptamer of the plurality of aptamers comprises:
contacting the individual aptamer with a reporter probe to hybridize a complementary region of the reporter probe to a region of the individual aptamer to form an aptamer-reporter probe complex, wherein the reporter probe comprises a nonhybridizing region extending from the complementary region, the nonhybridizing region comprising an identification sequence uniquely identifying for the individual aptamer;
capturing the aptamer-reporter probe complex to separate the aptamer-reporter probe complex from other reporter probes not complexed to aptamers; and
detecting the identification sequence of the captured reporter probe.
2. The method of claim 1, wherein capturing the aptamer-reporter probe complex comprises using an antibody with binding affinity for a label of the aptamer.
3. The method of claim 1, wherein capturing the aptamer-reporter probe complex comprises using an affinity tag capture molecule to capture an affinity tag of the aptamer.
4. The method of claim 3, wherein the affinity tag is biotin and the affinity tag capture molecule is avidin or streptavidin.

5. The method of claim 1, wherein detecting the identification sequence of the captured reporter probe comprises contacting the captured reporter probe with primers to generate an amplification product.
6. The method of claim 5, wherein the primers comprise a first primer that binds to a first primer binding region of the nonhybridizing region and a second primer that binds to a second primer binding region of the nonhybridizing region, wherein the first primer binding region and the second primer binding region flank the identification sequence.
7. The method of claim 6, wherein the first primer comprises a first sequencing primer and the second primer comprises a second sequencing primer such that the amplification product comprises the first sequencing primer and the second sequencing primer.
8. The method of claim 5, wherein detecting the identification sequence of the captured reporter probe comprises sequencing the amplification product.
9. The method of claim 1, wherein the aptamer comprises a sulfhydryl group, and wherein capturing the aptamer-reporter probe complex comprises using a sulfhydryl group binder.
10. The method of claim 1, further comprising modifying the aptamer using click chemistry to couple a capture bead to the aptamer.
11. The method of claim 1, further comprising ligating an oligonucleotide comprising an affinity tag to the aptamer and using the affinity tag to capture the aptamer-reporter probe complex.

12. The method of claim 1, further comprising extending from an end of the hybridized reporter probe to incorporate nucleotides comprising an affinity tag into the extended reporter probe and using the affinity tag to capture the aptamer-reporter probe complex.

13. The method of claim 1, wherein capturing the aptamer-reporter probe complex comprises using a capture bead.

14. The method of claim 13, wherein the capture bead is magnetic.

15. A method of aptamer detection, comprising:

contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and

detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting an individual aptamer of the plurality of aptamers comprises:

contacting the individual aptamer with a reporter probe to hybridize a complementary region of the reporter probe to a region of the individual aptamer to form an aptamer-reporter probe complex, wherein the reporter probe is coupled to an affinity tag;

capturing the aptamer-reporter probe complex using the affinity tag to separate the aptamer-reporter probe complex from other reporter probes not complexed to aptamers; and

detecting the sequence of the captured reporter probe.

16. The method of claim 15, wherein the reporter probe comprises adapter sequences flanking the complementary region that do not hybridize to the aptamer.

17. The method of claim 16, wherein the adapter sequences are universal adapter sequences that are conserved between different reporter probes that bind to different aptamers.

18. The method of claim 15, comprising hybridizing a degenerate probe to the aptamer and ligating the degenerate probe to an end of the reporter probe, wherein the degenerate probe comprises an adapter sequence.

19. The method of claim 15, wherein the reporter probe comprises one or more internal hairpins that do not bind to the aptamer such that the complementary region is a discontinuous complementary region.

20. A method of aptamer detection, comprising:

contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and

detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting an individual aptamer of the plurality of aptamers comprises:

contacting the individual aptamer with a reporter probe to hybridize a complementary region of the reporter probe to a region of the individual aptamer to form an aptamer-reporter probe complex, wherein the reporter probe comprises a 5' adapter sequence that does not hybridize to the aptamer;

incorporating a 3' adapter sequence at an end of the reporter probe;

and

using the 5' adapter sequence and the 3' adapter sequence to sequence the extended reporter probe.

21. The method of claim 20, comprising:

extending from the complementary region of the reporter probe using the aptamer as a template with a polymerase;

wherein incorporating the 3' adapter sequence comprises ligating the 3' adapter sequence to an end of the extended reporter probe.

22. The method of claim 20, wherein incorporating the 3' adapter sequence comprises incorporating an oligonucleotide comprising the 3' adapter sequence linked to a modified nucleotide at the 3' end.

23. The method of claim 20, comprising:
ligating the 3' adapter sequence to an end of the reporter probe using the aptamer as a splint.

24. A method of aptamer detection, comprising:
contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and
detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting an individual aptamer of the aptamers comprises:
truncating or fragmenting the individual aptamer; and
detecting a sequence of the truncated aptamer.

25. The method of claim 24, wherein truncating the individual aptamer comprises:
contacting the individual aptamer with oligonucleotides that hybridize to ends of the individual aptamer such that the individual aptamer forms a partially double-stranded structure; and
contacting the partially double-stranded structure with a double-stranded exonuclease to digest the ends of the individual aptamer and the oligonucleotides to form a truncated aptamer

26. The method of claim 24, wherein the truncated aptamer is used to form a fragment of a sequencing library to sequence the truncated aptamer.

27. The method of claim 24, wherein adapters are incorporated onto the truncated aptamer to sequence the truncated aptamer.

28. A method of aptamer detection, comprising:
contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and
detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting an individual aptamer of the plurality of aptamers comprises:
contacting the aptamers with reporter probes to hybridize a complementary region of an individual reporter probe to a region of the individual aptamer to form an aptamer-reporter probe complex, wherein the reporter probes are in a first configuration when bound to the aptamers and in a second configuration when not bound to the aptamers;
removing the reporter probes in the second configuration; and
detecting a sequence of the reporter probes in the second configuration.

29. The method of claim 28, wherein the first configuration is circularized and the second configuration is linear.

30. The method of claim 86, wherein the second configuration comprises an assembled restriction site.

31. A method of aptamer detection, comprising:

contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and

detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting an individual aptamer of the plurality of aptamers comprises:

contacting the individual aptamer with a reporter probe to hybridize a complementary region of the reporter probe to a region of the individual aptamer to form an aptamer-reporter probe complex, wherein the reporter probe comprises a nonhybridizing region extending from the complementary region, the nonhybridizing region comprising an identification sequence uniquely identifying for the individual aptamer;

assembling the nonhybridizing region of the individual reporter probe with nonhybridizing regions of other reporter probes to form an assembled fragment; and

detecting the identification sequence and other identification sequences of the assembled fragment.

32. A method of aptamer detection, comprising:

contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and

detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting the aptamers comprises:

generating double-stranded fragments from the aptamers;

assembling the double-stranded fragments into an assembled fragment; and

detecting the aptamers of the assembled fragment.

33. A method of aptamer detection, comprising:

contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and

detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting an individual aptamer of the plurality of aptamers comprises:

contacting the individual aptamer with a reporter probe to hybridize a complementary region of the reporter probe to a region of the individual aptamer to form an aptamer-reporter probe complex;

using one or more oligonucleotides as a split to ligate the reporter probe to the aptamer or a first portion of the reporter probe to a second portion of the reporter probe; and

detecting an identification sequence of the reporter probe after the ligating.

34. A method of aptamer detection, comprising:

contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and

detecting the analytes by detecting aptamers of the analyte-aptamer complexes, wherein detecting the aptamers comprises:

generating amplification products from the aptamers; and
sequencing the amplification products.

35. The method of claim 34, wherein the aptamer comprises adapter sequences.

36. The method of claim 34, wherein generating the amplification products from the aptamers comprises using primers comprising adapters.

37. A method of aptamer detection, comprising:
contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes; and
detecting the analytes by sensing a conformational change of a sensor upon binding of an aptamer to the sensor.

38. A method of aptamer detection, comprising:
contacting analytes of a sample with a plurality of aptamers under conditions that permit analyte-aptamer complexes to form, wherein different aptamers of the plurality of aptamers have specific affinity for respective different analytes of the analytes, wherein a portion of the aptamers comprise a cleavage group and a portion of the aptamers do not comprises the cleavage group.

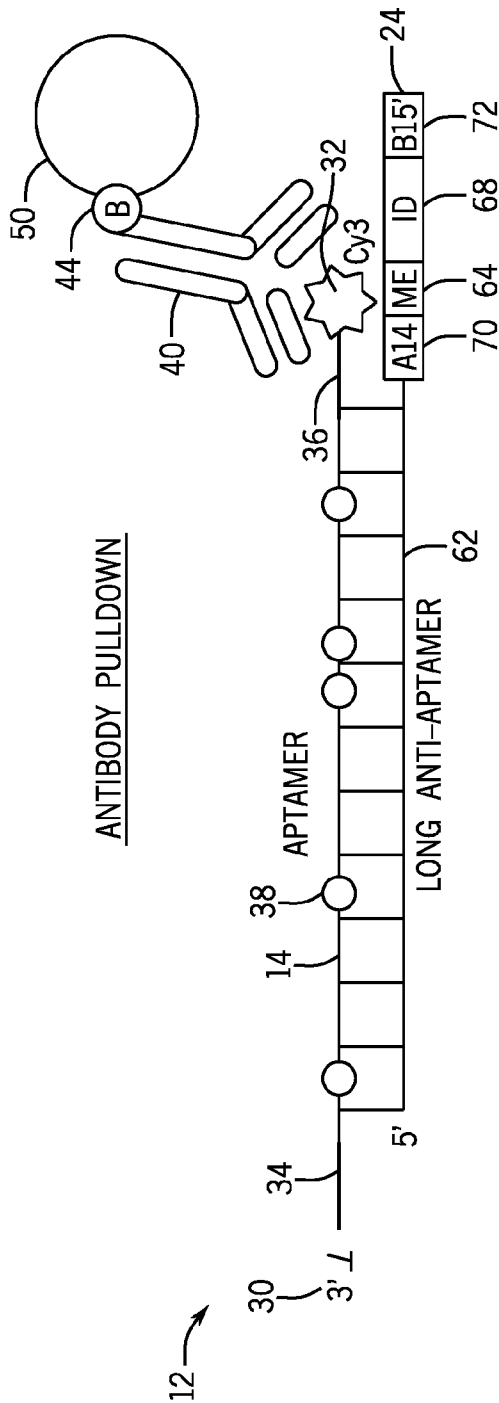


FIG. 1

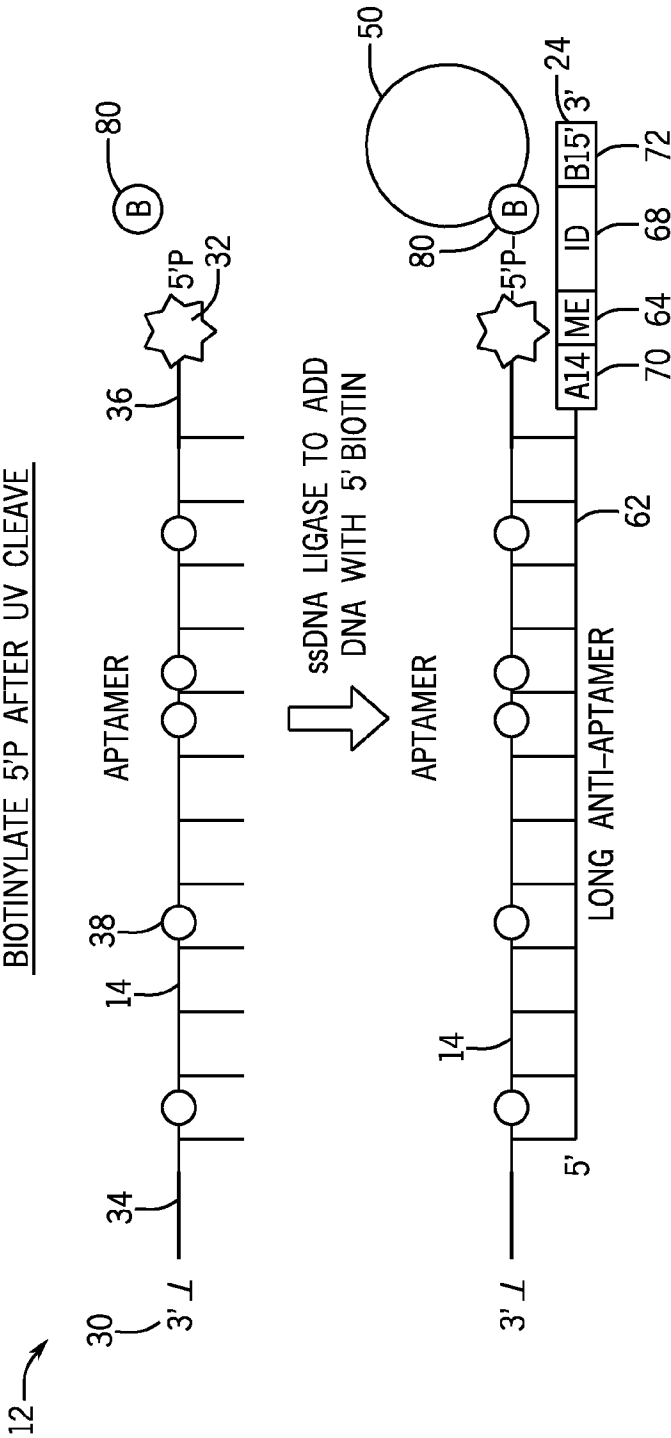


FIG. 2

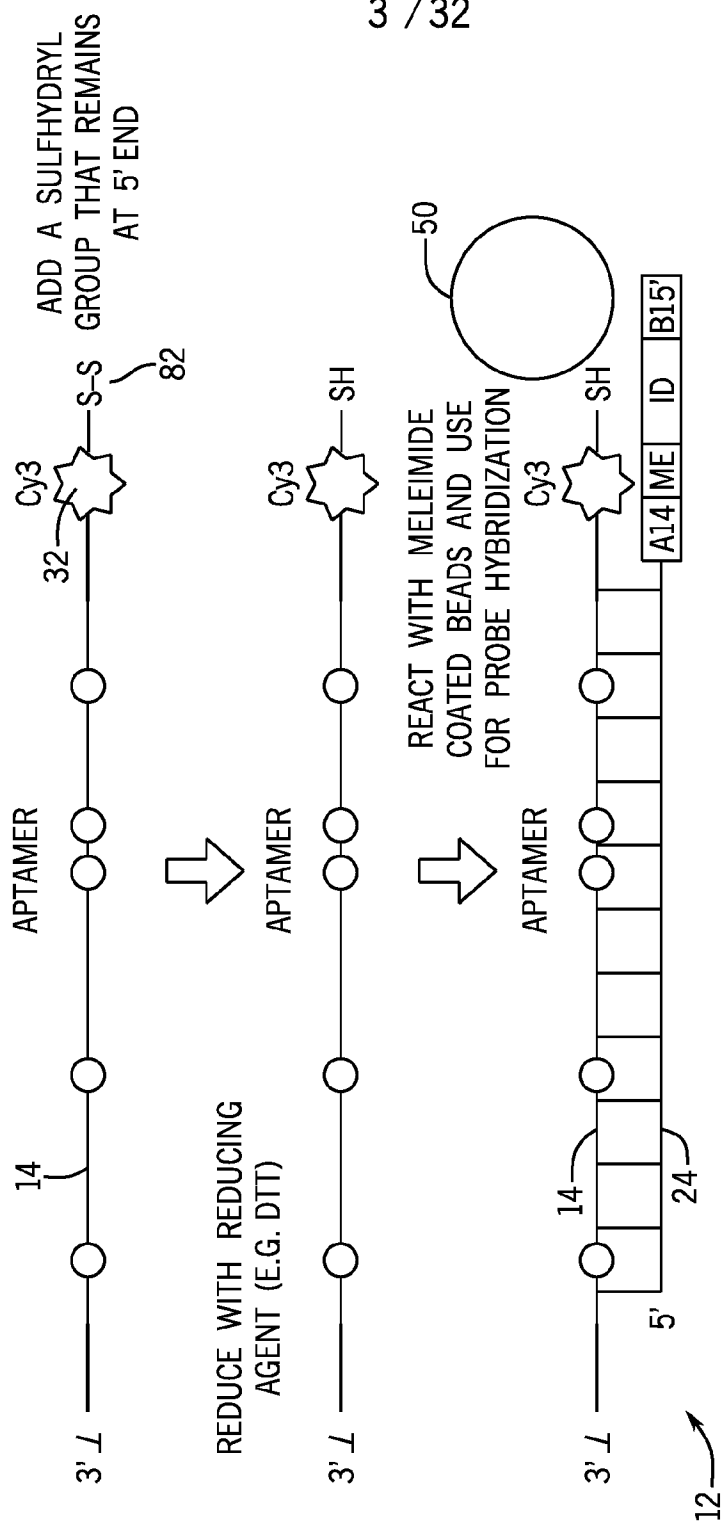


FIG. 3

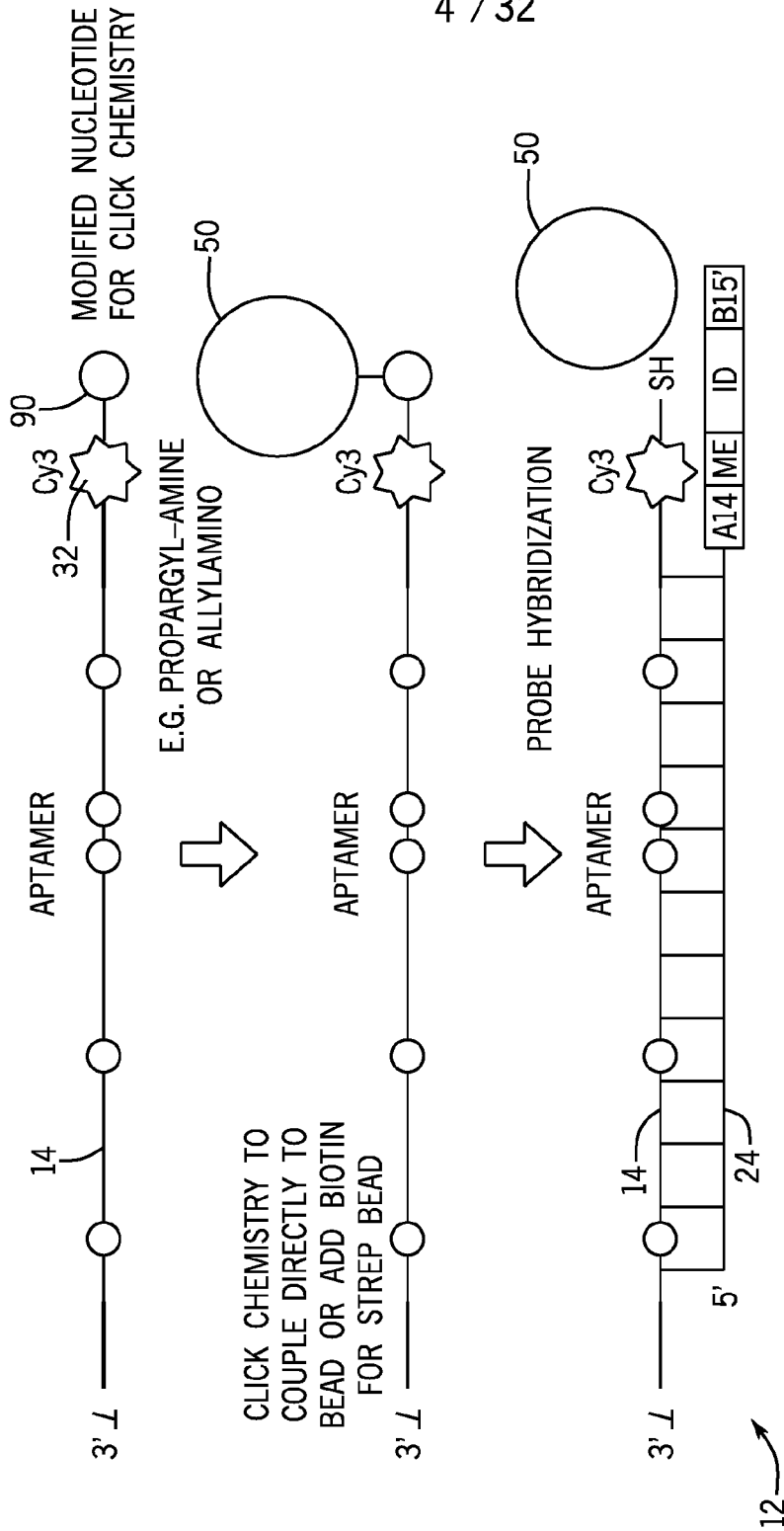


FIG. 4

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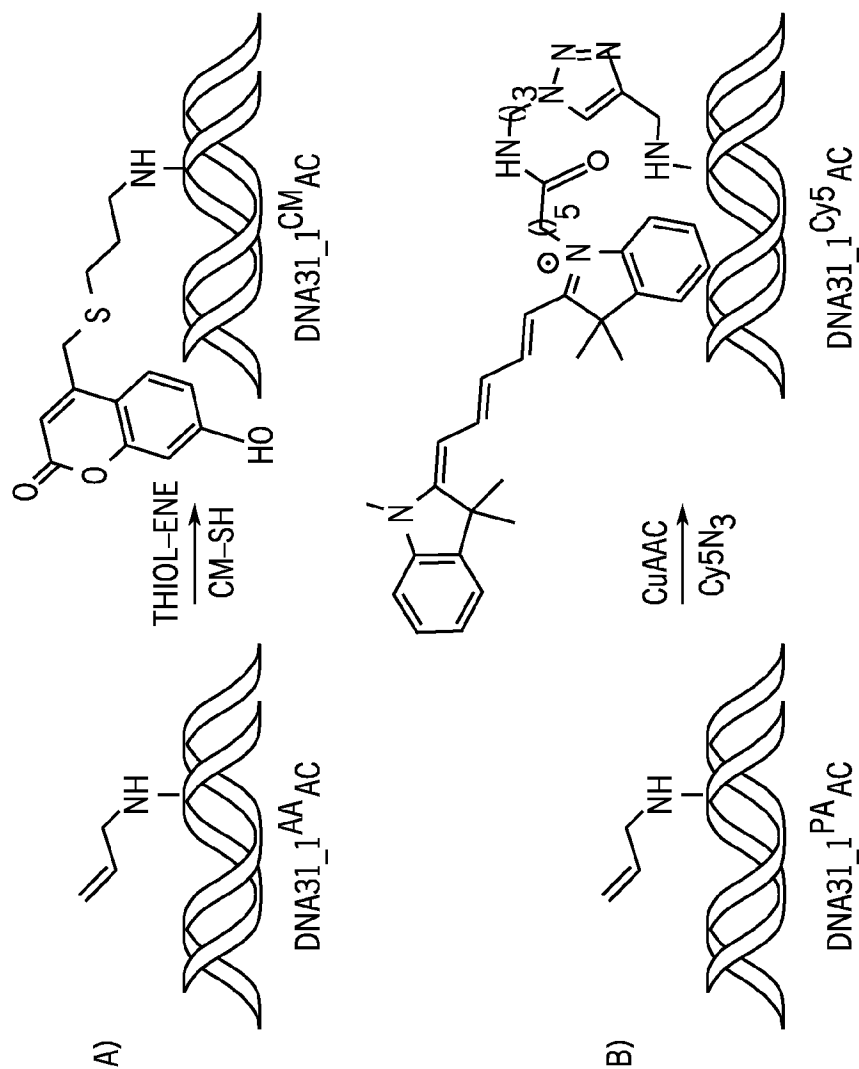


FIG. 5

REDESIGN APTAMERS WITHOUT INVERTED dT OR 5' PHOSPHATE TO ADD BIOTIN GROUP

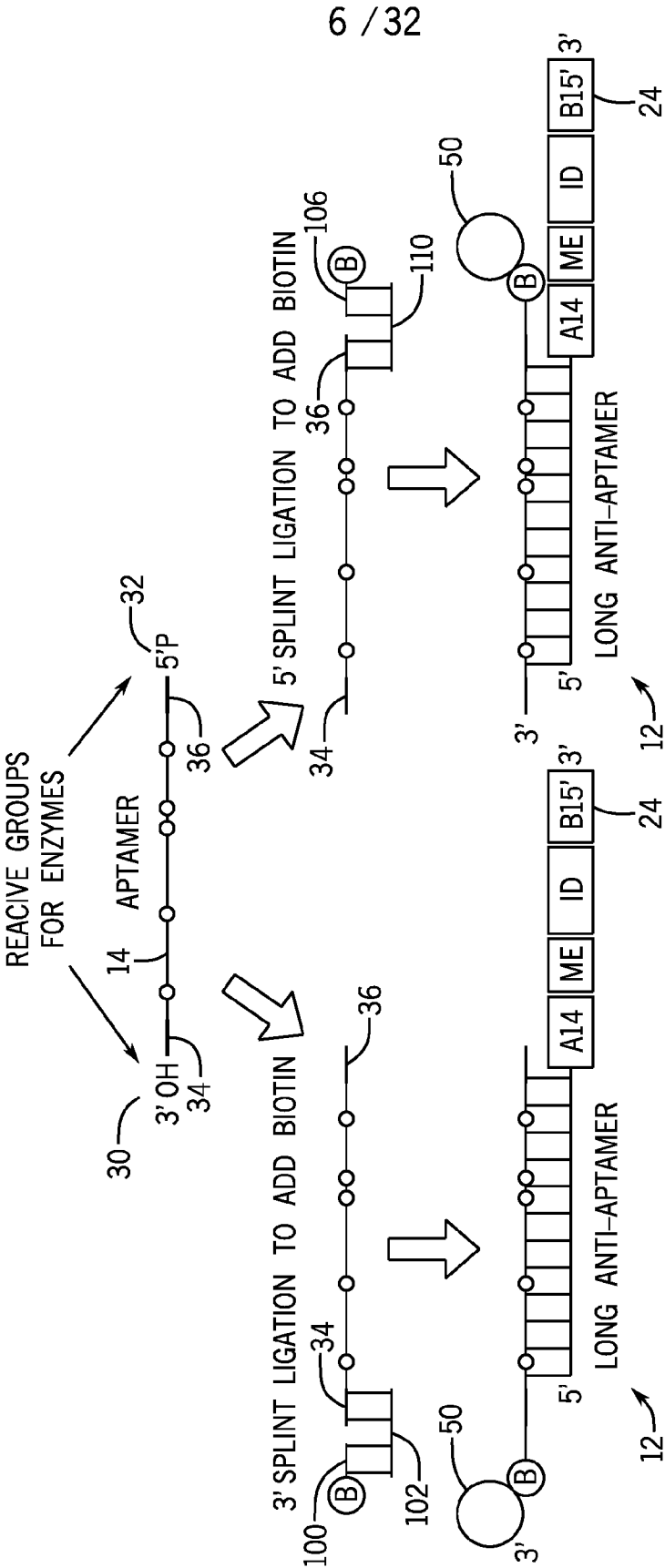


FIG. 6

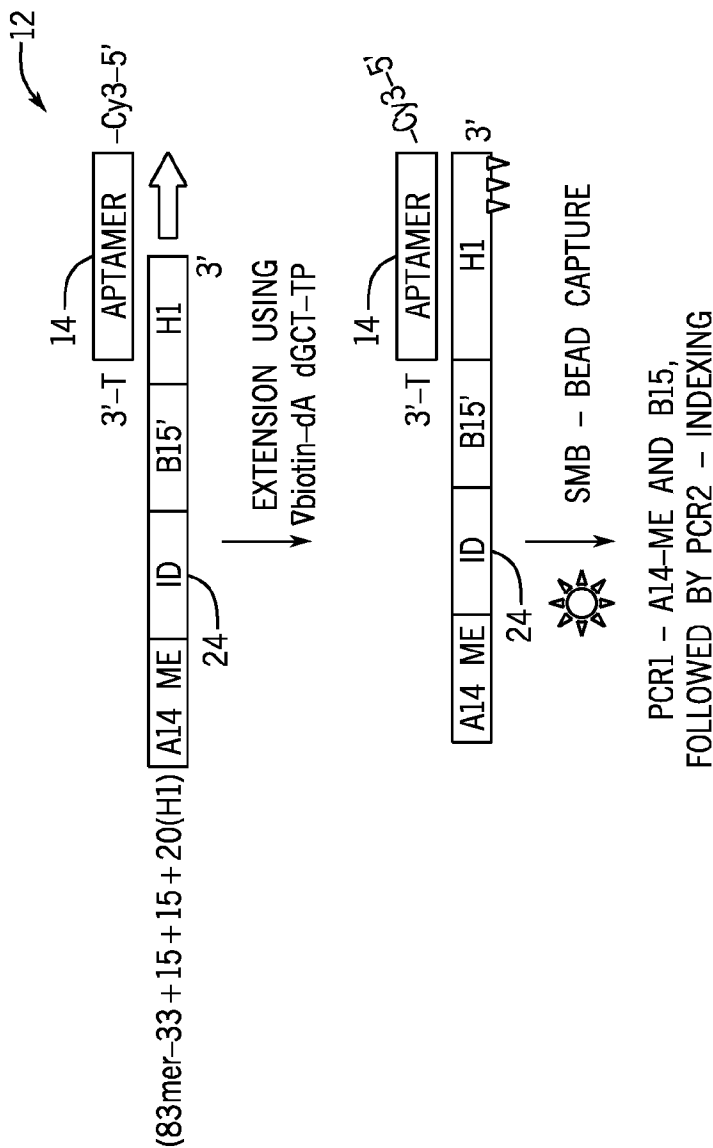


FIG. 7

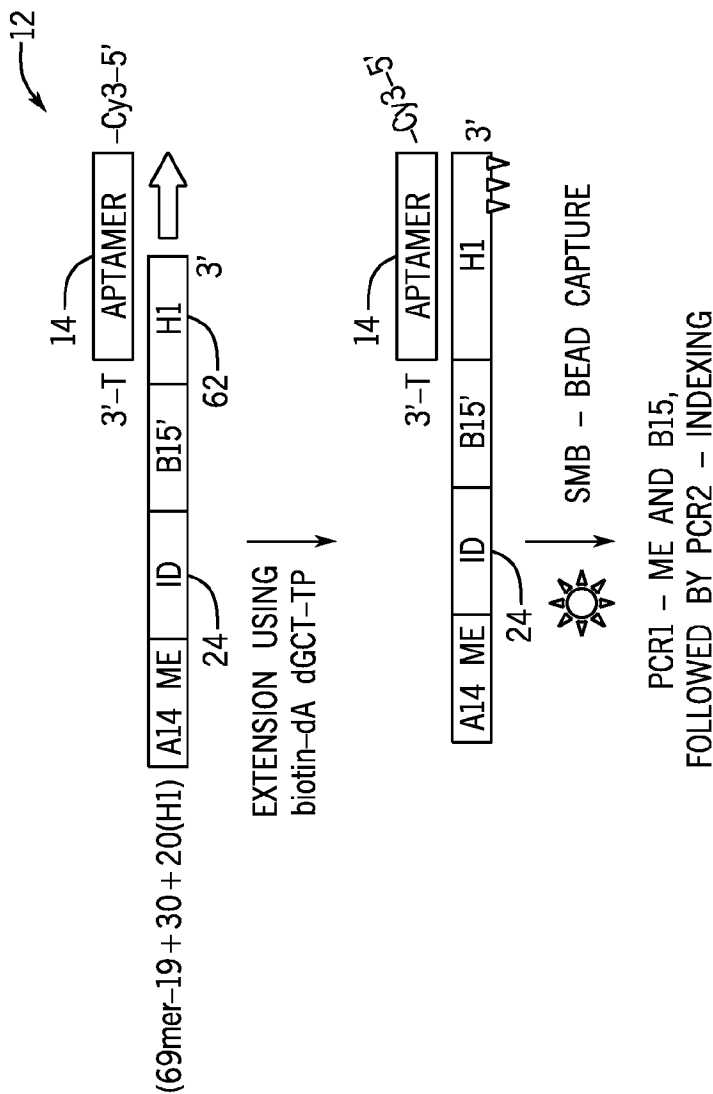


FIG. 8

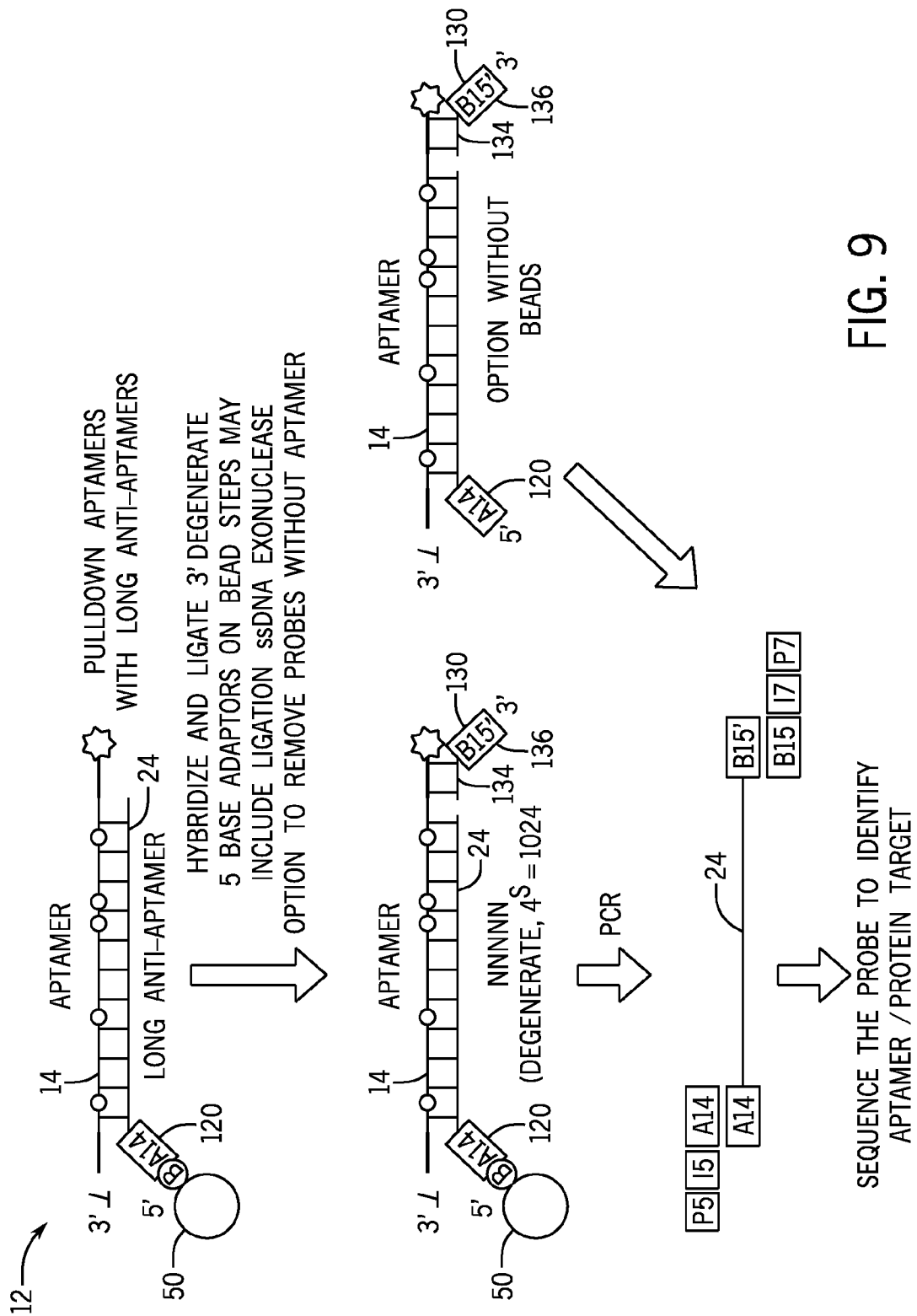


FIG. 9

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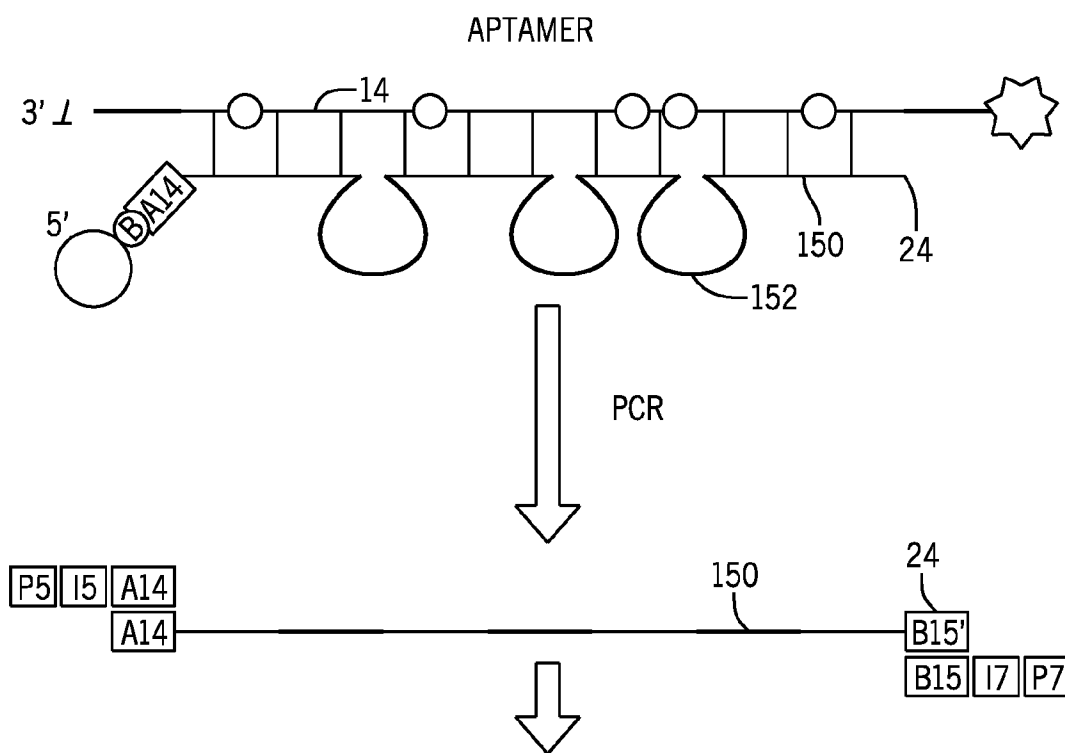


FIG. 10

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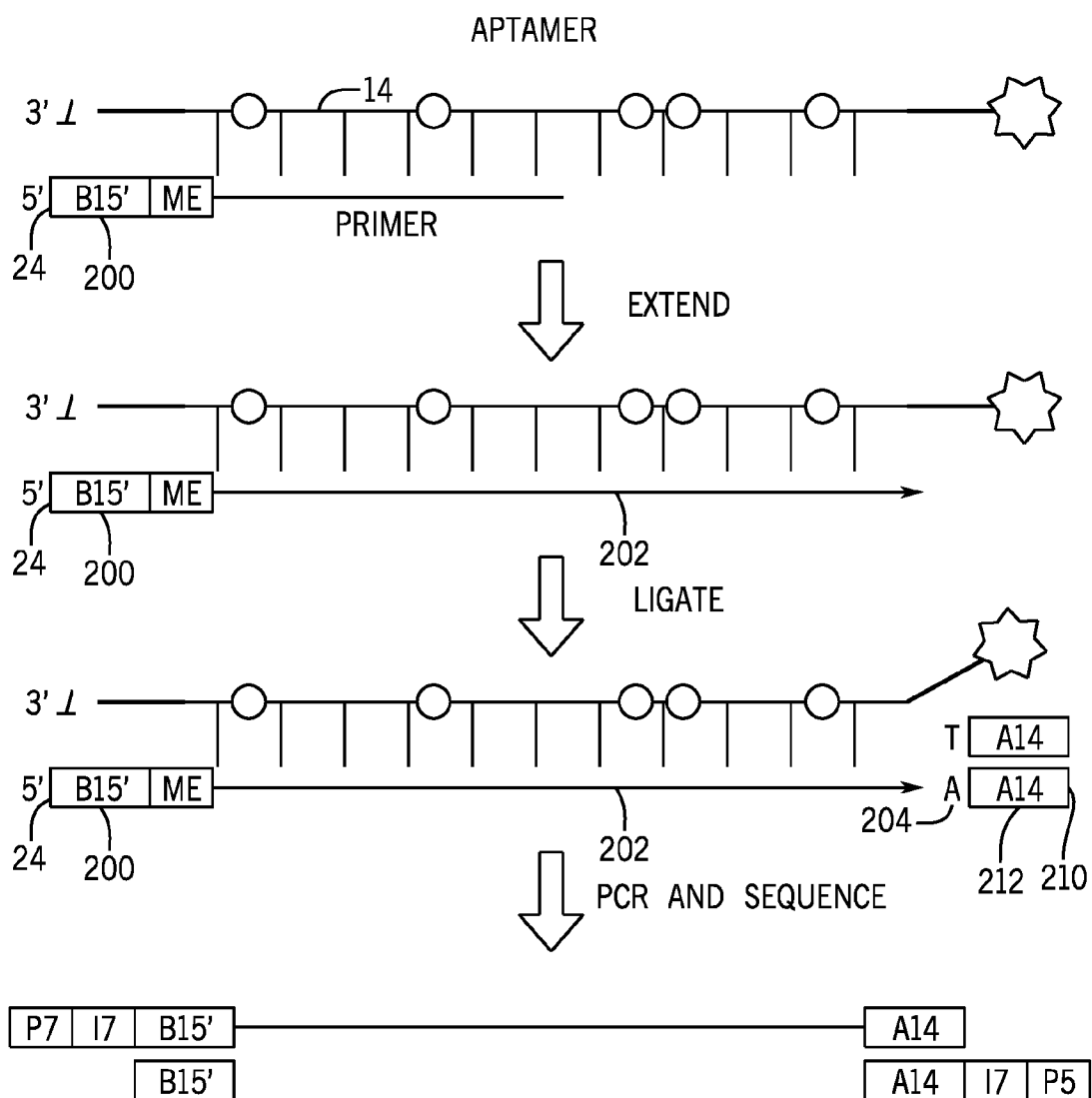


FIG. 11

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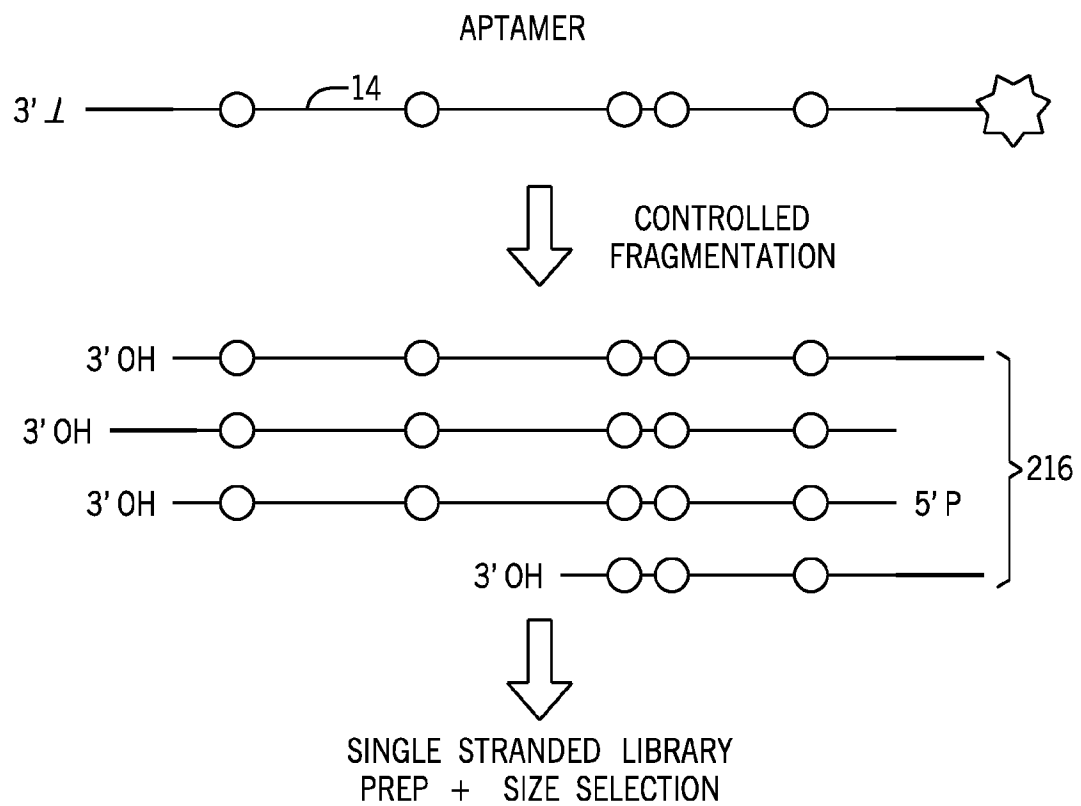


FIG. 12

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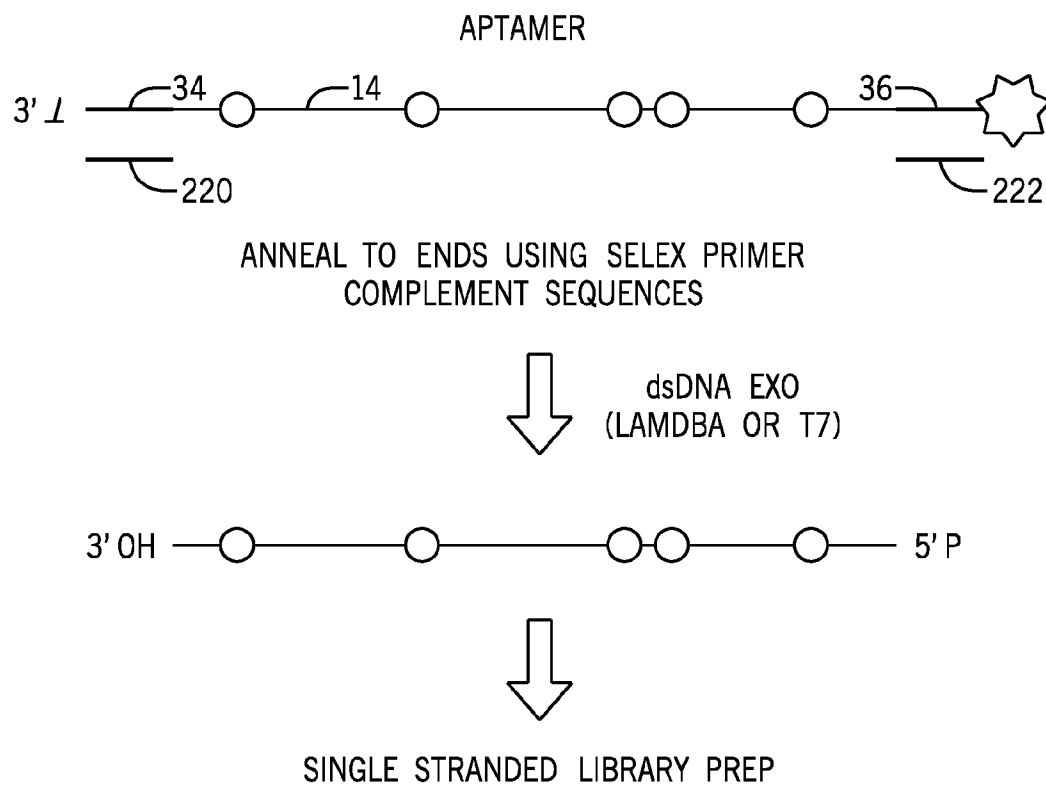


FIG. 13

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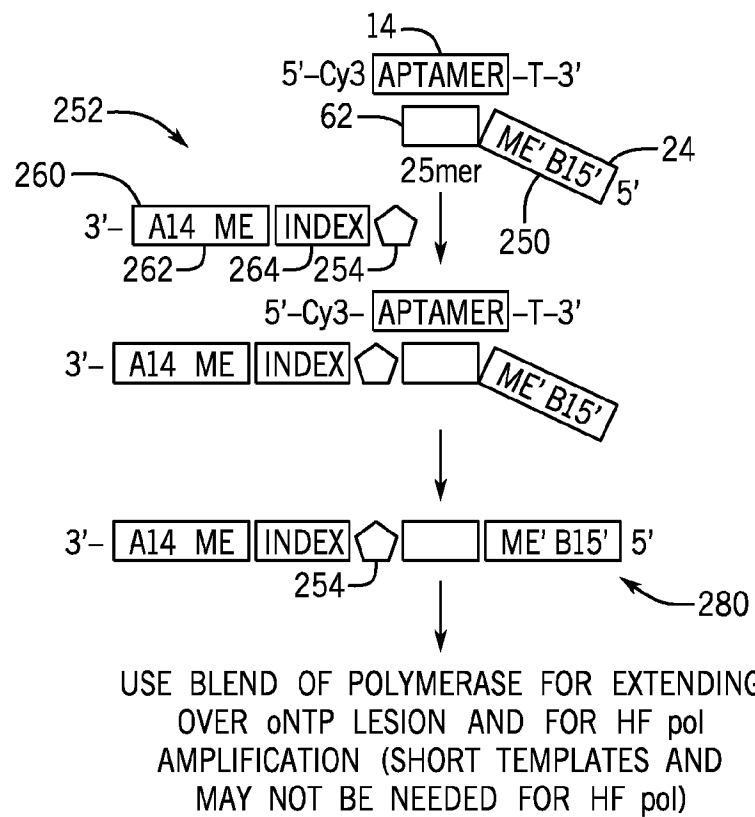


FIG. 14

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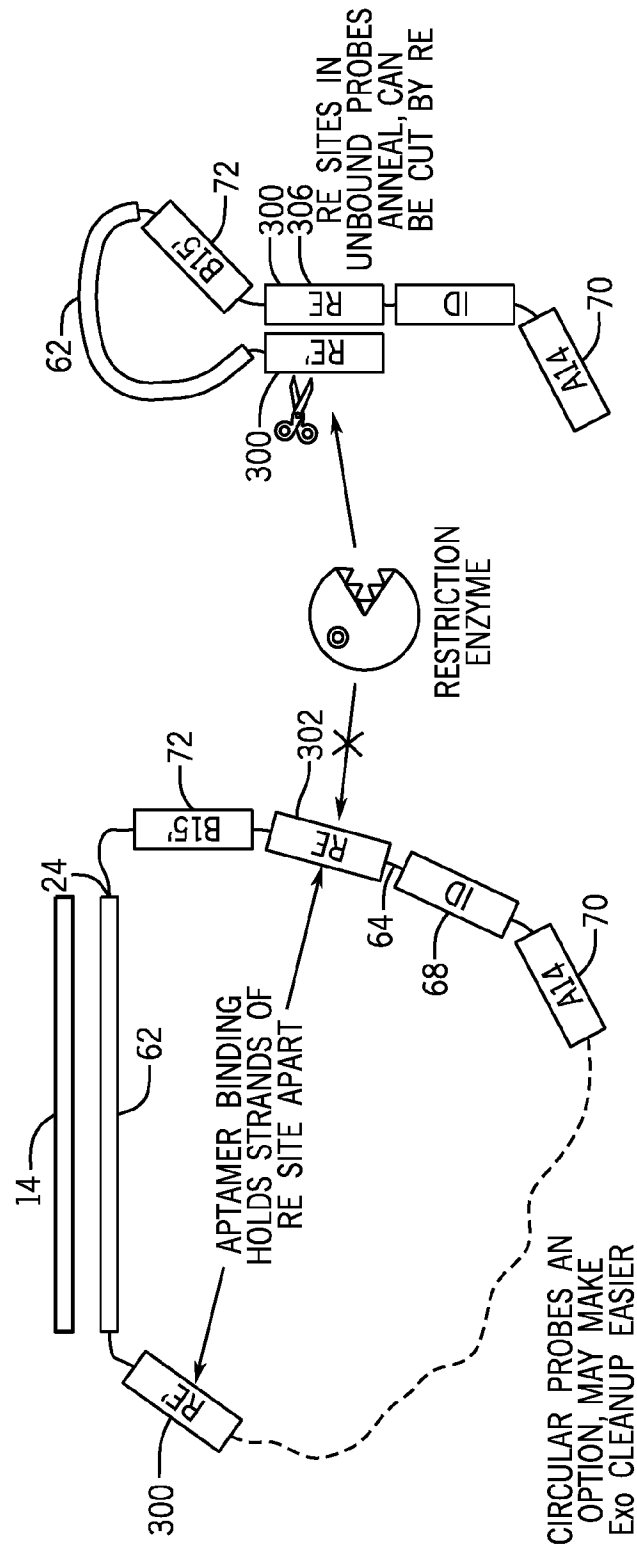
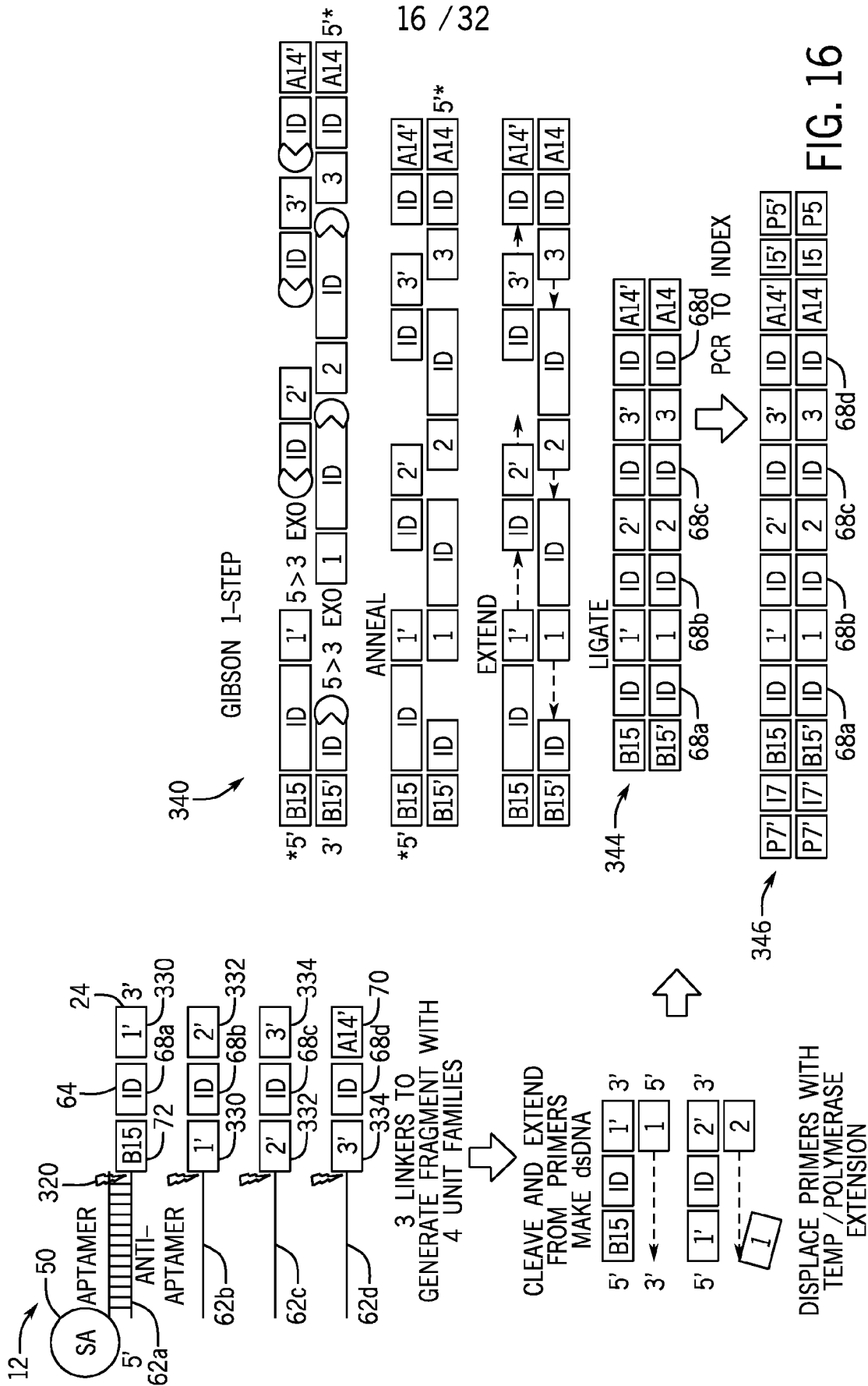


FIG. 15



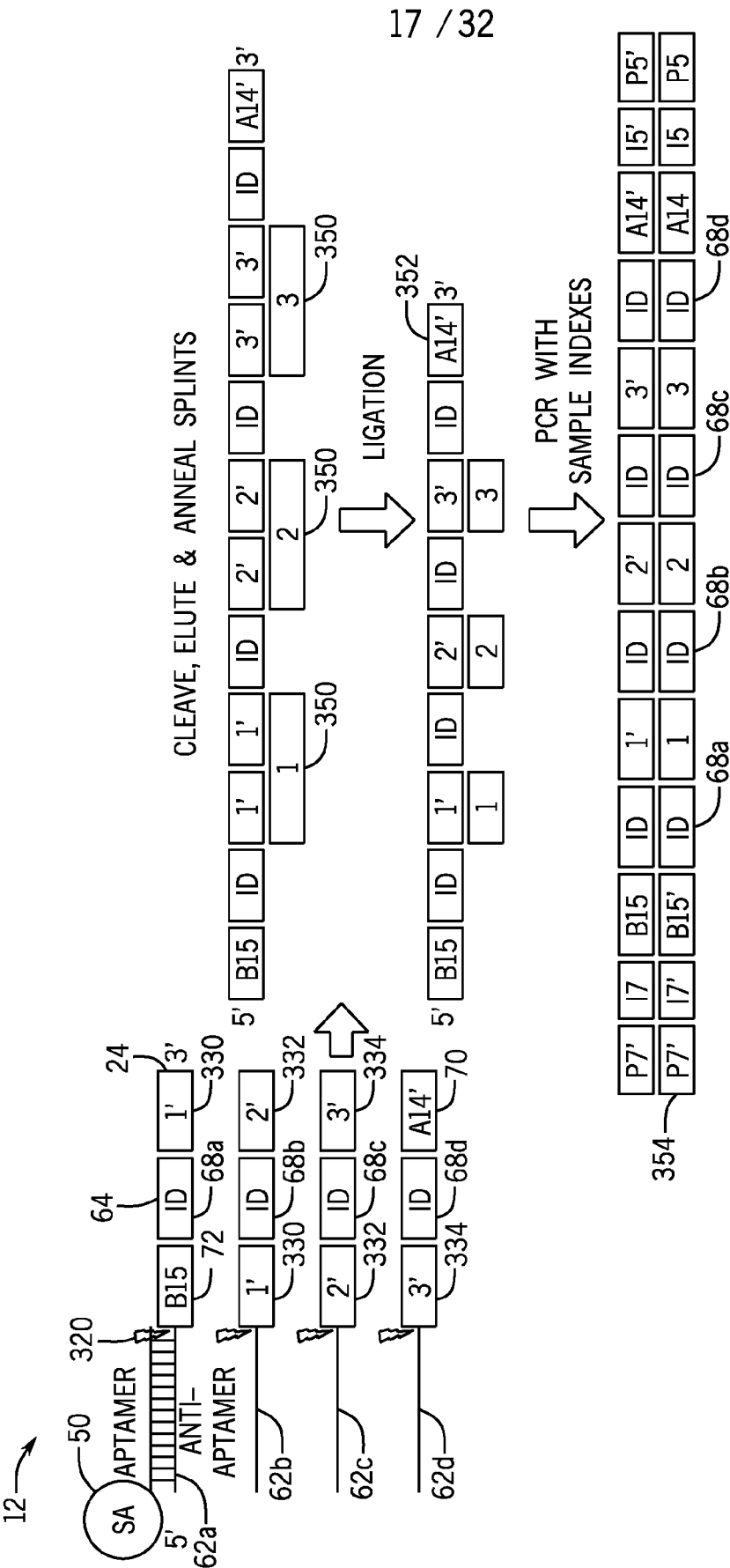
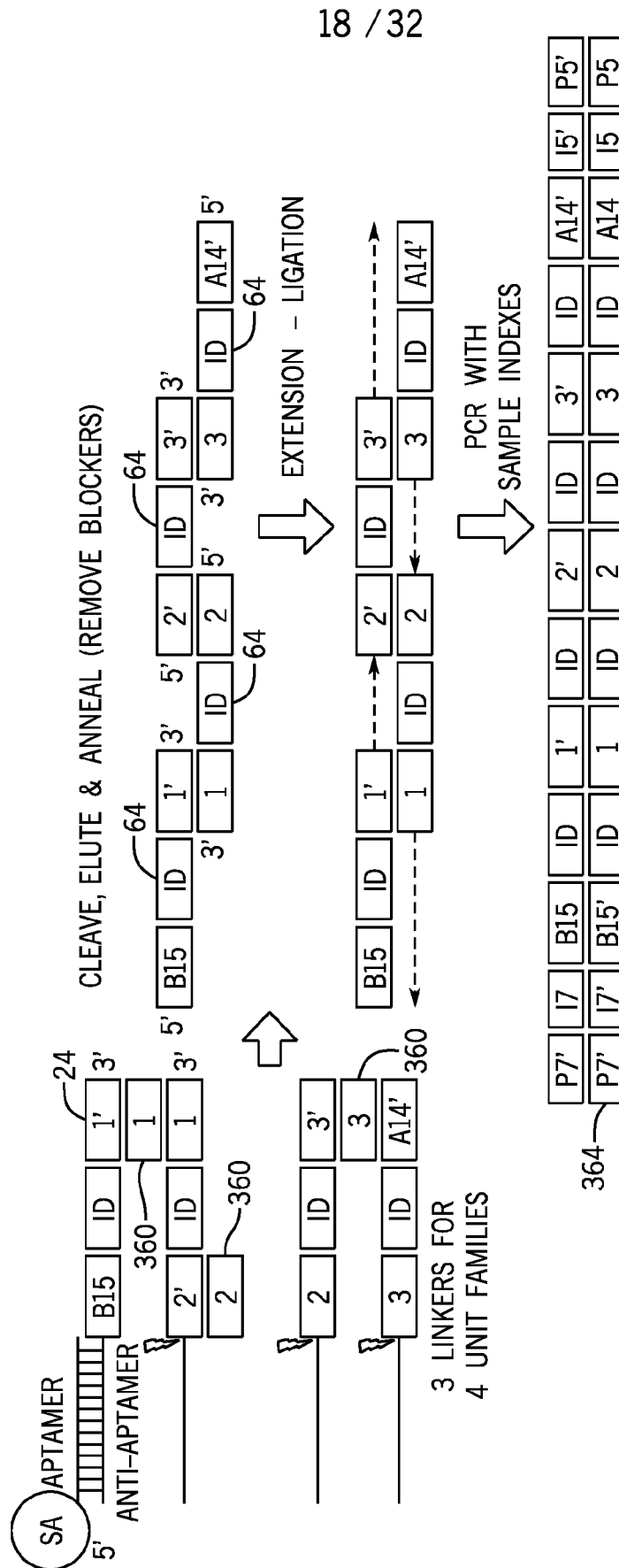


FIG. 17



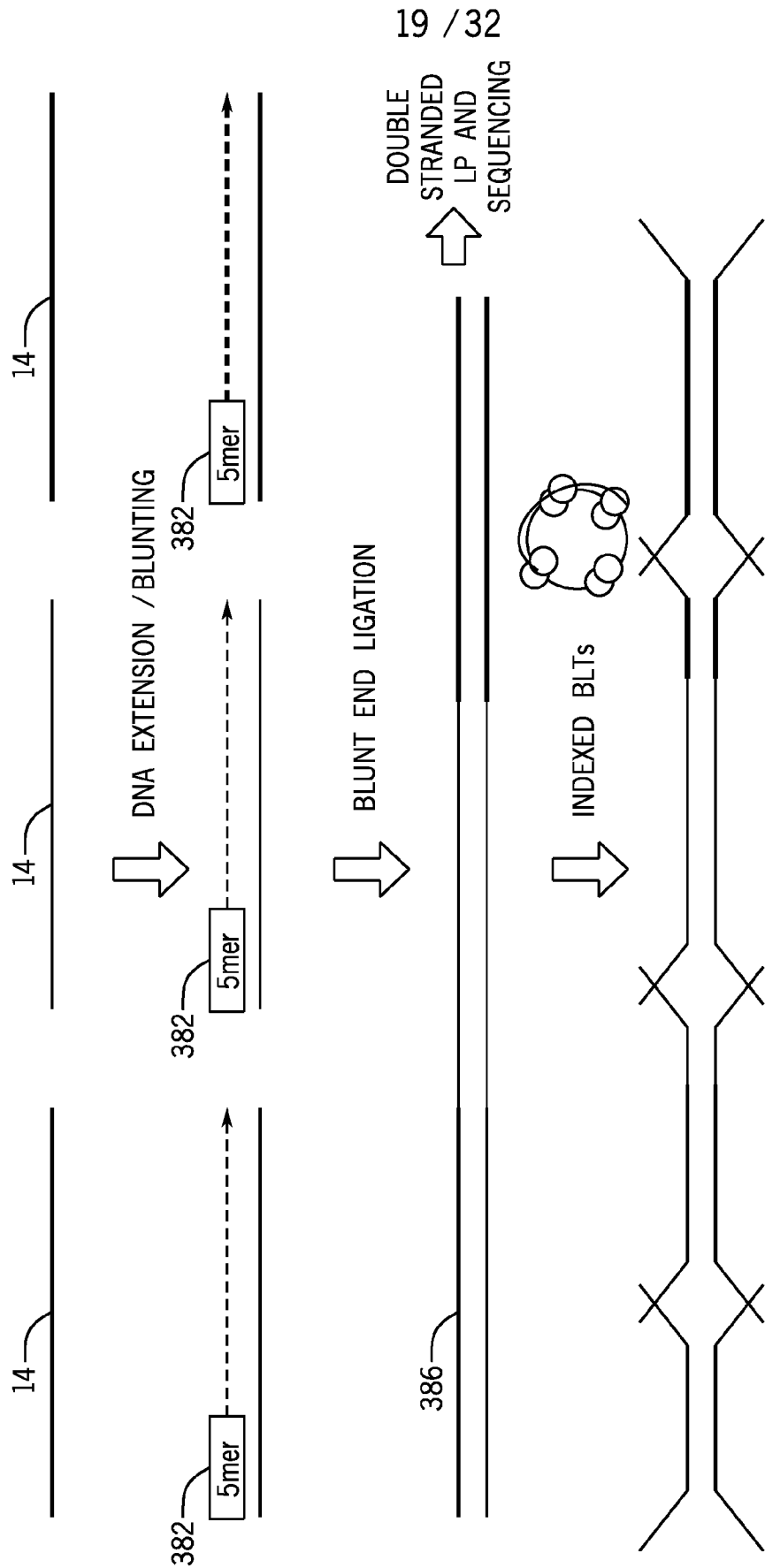


FIG. 19

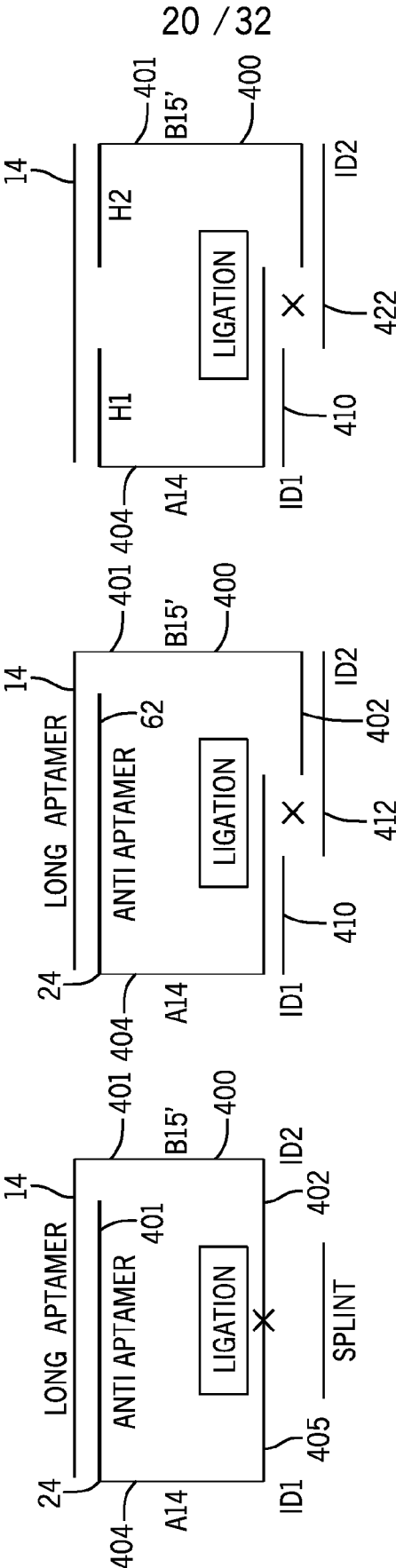


FIG. 20

FIG. 21

FIG. 22

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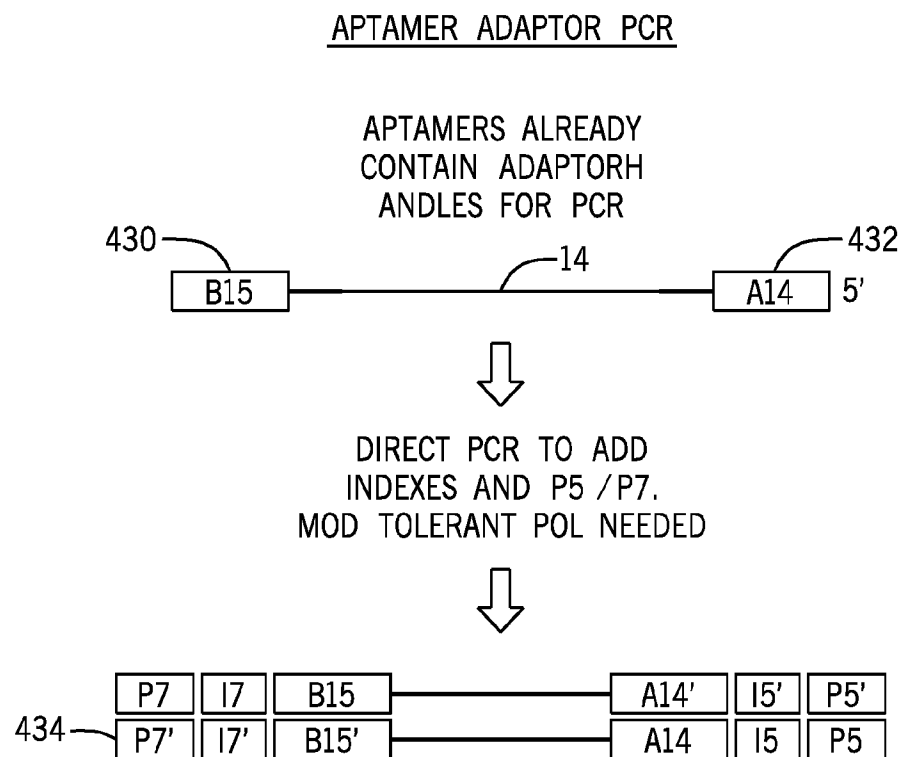


FIG. 23

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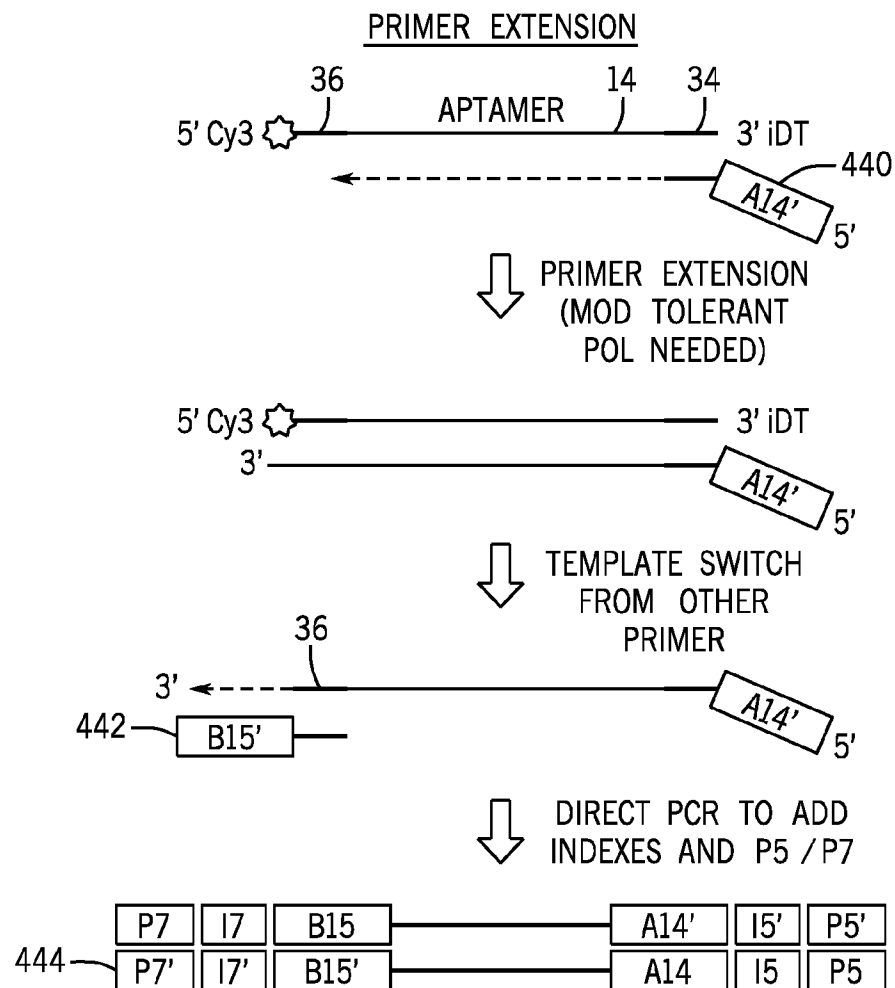


FIG. 24

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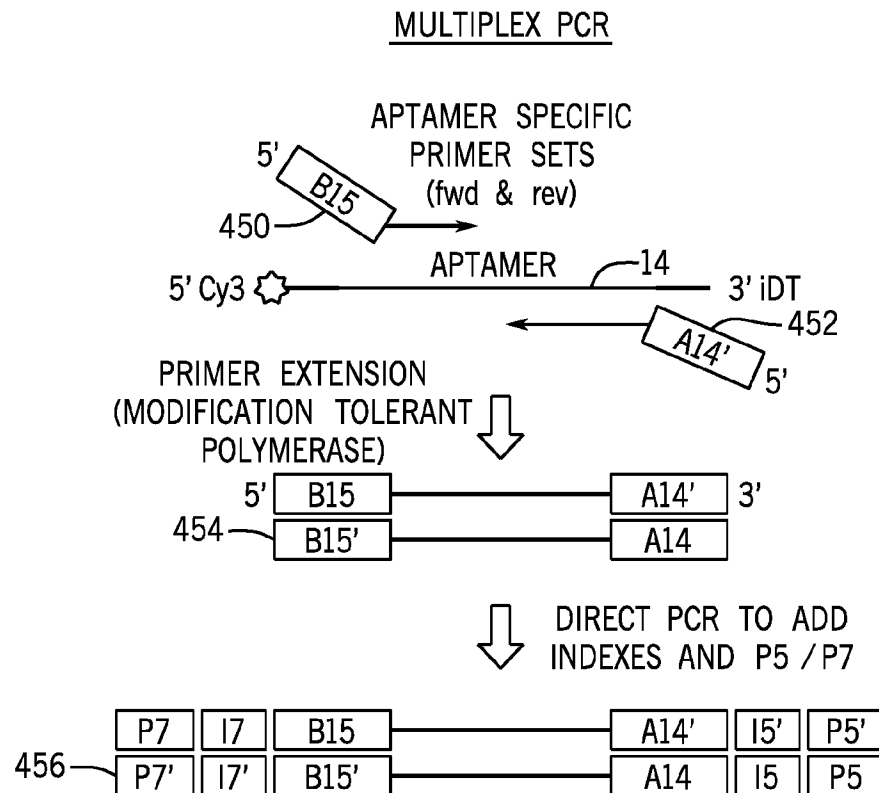


FIG. 25

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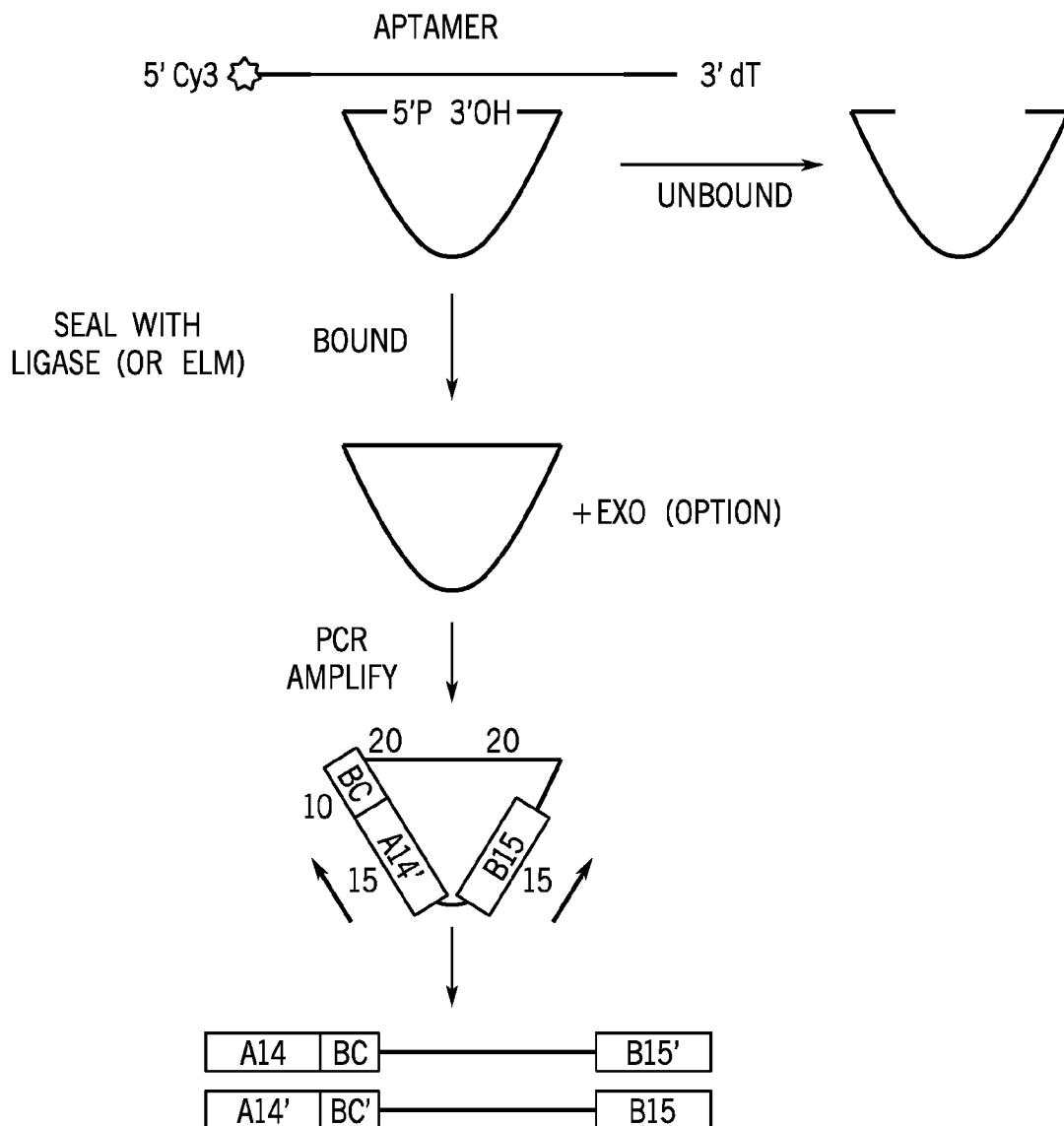


FIG. 26

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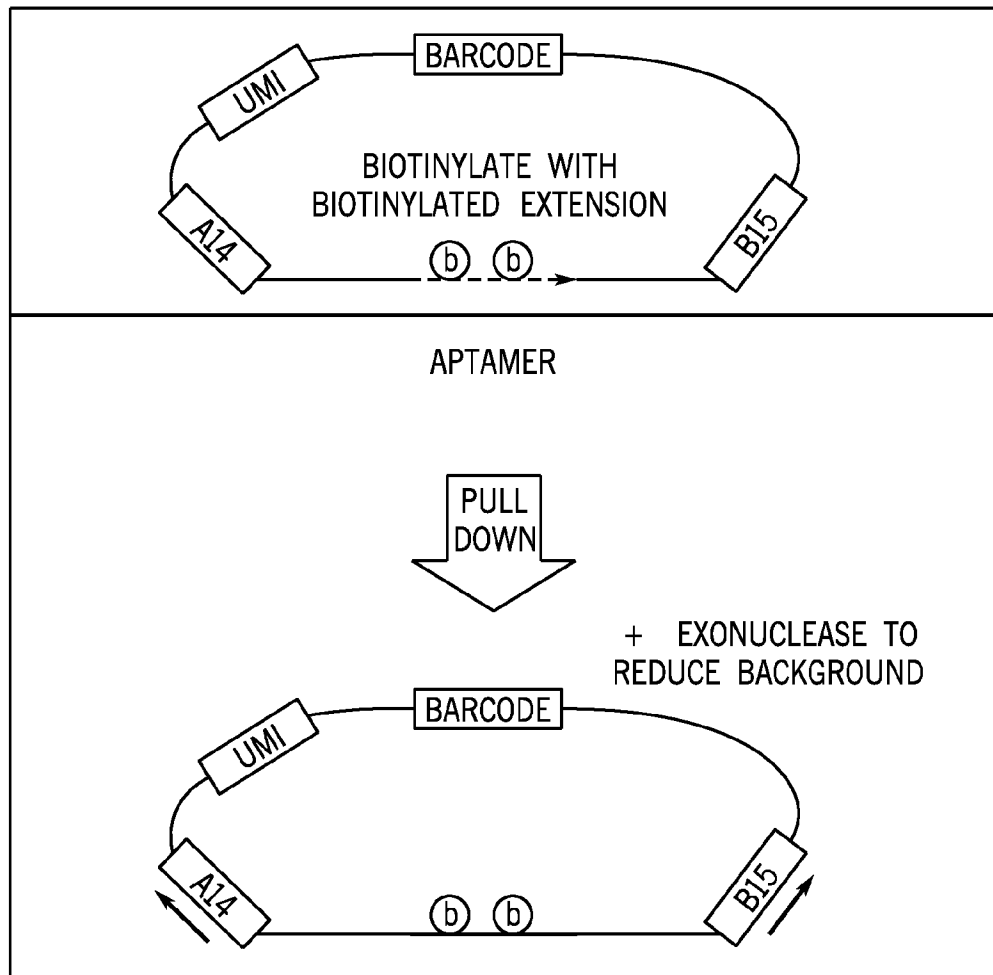


FIG. 27

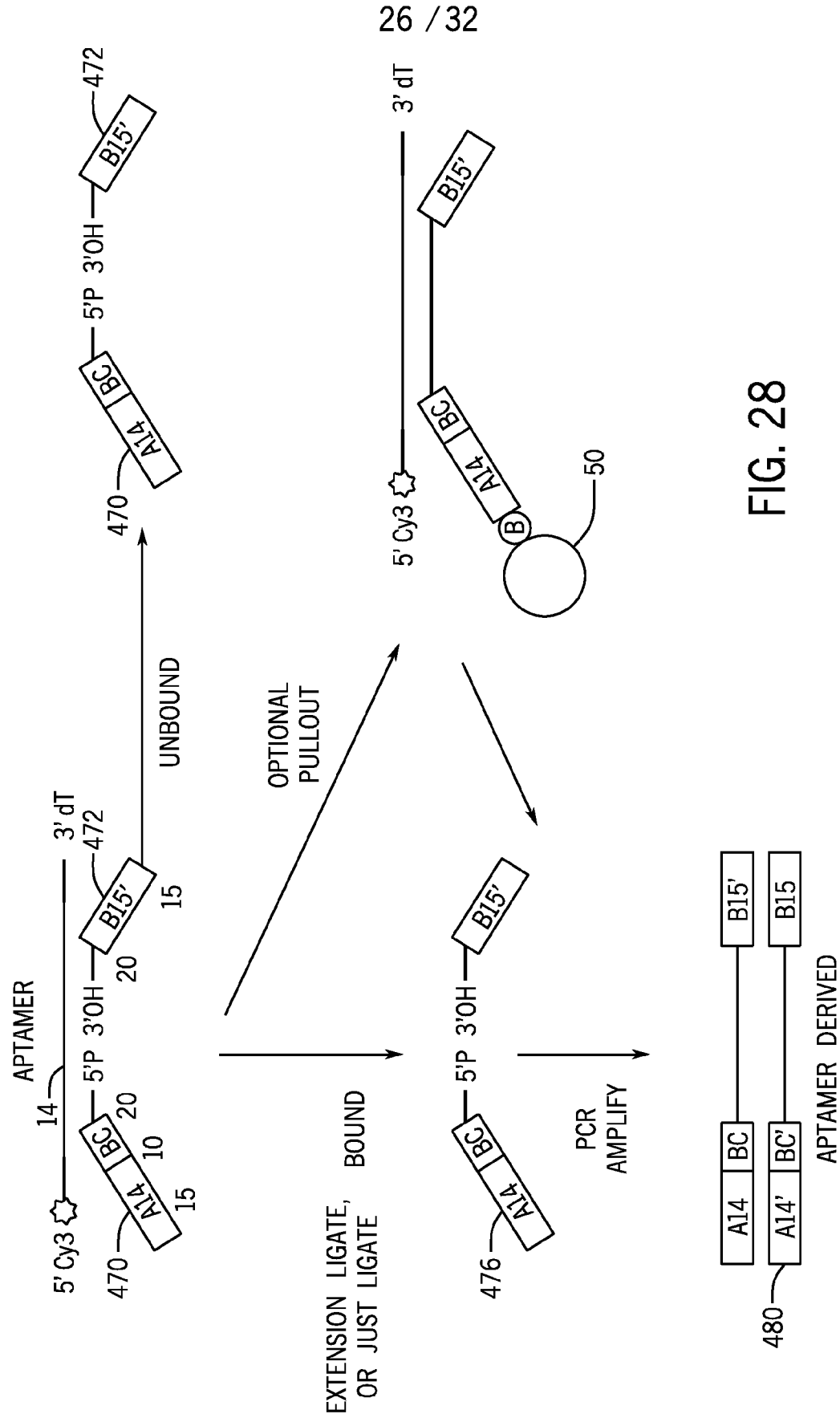


FIG. 28

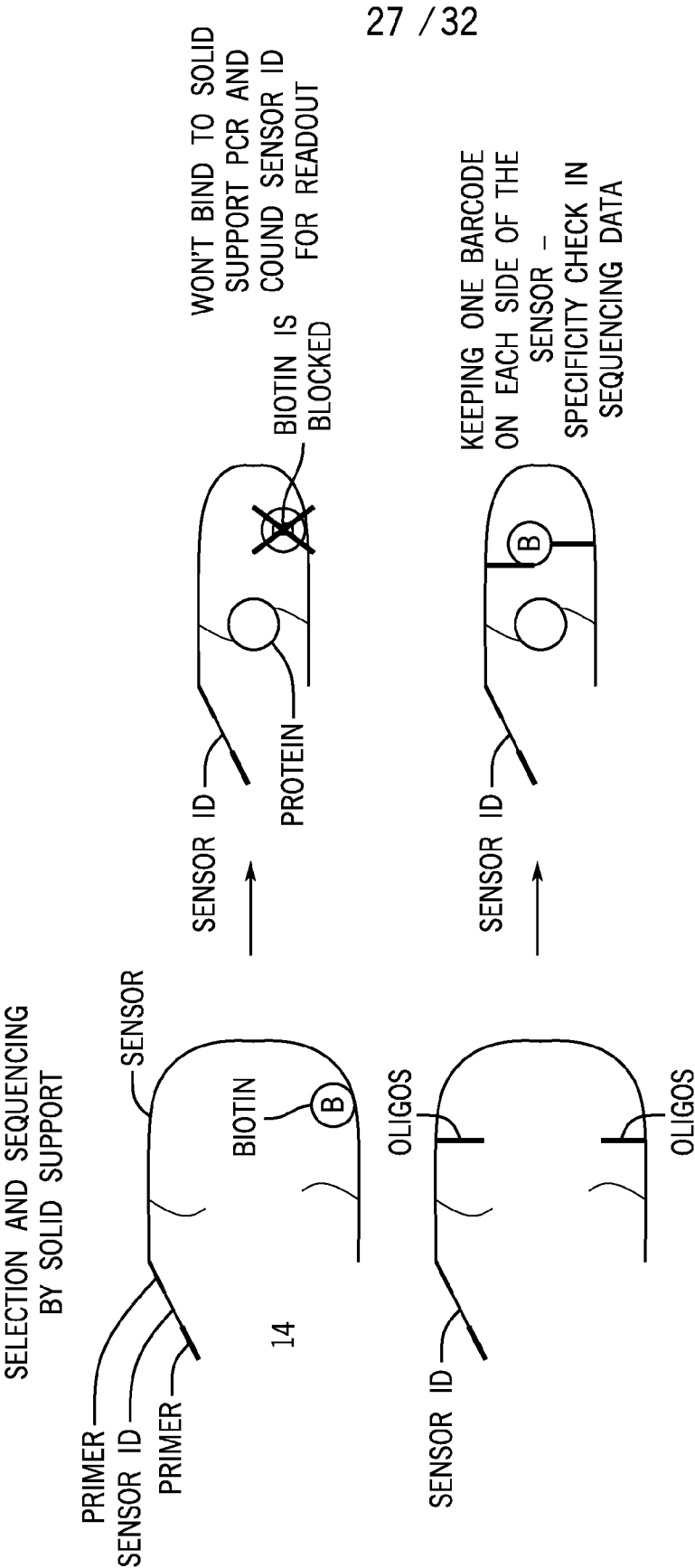


FIG. 29

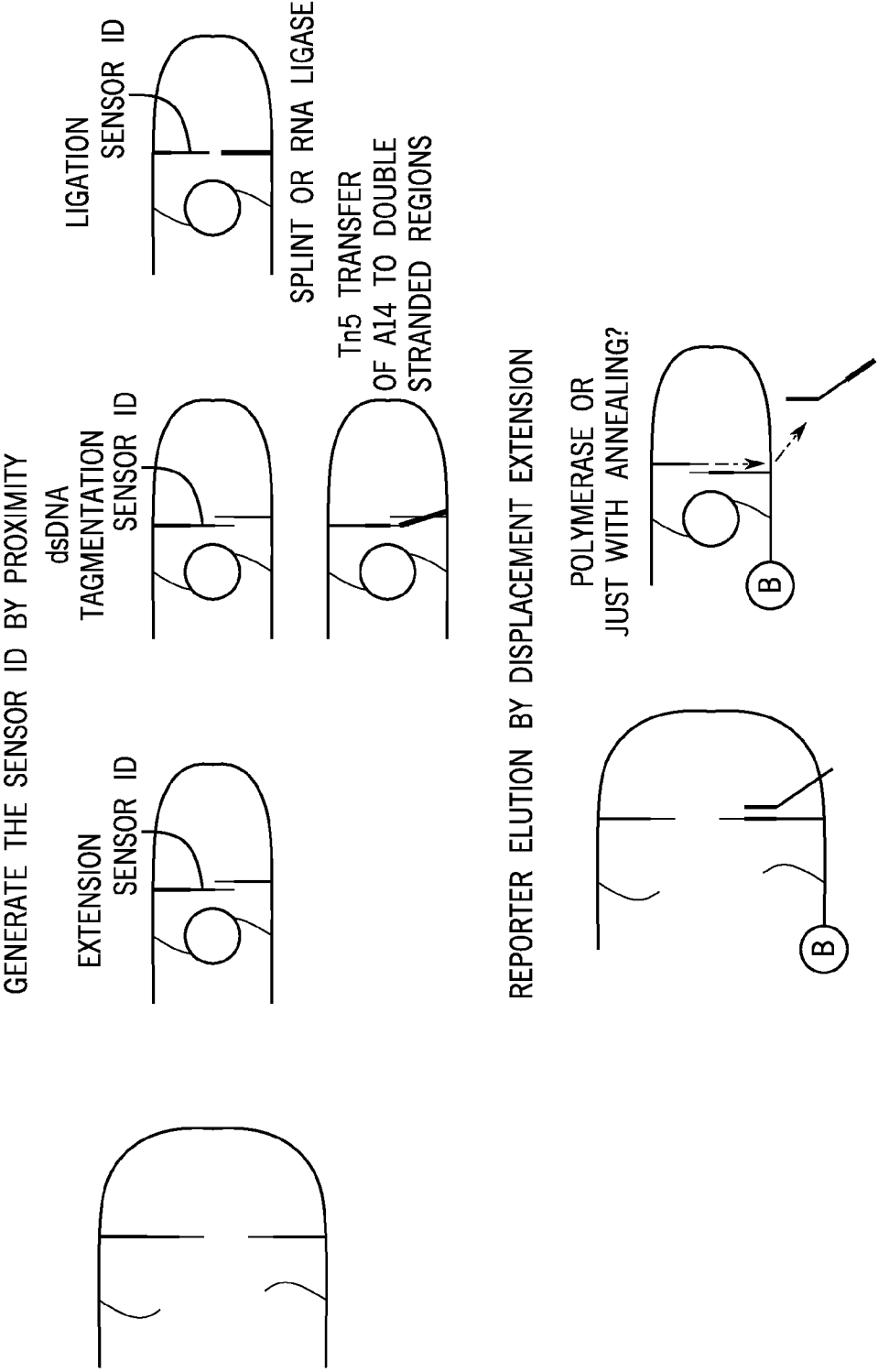


FIG. 30

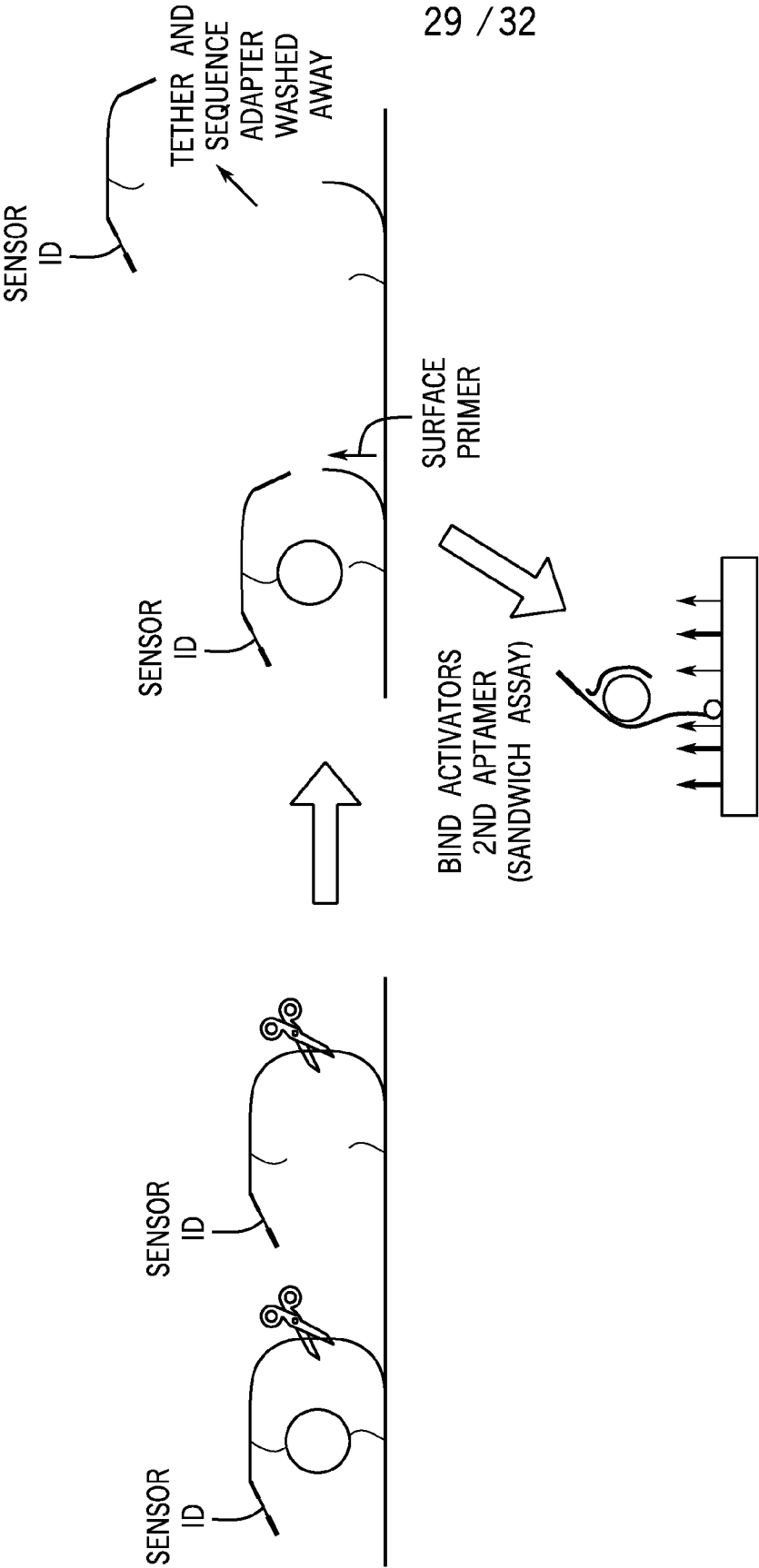


FIG. 31

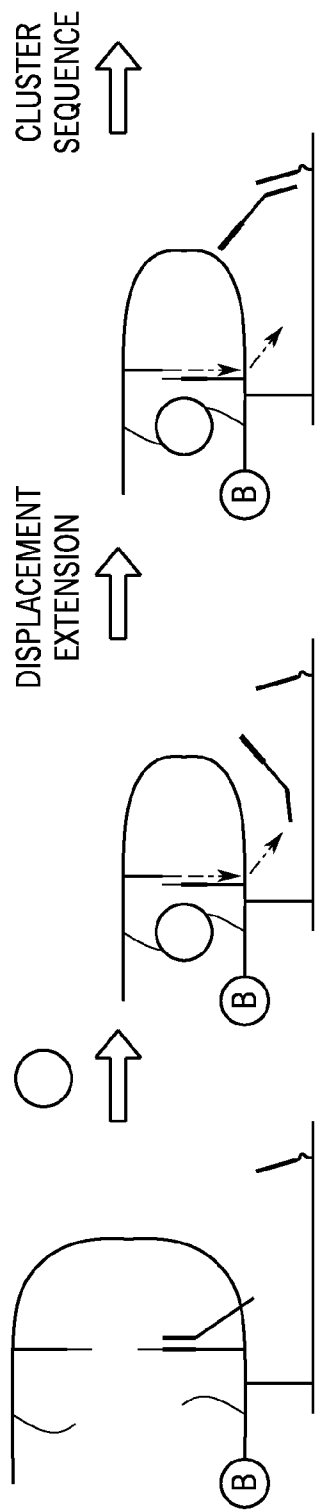


FIG. 32

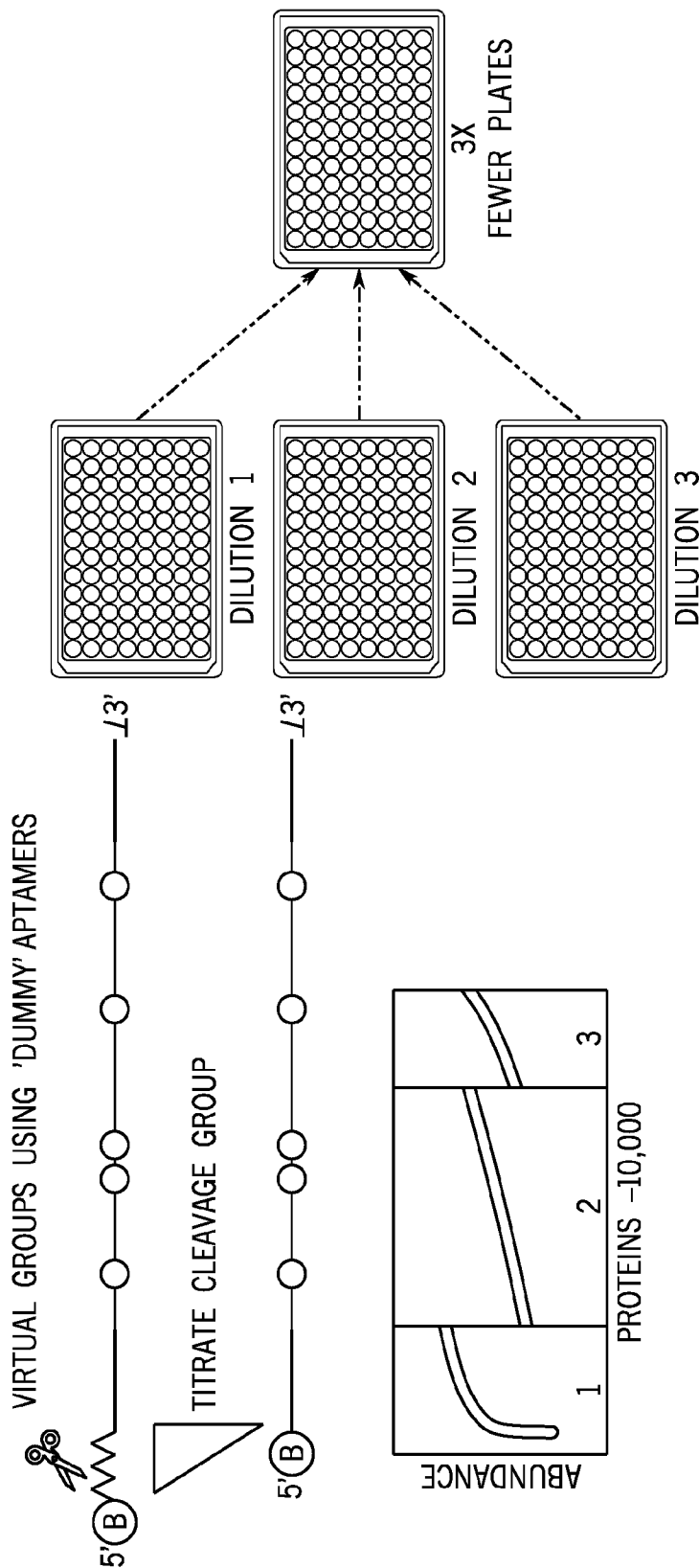


FIG. 33

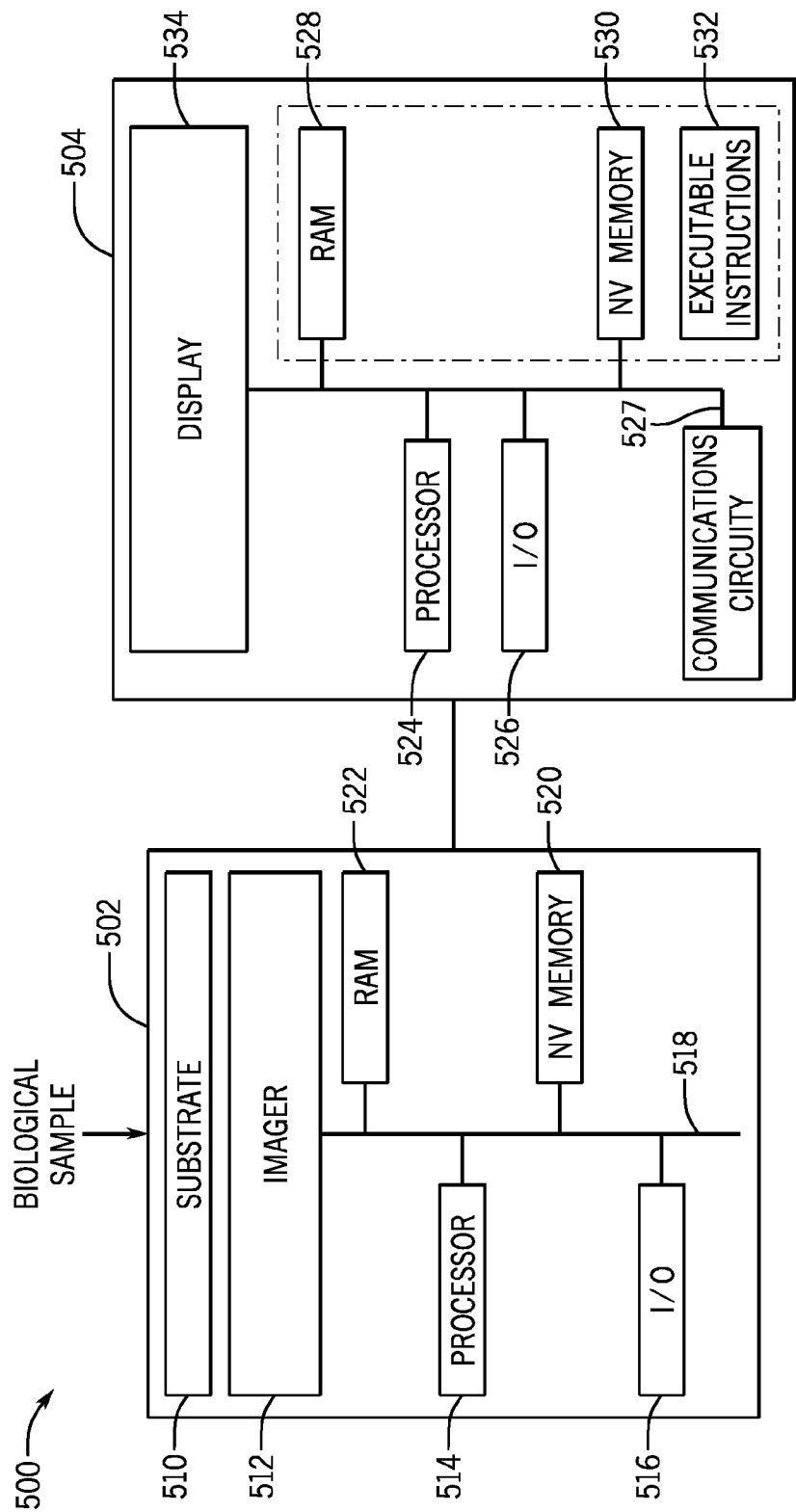


FIG. 34