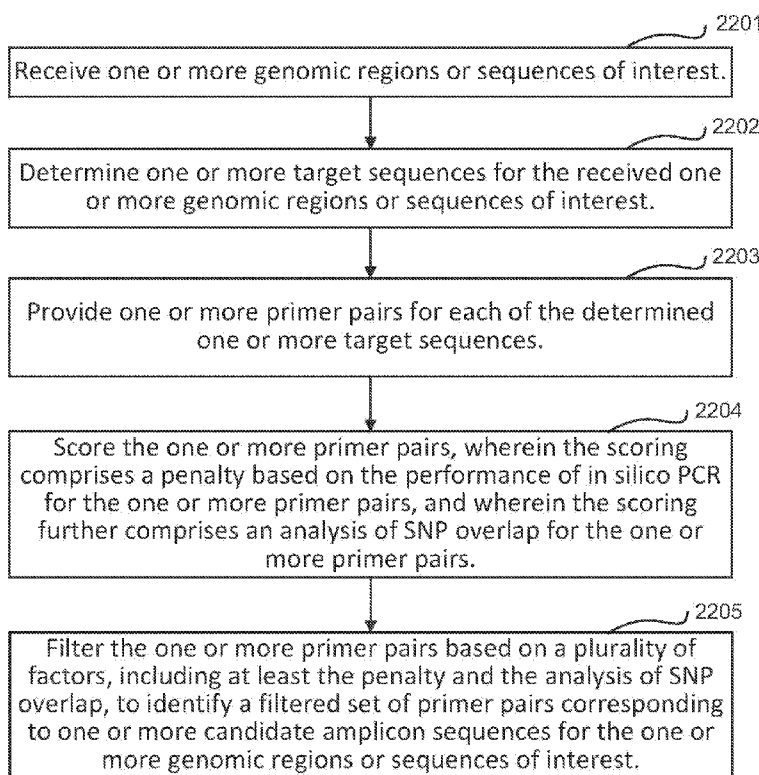




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[Continued on next page]

(54) Title: HUMAN IDENTIFICATION USING A PANEL OF SNPS



(57) Abstract: The present invention provides methods, compositions, kits, systems and apparatus that are useful for multiplex PCR of one or more nucleic acids which belong to a panel of single nucleotide polymorphisms (SNPs) useful to identify a human. In particular, various target-specific primers are provided that allow for the selective amplification of one or more target sequences in the panel. In some aspects, amplified target sequences obtained using the disclosed methods, kits, systems and apparatuses can be used in various downstream processes including nucleic acid sequencing and used to identify a human.

FIG. 8

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Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

HUMAN IDENTIFICATION USING A PANEL OF SNPS

[0001] CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application incorporates by reference in its entirety the disclosure of U.S. Application Serial No. 13/458,739, entitled "Methods and Composition for Multiplex PCR", inventors John Leamon, Mark Andersen, and Michael Thornton, filed on April 27, 2012.

[0003] TECHNICAL FIELD

[0004] In some embodiments, the disclosure relates generally to methods, compositions, systems, apparatuses and kits for amplifying one or more target sequences within a sample containing a plurality of target sequences in order to identify a human. Optionally, a plurality of target sequences, for example at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 100, 120, 130 or more sequences, are amplified within a single amplification reaction. In some embodiments, the disclosure relates generally to methods, compositions, systems, apparatuses and kits for amplifying one or more target sequences from a single source, such as genomic DNA, formalin-fixed paraffin-embedded (FFPE) DNA, or a forensic sample. In particular, methods, kits, systems apparatuses and compositions useful for amplifying one or more target sequences using primers having a cleavable group are disclosed.

[0005] BACKGROUND

[0006] Several biological applications involve the selective amplification of nucleic acid molecules within a population. For example, next-generation sequencing methods can involve the analysis of selected targets within a large population of nucleic acid molecules. For such applications, it can be useful to increase the total number of targets that can be selectively amplified from a population within a single amplification reaction. Such selective amplification is typically achieved through use of one or more primers that can selectively hybridize to, or selectively promote the amplification of, a particular target nucleic acid molecule. Such selective amplification can be complicated by the formation of amplification artifacts, such as primer-dimers and the like. The formation of such amplification artifacts (also referred to herein as nonspecific amplification products) can consume critical amplification reagents, e.g., nucleotides, polymerase, primers, etc. Furthermore, such artifacts can frequently have shorter length relative to the intended product and in such situations can amplify more efficiently than the intended products and dominate the reaction output. Selective amplification can also be complicated by the formation of 'superamplicons', i.e., the formation of a extended amplicon, which can occur when extension of a first primer is extended through an adjacent

target nucleic acid sequence, thereby creating a long non-specific amplification product, which can act as a template for extension with a second primer. The formation of such artifacts in amplification reactions, even when only a single pair of primers is employed, can complicate downstream applications such as qPCR, cloning, gene expression analysis and sample preparation for next-generation sequencing. In some downstream applications, including several next-generation sequencing methods, this problem can be compounded by the requirement to practice a secondary amplification step, since the artifacts can be further amplified during the secondary amplification. For example, downstream sequencing applications can involve the generation of clonally amplified nucleic acid populations that are individually attached to separate supports, such as beads, using emulsion PCR ("emPCR") and enrichment for clonal amplicons performed via positive selection. In such applications, the artifacts can be carried all the way through the library generation process to the emPCR stage, producing DNA capture beads that include non-specific amplification products. These artifact-containing beads can be selected for during the enrichment process with the template containing beads but are genetically non-informative.

[0007] Nucleic acid molecules amplified in a multiplex PCR reaction can be used in many downstream analysis or assays with, or without, further purification or manipulation. For example, the products of a multiplex PCR reaction (amplicons) when obtained in sufficient yield can be used for single nucleotide polymorphism (SNP) analysis, genotyping, copy number variation analysis, epigenetic analysis, gene expression analysis, hybridization arrays, analysis of gene mutations including but not limited to detection, prognosis and/or diagnosis of disease states, detection and analysis of rare or low frequency allele mutations, nucleic acid sequencing including but not limited to *de novo* sequencing or targeted resequencing, and the like.

[0008] Exemplary next-generation sequencing systems include the Ion Torrent PGM™ sequencer (Life Technologies) and the Ion Torrent Proton™ Sequencer (Life Technologies), which are ion-based sequencing systems that sequence nucleic acid templates by detecting ions produced as a byproduct of nucleotide incorporation. Typically, hydrogen ions are released as byproducts of nucleotide incorporations occurring during template-dependent nucleic acid synthesis by a polymerase. The Ion Torrent PGM™ sequencer and Ion Proton™ Sequencer detect the nucleotide incorporations by detecting the hydrogen ion byproducts of the nucleotide incorporations. The Ion Torrent PGM™ sequencer and Ion Proton™ sequencer include a plurality of nucleic acid templates to be sequenced, each template disposed within a respective sequencing reaction well in an array. The wells of the array are each coupled to at least one ion sensor that can detect the release of

H⁺ ions or changes in solution pH produced as a byproduct of nucleotide incorporation. The ion sensor comprises a field effect transistor (FET) coupled to an ion-sensitive detection layer that can sense the presence of H⁺ ions or changes in solution pH. The ion sensor provides output signals indicative of nucleotide incorporation which can be represented as voltage changes whose magnitude correlates with the H⁺ ion concentration in a respective well or reaction chamber. Different nucleotide types are flowed serially into the reaction chamber, and are incorporated by the polymerase into an extending primer (or polymerization site) in an order determined by the sequence of the template. Each nucleotide incorporation is accompanied by the release of H⁺ ions in the reaction well, along with a concomitant change in the localized pH. The release of H⁺ ions is registered by the FET of the sensor, which produces signals indicating the occurrence of the nucleotide incorporation. Nucleotides that are not incorporated during a particular nucleotide flow will not produce signals. The amplitude of the signals from the FET may also be correlated with the number of nucleotides of a particular type incorporated into the extending nucleic acid molecule thereby permitting homopolymer regions to be resolved. Thus, during a run of the sequencer multiple nucleotide flows into the reaction chamber along with incorporation monitoring across a multiplicity of wells or reaction chambers permit the instrument to resolve the sequence of many nucleic acid templates simultaneously. Further details regarding the compositions, design and operation of the Ion Torrent PGM™ sequencer can be found, for example, in U.S. Patent Application Ser. No. 12/002781, now published as U.S. Patent Publication No. 2009/0026082; U.S. Patent Application Ser. No. 12/474897, now published as U.S. Patent Publication No. 2010/0137143; and U.S. Patent Application Ser. No. 12/492844, now published as U.S. Patent Publication No. 2010/0282617, all of which applications are incorporated by reference herein in their entireties. In some embodiments, amplicons can be manipulated or amplified through bridge amplification or emPCR to generate a plurality of clonal templates that are suitable for a variety of downstream processes including nucleic acid sequencing. In one embodiment, nucleic acid templates to be sequenced using the Ion Torrent PGM™ or Ion Torrent Proton™ system can be prepared from a population of nucleic acid molecules using one or more of the target-specific amplification techniques outlined herein. Optionally, following target-specific amplification a secondary and/or tertiary amplification process including, but not limited to a library amplification step and/or a clonal amplification step such as emPCR can be performed.

[0009] As the number of nucleic acid targets desired to be amplified within a sample nucleic acid population increases, the challenge of selectively amplifying these targets while avoiding the

formation of undesirable amplification artifacts can correspondingly increase. For example, the formation of artifacts including primer-dimers and superamplicons can be a greater issue in multiplex PCR reactions where PCR primer pairs for multiple targets are combined in a single reaction tube and co-amplified. In multiplex PCR, the presence of additional primer pairs at elevated concentrations relative to the template DNA makes primer-primer interactions, and the formation of primer-dimers and other artifacts, more likely.

[0010] Current methods for avoiding or reducing the formation of artifacts, such as primer-dimers, during nucleic acid amplification center around the primer design process and often utilize dedicated software packages (e.g., DNAsoftware's Visual OMP, MultiPLX, ABI's Primer Express, etc.) to design primer pairs that are predicted to exhibit minimal interaction between the other primers in the pool during amplification. Through the use of such software, primers can be designed to be as target-specific or amplicon-specific as possible, and often are grouped into subsets to minimize primer-primer interactions, primer-dimer formation and superamplicons. Stringent design parameters, however, limit the number of amplicons that can be co-amplified simultaneously and in some cases may prevent the amplification of some amplicons altogether. Other current methods require the use of multiple PCR primer pools to segregate primers into non-overlapping pools to minimize or prevent primer artifacts during the amplification step. Other methods include the use of multiple primer pools or single plex reactions to enhance the overall yield of amplification product per reaction. In a multiplex PCR reaction, each primer pair competes in the amplification reaction with additional primer pairs for a finite amount of dNTPs, polymerase and other reagents. There is therefore a need for improved methods, compositions, systems, apparatuses and kits that allow for the selective amplification of multiple target nucleic acid molecules within a population of nucleic acid molecules while avoiding, or minimizing, the formation of artifacts (also referred to as non-specific amplification products), including primer dimers. There is also a need for improved methods, compositions, systems, apparatuses and kits that allow for the selective amplification of multiple target nucleic acid molecules from a single nucleic acid sample, such as genomic DNA and/or formalin-fixed paraffin embedded (FFPE) DNA while avoiding, or minimizing, the formation of artifacts. There is also a need in the art for improved methods, compositions, systems and kits that allow for the simultaneous amplification of thousands of target-specific nucleic acid molecules in a single reaction, which can be used in any applicable downstream assay or analysis.

[0011] The practice of the present subject matter may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, molecular biology (including

recombinant techniques), cell biology, and biochemistry, which are within the skill of the art. Such conventional techniques include, but are not limited to, preparation of synthetic polynucleotides, polymerization techniques, chemical and physical analysis of polymer particles, preparation of nucleic acid libraries, nucleic acid sequencing and analysis, and the like. Specific illustrations of suitable techniques can be used by reference to the examples provided herein. Other equivalent conventional procedures can also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *PCR Primer: A Laboratory Manual*, and *Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Hermanson, *Bioconjugate Techniques*, Second Edition (Academic Press, 2008); Merkus, *Particle Size Measurements* (Springer, 2009); Rubinstein and Colby, *Polymer Physics* (Oxford University Press, 2003); and the like.

[0012] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which these inventions belong. All patents, patent applications, published applications, treatises and other publications referred to herein, both *supra* and *infra*, are incorporated by reference in their entirety. If a definition and/or description is set forth herein that is contrary to or otherwise inconsistent with any definition set forth in the patents, patent applications, published applications, and other publications that are herein incorporated by reference, the definition and/or description set forth herein prevails over the definition that is incorporated by reference.

[0013] As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having” or any other variation thereof, are intended to cover a non-exclusive inclusion. For example, a process, method, article, or apparatus that comprises a list of features is not necessarily limited only to those features but may include other features not expressly listed or inherent to such process, method, article, or apparatus. Further, unless expressly stated to the contrary, “or” refers to an inclusive-or and not to an exclusive-or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

[0014] The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way.

SUMMARY

[0015] In one aspect of the invention, a plurality of primer pairs is provided that are configured to specifically hybridize to a panel of SNP regions of a human genome, where the panel of SNP regions are selected from the SNP regions of Table 1, and wherein at least one primer of at least one primer pair comprises a cleavable nucleotide. In some embodiments, the plurality of primer pairs are configured to hybridize to the panel of SNPs which are entries 1-136 of Table 1. In other embodiments, the plurality of primer pairs are configured to hybridize to the panel of SNPs which are entries 1-103 of Table 1. In yet other embodiments, the at least one primer of the at least one primer pair comprises an uracil cleavable nucleotide.

[0016] In another aspect of the invention, a method for amplifying a plurality of different target sequences within a sample is provided, including the steps of: amplifying within a single amplification reaction mixture a plurality of different target sequences from a sample including the plurality of different target sequences, wherein the amplifying includes contacting at least some portion of the sample with a plurality of target-specific primers, and a polymerase under amplification conditions, thereby producing an amplified plurality of target sequences, wherein at least two of the different amplified target sequences are less than 50% complementary to each other and wherein at least one of the plurality of target-specific primers and at least one of the amplified target sequences includes a cleavable group; cleaving a cleavable group of at least one amplified target sequence of the amplified plurality of target sequences; ligating at least one adapter to at least one amplified target sequence in a blunt-ended ligation reaction, thereby producing one or more adapter-ligated amplified target sequences, and reamplifying at least one of the adapter-ligated amplified target sequences.

[0017] In some embodiments, one or more of the at least one adapter is not substantially complementary to at least one amplified target sequence. In other embodiments, the reamplifying includes contacting the at least one adapter-ligated amplified target sequence with one or more primers including a sequence that is complementary to at least one of the adapters or their complement, and a polymerase under amplification conditions, thereby producing at least one reamplified adapter-ligated amplified target sequence. In yet other embodiments, at least one of the one or more adapters or their complements is not substantially complementary to at least one amplified target sequence. The invention further provides at least one target-specific primer that is substantially complementary to at least a portion of a corresponding target sequence in the sample. In other embodiments, an adapter that is ligated to at least one of the amplified target sequences is susceptible to exonuclease digestion. In yet other embodiments, an adapter that is ligated to at least

one of the amplified target sequences does not include a protecting group. In yet other embodiments, the ligating step includes contacting at least one amplified target sequence having a 3' end and a 5' end with a ligation reaction mixture including one or more adapters and a ligase under ligation conditions, wherein none of the adapters in the ligation reaction mixture includes, prior to the ligating, a target-specific sequence. The invention also provides that the ligating step includes contacting at least one amplified target sequence with a ligation reaction mixture including one or more adapters and a ligase under ligation conditions, wherein the ligation reaction mixture does not include one or more additional oligonucleotide adapters prior to ligating the one or more adapters to at least one amplified target sequence. In other embodiment, the amplifying step further includes a digestion step prior to the ligating step, thereby producing a plurality of blunt-end amplified target sequences possessing a 5' phosphate group.

[0018] In some embodiments, the disclosure relates generally to methods, compositions, systems, apparatuses and kits for performing multiplex amplification of nucleic acids. In some embodiments, the method includes amplifying a plurality of target sequences within a sample including two or more target sequences. Optionally, multiple target sequences of interest from a sample can be amplified using one or more target-specific primers in the presence of a polymerase under amplification conditions to produce a plurality of amplified target sequences. The amplifying optionally includes contacting a nucleic acid molecule including at least one target sequence with one or more target-specific primers and at least one polymerase under amplification conditions. The contacting can produce one or more amplified target sequences.

[0019] In some embodiments, the disclosure relates generally to a composition comprising a plurality of target-specific primers of about 15 nucleotides to about 40 nucleotides in length having at least two or more following criteria: a cleavable group located at a 3' end of substantially all of the plurality of primers, a cleavable group located near or about a central nucleotide of substantially all of the plurality of primers, substantially all of the plurality of primers at a 5' end including only non-cleavable nucleotides, minimal cross-hybridization to substantially all of the primers in the plurality of primers, minimal cross-hybridization to non-specific sequences present in a sample, minimal self-complementarity, and minimal nucleotide sequence overlap at a 3' end or a 5' end of substantially all of the primers in the plurality of primers. In some embodiments, the composition can include any 3, 4, 5, 6 or 7 of the above criteria.

[0020] In some embodiments, the disclosure relates generally to a composition comprising a plurality of at least 2 target-specific primers of about 15 nucleotides to about 40 nucleotides in length

having two or more of the following criteria, a cleavable group located near or about a central nucleotide of substantially all of the plurality of primers, substantially all of the plurality of primers at a 5' end including only non-cleavable nucleotides, substantially all of the plurality of primers having less than 20% of the nucleotides across the primer's entire length containing a cleavable group, at least one primer having a complementary nucleic acid sequence across its entire length to a target sequence present in a sample, minimal cross-hybridization to substantially all of the primers in the plurality of primers, minimal cross-hybridization to non-specific sequences present in a sample, and minimal nucleotide sequence overlap at a 3' end or a 5' end of substantially all of the primers in the plurality of primers. In some embodiments, the composition can include any 3, 4, 5, 6 or 7 of the above criteria.

[0021] In some embodiments, the disclosure is generally related to an amplification product generated by amplifying at least one target sequence present in a sample with one or more target-specific primers disclosed herein or one or more target-specific primers designed using the primer selection criteria disclosed herein. In some embodiments, the disclosure is generally related to an amplification product generated by contacting at least one target sequence in a sample with one or more target-specific primers disclosed herein or one or more target-specific primers designed using the primer selection criteria disclosed herein under amplification conditions. In some embodiments, the amplification product can include one or more mutations associated with cancer or inherited disease. For example, a sample suspected of containing one or more mutations associated with at least one cancer can be subjected to any one of the amplification methods disclosed herein. The amplification products obtained from the selected amplification method can optionally be compared to a normal or matched sample known to be noncancerous with respect to the at least one cancer, and can therefore be used as a reference sample. In some embodiments, the amplification products obtained by the methods disclosed herein can be optionally sequenced using any suitable nucleic acid sequencing platform to determine the nucleic acid sequence of the amplification products, and optionally compared to sequencing information from the normal or non-cancerous sample. In some embodiments, amplification products can include one or more markers associated with antibiotic resistance, pathogenicity or genetic modification. In some embodiments, nucleic acid sequences of one or more amplification products obtained by contacting at least one target sequence with at least one target-specific primer under amplification conditions can be used to determine the presence or absence of a genetic variant within the one or more amplification products.

[0022] In some embodiments, the disclosure generally relates to compositions (as well as related kits, methods, systems and apparatuses using the disclosed compositions) for performing nucleic acid amplification and nucleic acid synthesis. In some embodiments, the composition includes a plurality of target-specific primer pairs, at least one target-specific primer pair including a target-specific forward primer and a target-specific reverse primer. In some embodiments, the composition includes at least 100, 200, 500, 750, 1000, 2500, 5000, 7500, 10000, 12000, 15000, 17500, 20000 or 50000 different primer pairs, some or all of which can be target-specific. Optionally at least two of the different target-specific primer pairs are directed to (i.e., are specific for) different target sequences.

[0023] In some embodiments, the composition includes at least one target-specific primer pair that can be specific for at least one amplified target sequence. In some embodiments, the composition includes a plurality of target-specific primer pairs, at least two target-specific primer pairs being specific for at different amplified target sequences. In some embodiments, the composition includes a target-specific primer pair, which each member of the primer pair includes a target-specific primer that can hybridize to at least a portion of a first amplified sequence or its complement, and that is substantially non-complementary to the 3' end or the 5' end of any other amplified sequence in the sample. In some embodiments, the composition includes at least one target-specific primer pair that can be substantially non-complementary to a portion of any other nucleic acid molecule in the sample. In some embodiments, the compositions include a plurality of target-specific primer pairs that include one or more cleavable groups at one or more locations within the target-specific primer pair.

[0024] In some embodiments, the composition includes one or more target-specific primer pairs that can amplify a nucleotide polymorphism or portion thereof. For example, a plurality of target-specific primer pairs can uniformly amplify one gene, exon, coding region, exome or portion thereof. In some embodiments, the compositions include target-specific primer pairs designed to minimize overlap of nucleotide sequences amplified using the one or more target-specific primer pairs. In some embodiments, the nucleotide sequence overlap between one or more target-specific primers can be minimized at the 3' end, the 5' end, or both. In some embodiments, at least one primer in a plurality of target-specific primers includes less than 5 nucleotides of nucleotide sequence overlap at the 3' end, 5' end or both. In some embodiments, at least one target-specific primer of a plurality of target-specific primers includes a nucleotide sequence gap of at least one nucleotide, as compared to the plurality of target-specific primers. In some embodiments, the compositions include one or more target-specific primer pairs designed to comprehensively amplify one or more genes or exons. For

example, a plurality of target-specific primer pairs can be designed to uniformly amplify (i.e., provide 100% representation of all nucleotides) in a single gene or exon

[0025] In some embodiments, the disclosure relates generally to a kit for performing multiplex nucleic acid amplification or multiplex nucleic acid synthesis. In some embodiments, the kit comprises a plurality of target-specific primers. In some embodiments, the kit can further include a polymerase, at least one adapter and/or a cleaving reagent. In some embodiments, the kit can also include dATP, dCTP, dGTP, dTTP and/or an antibody. In some embodiments, the cleaving reagent is any reagent that can cleave one or more cleaving groups present in one or more target-specific primers. In some embodiments the cleaving reagent can include an enzyme or chemical reagent. In some embodiments, the cleaving reagent can include an enzyme with an affinity for apurinic bases. In some embodiments, the cleaving reagent can include a first enzyme with an affinity for a first cleavable group and can further include a second enzyme with an affinity for a second cleavable group. In some embodiments, the kit can further include an enzyme with an affinity for abasic sites. In some embodiments, the polymerase is a thermostable polymerase. In some embodiments, the kits can include one or more preservatives, adjuvants or nucleic acid sequencing barcodes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] The accompanying drawings, which are incorporated into and form a part of the specification, illustrate one or more exemplary embodiments and serve to explain the principles of various exemplary embodiments. The drawings are exemplary and explanatory only and are not to be construed as limiting or restrictive in any way.

[0027] FIG. 1 is a schematic outlining an exemplary embodiment of a method utilizing degradable amplification primers according to the disclosure.

[0028] FIG. 2 is a schematic outlining an exemplary embodiment of a method obtaining a target-specific amplicon library according to the disclosure.

[0029] FIG. 3 illustrates a system for designing primers or assays according to an exemplary embodiment.

[0030] FIG. 4 illustrates a system for designing primers or assays according to an exemplary embodiment.

[0031] FIG. 5 illustrates an amplicon sequence including an insert sequence surrounded by a pair of primers designed according to an exemplary embodiment.

[0032] FIG. 6 illustrates PCR amplification of an amplicon sequence (which may be referred to as “tile” herein) including an insert surrounded by a pair of primers designed according to an exemplary embodiment.

[0033] FIG. 7 illustrates a set of candidate amplicons for a given target region, each including an insert surrounded by a pair of primers, for tiling and pooling according to an exemplary embodiment.

[0034] FIG. 8 illustrates a method according to an exemplary embodiment.

DETAILED DESCRIPTION

[0035] The following description of various exemplary embodiments is exemplary and explanatory only and is not to be construed as limiting or restrictive in any way. Other embodiments, features, objects, and advantages of the present teachings will be apparent from the description and accompanying drawings, and from the claims.

[0036] As used herein, “amplify”, “amplifying” or “amplification reaction” and their derivatives, refer generally to any action or process whereby at least a portion of a nucleic acid molecule (referred to as a template nucleic acid molecule) is replicated or copied into at least one additional nucleic acid molecule. The additional nucleic acid molecule optionally includes sequence that is substantially identical or substantially complementary to at least some portion of the template nucleic acid molecule. The template nucleic acid molecule can be single-stranded or double-stranded and the additional nucleic acid molecule can independently be single-stranded or double-stranded. In some embodiments, amplification includes a template-dependent in vitro enzyme-catalyzed reaction for the production of at least one copy of at least some portion of the nucleic acid molecule or the production of at least one copy of a nucleic acid sequence that is complementary to at least some portion of the nucleic acid molecule. Amplification optionally includes linear or exponential replication of a nucleic acid molecule. In some embodiments, such amplification is performed using isothermal conditions; in other embodiments, such amplification can include thermocycling. In some embodiments, the amplification is a multiplex amplification that includes the simultaneous amplification of a plurality of target sequences in a single amplification reaction. At least some of the target sequences can be situated on the same nucleic acid molecule or on different target nucleic acid molecules included in the single amplification reaction. In some embodiments, “amplification” includes amplification of at least some portion of DNA- and RNA-based nucleic acids alone, or in combination. The amplification reaction can include single or double-stranded nucleic acid substrates

and can further including any of the amplification processes known to one of ordinary skill in the art. In some embodiments, the amplification reaction includes polymerase chain reaction (PCR).

[0037] As used herein, "amplification conditions" and its derivatives, generally refers to conditions suitable for amplifying one or more nucleic acid sequences. Such amplification can be linear or exponential. In some embodiments, the amplification conditions can include isothermal conditions or alternatively can include thermocycling conditions, or a combination of isothermal and thermocycling conditions. In some embodiments, the conditions suitable for amplifying one or more nucleic acid sequences includes polymerase chain reaction (PCR) conditions. Typically, the amplification conditions refer to a reaction mixture that is sufficient to amplify nucleic acids such as one or more target sequences, or to amplify an amplified target sequence ligated to one or more adapters, e.g., an adapter-ligated amplified target sequence. Generally, the amplification conditions include a catalyst for amplification or for nucleic acid synthesis, for example a polymerase; a primer that possesses some degree of complementarity to the nucleic acid to be amplified; and nucleotides, such as deoxyribonucleotide triphosphates (dNTPs) to promote extension of the primer once hybridized to the nucleic acid. The amplification conditions can require hybridization or annealing of a primer to a nucleic acid, extension of the primer and a denaturing step in which the extended primer is separated from the nucleic acid sequence undergoing amplification. Typically, but not necessarily, amplification conditions can include thermocycling; in some embodiments, amplification conditions include a plurality of cycles where the steps of annealing, extending and separating are repeated. Typically, the amplification conditions include cations such as Mg^{++} or Mn^{++} (e.g., $MgCl_2$, etc) and can also include various modifiers of ionic strength.

[0038] As used herein, "target sequence" or "target sequence of interest" and its derivatives, refers generally to any single or double-stranded nucleic acid sequence that can be amplified or synthesized according to the disclosure, including any nucleic acid sequence suspected or expected to be present in a sample. In some embodiments, the target sequence is present in double-stranded form and includes at least a portion of the particular nucleotide sequence to be amplified or synthesized, or its complement, prior to the addition of target-specific primers or appended adapters. Target sequences can include the nucleic acids to which primers useful in the amplification or synthesis reaction can hybridize prior to extension by a polymerase. In some embodiments, the term refers to a nucleic acid sequence whose sequence identity, ordering or location of nucleotides is determined by one or more of the methods of the disclosure.

[0039] As defined herein, “sample” and its derivatives, is used in its broadest sense and includes any specimen, culture and the like that is suspected of including a target. In some embodiments, the sample comprises DNA, RNA, PNA, LNA, chimeric, hybrid, or multiplex-forms of nucleic acids. The sample can include any biological, clinical, surgical, agricultural, atmospheric or aquatic-based specimen containing one or more nucleic acids. The term also includes any isolated nucleic acid sample such a genomic DNA, fresh-frozen or formalin-fixed paraffin-embedded nucleic acid specimen.

[0040] As used herein, “contacting” and its derivatives, when used in reference to two or more components, refers generally to any process whereby the approach, proximity, mixture or commingling of the referenced components is promoted or achieved without necessarily requiring physical contact of such components, and includes mixing of solutions containing any one or more of the referenced components with each other. The referenced components may be contacted in any particular order or combination and the particular order of recitation of components is not limiting. For example, “contacting A with B and C” encompasses embodiments where A is first contacted with B then C, as well as embodiments where C is contacted with A then B, as well as embodiments where a mixture of A and C is contacted with B, and the like. Furthermore, such contacting does not necessarily require that the end result of the contacting process be a mixture including all of the referenced components, as long as at some point during the contacting process all of the referenced components are simultaneously present or simultaneously included in the same mixture or solution. For example, “contacting A with B and C” can include embodiments wherein C is first contacted with A to form a first mixture, which first mixture is then contacted with B to form a second mixture, following which C is removed from the second mixture; optionally A can then also be removed, leaving only B. Where one or more of the referenced components to be contacted includes a plurality (e.g., “contacting a target sequence with a plurality of target-specific primers and a polymerase”), then each member of the plurality can be viewed as an individual component of the contacting process, such that the contacting can include contacting of any one or more members of the plurality with any other member of the plurality and/or with any other referenced component (e.g., some but not all of the plurality of target specific primers can be contacted with a target sequence, then a polymerase, and then with other members of the plurality of target-specific primers) in any order or combination.

[0041] As used herein, the term “primer” and its derivatives refer generally to any polynucleotide that can hybridize to a target sequence of interest. In some embodiments, the primer can also serve to

prime nucleic acid synthesis. Typically, the primer functions as a substrate onto which nucleotides can be polymerized by a polymerase; in some embodiments, however, the primer can become incorporated into the synthesized nucleic acid strand and provide a site to which another primer can hybridize to prime synthesis of a new strand that is complementary to the synthesized nucleic acid molecule. The primer may be comprised of any combination of nucleotides or analogs thereof, which may be optionally linked to form a linear polymer of any suitable length. In some embodiments, the primer is a single-stranded oligonucleotide or polynucleotide. (For purposes of this disclosure, the terms "polynucleotide" and "oligonucleotide" are used interchangeably herein and do not necessarily indicate any difference in length between the two). In some embodiments, the primer is single-stranded but it can also be double-stranded. The primer optionally occurs naturally, as in a purified restriction digest, or can be produced synthetically. In some embodiments, the primer acts as a point of initiation for amplification or synthesis when exposed to amplification or synthesis conditions; such amplification or synthesis can occur in a template-dependent fashion and optionally results in formation of a primer extension product that is complementary to at least a portion of the target sequence. Exemplary amplification or synthesis conditions can include contacting the primer with a polynucleotide template (e.g., a template including a target sequence), nucleotides and an inducing agent such as a polymerase at a suitable temperature and pH to induce polymerization of nucleotides onto an end of the target-specific primer. If double-stranded, the primer can optionally be treated to separate its strands before being used to prepare primer extension products. In some embodiments, the primer is an oligodeoxyribonucleotide or an oligoribonucleotide. In some embodiments, the primer can include one or more nucleotide analogs. The exact length and/or composition, including sequence, of the target-specific primer can influence many properties, including melting temperature (T_m), GC content, formation of secondary structures, repeat nucleotide motifs, length of predicted primer extension products, extent of coverage across a nucleic acid molecule of interest, number of primers present in a single amplification or synthesis reaction, presence of nucleotide analogs or modified nucleotides within the primers, and the like. In some embodiments, a primer can be paired with a compatible primer within an amplification or synthesis reaction to form a primer pair consisting of a forward primer and a reverse primer. In some embodiments, the forward primer of the primer pair includes a sequence that is substantially complementary to at least a portion of a strand of a nucleic acid molecule, and the reverse primer of the primer of the primer pair includes a sequence that is substantially identical to at least a portion of the strand. In some embodiments, the forward primer and the reverse primer are capable of

hybridizing to opposite strands of a nucleic acid duplex. Optionally, the forward primer primes synthesis of a first nucleic acid strand, and the reverse primer primes synthesis of a second nucleic acid strand, wherein the first and second strands are substantially complementary to each other, or can hybridize to form a double-stranded nucleic acid molecule. In some embodiments, one end of an amplification or synthesis product is defined by the forward primer and the other end of the amplification or synthesis product is defined by the reverse primer. In some embodiments, where the amplification or synthesis of lengthy primer extension products is required, such as amplifying an exon, coding region, or gene, several primer pairs can be created that span the desired length to enable sufficient amplification of the region. In some embodiments, a primer can include one or more cleavable groups. In some embodiments, primer lengths are in the range of about 10 to about 60 nucleotides, about 12 to about 50 nucleotides and about 15 to about 40 nucleotides in length. Typically, a primer is capable of hybridizing to a corresponding target sequence and undergoing primer extension when exposed to amplification conditions in the presence of dNTPS and a polymerase. In some instances, the particular nucleotide sequence or a portion of the primer is known at the outset of the amplification reaction or can be determined by one or more of the methods disclosed herein. In some embodiments, the primer includes one or more cleavable groups at one or more locations within the primer.

[0042] As used herein, "target-specific primer" and its derivatives, refers generally to a single stranded or double-stranded polynucleotide, typically an oligonucleotide, that includes at least one sequence that is at least 50% complementary, typically at least 75% complementary or at least 85% complementary, more typically at least 90% complementary, more typically at least 95% complementary, more typically at least 98% or at least 99% complementary, or identical, to at least a portion of a nucleic acid molecule that includes a target sequence. In such instances, the target-specific primer and target sequence are described as "corresponding" to each other. In some embodiments, the target-specific primer is capable of hybridizing to at least a portion of its corresponding target sequence (or to a complement of the target sequence); such hybridization can optionally be performed under standard hybridization conditions or under stringent hybridization conditions. In some embodiments, the target-specific primer is not capable of hybridizing to the target sequence, or to its complement, but is capable of hybridizing to a portion of a nucleic acid strand including the target sequence, or to its complement. In some embodiments, the target-specific primer includes at least one sequence that is at least 75% complementary, typically at least 85% complementary, more typically at least 90% complementary, more typically at least 95%

complementary, more typically at least 98% complementary, or more typically at least 99% complementary, to at least a portion of the target sequence itself; in other embodiments, the target-specific primer includes at least one sequence that is at least 75% complementary, typically at least 85% complementary, more typically at least 90% complementary, more typically at least 95% complementary, more typically at least 98% complementary, or more typically at least 99% complementary, to at least a portion of the nucleic acid molecule other than the target sequence. In some embodiments, the target-specific primer is substantially non-complementary to other target sequences present in the sample; optionally, the target-specific primer is substantially non-complementary to other nucleic acid molecules present in the sample. In some embodiments, nucleic acid molecules present in the sample that do not include or correspond to a target sequence (or to a complement of the target sequence) are referred to as "non-specific" sequences or "non-specific nucleic acids". In some embodiments, the target-specific primer is designed to include a nucleotide sequence that is substantially complementary to at least a portion of its corresponding target sequence. In some embodiments, a target-specific primer is at least 95% complementary, or at least 99% complementary, or identical, across its entire length to at least a portion of a nucleic acid molecule that includes its corresponding target sequence. In some embodiments, a target-specific primer can be at least 90%, at least 95% complementary, at least 98% complementary or at least 99% complementary, or identical, across its entire length to at least a portion of its corresponding target sequence. In some embodiments, a forward target-specific primer and a reverse target-specific primer define a target-specific primer pair that can be used to amplify the target sequence via template-dependent primer extension. Typically, each primer of a target-specific primer pair includes at least one sequence that is substantially complementary to at least a portion of a nucleic acid molecule including a corresponding target sequence but that is less than 50% complementary to at least one other target sequence in the sample. In some embodiments, amplification can be performed using multiple target-specific primer pairs in a single amplification reaction, wherein each primer pair includes a forward target-specific primer and a reverse target-specific primer, each including at least one sequence that substantially complementary or substantially identical to a corresponding target sequence in the sample, and each primer pair having a different corresponding target sequence. In some embodiments, the target-specific primer can be substantially non-complementary at its 3' end or its 5' end to any other target-specific primer present in an amplification reaction. In some embodiments, the target-specific primer can include minimal cross hybridization to other target-specific primers in the amplification reaction. In some embodiments,

target-specific primers include minimal cross-hybridization to non-specific sequences in the amplification reaction mixture. In some embodiments, the target-specific primers include minimal self-complementarity. In some embodiments, the target-specific primers can include one or more cleavable groups located at the 3' end. In some embodiments, the target-specific primers can include one or more cleavable groups located near or about a central nucleotide of the target-specific primer. In some embodiments, one of more targets-specific primers includes only non-cleavable nucleotides at the 5' end of the target-specific primer. In some embodiments, a target specific primer includes minimal nucleotide sequence overlap at the 3' end or the 5' end of the primer as compared to one or more different target-specific primers, optionally in the same amplification reaction. In some embodiments 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more, target-specific primers in a single reaction mixture include one or more of the above embodiments. In some embodiments, substantially all of the plurality of target-specific primers in a single reaction mixture includes one or more of the above embodiments.

[0043] As used herein, "polymerase" and its derivatives, generally refers to any enzyme that can catalyze the polymerization of nucleotides (including analogs thereof) into a nucleic acid strand. Typically but not necessarily, such nucleotide polymerization can occur in a template-dependent fashion. Such polymerases can include without limitation naturally occurring polymerases and any subunits and truncations thereof, mutant polymerases, variant polymerases, recombinant, fusion or otherwise engineered polymerases, chemically modified polymerases, synthetic molecules or assemblies, and any analogs, derivatives or fragments thereof that retain the ability to catalyze such polymerization. Optionally, the polymerase can be a mutant polymerase comprising one or more mutations involving the replacement of one or more amino acids with other amino acids, the insertion or deletion of one or more amino acids from the polymerase, or the linkage of parts of two or more polymerases. Typically, the polymerase comprises one or more active sites at which nucleotide binding and/or catalysis of nucleotide polymerization can occur. Some exemplary polymerases include without limitation DNA polymerases and RNA polymerases. The term "polymerase" and its variants, as used herein, also refers to fusion proteins comprising at least two portions linked to each other, where the first portion comprises a peptide that can catalyze the polymerization of nucleotides into a nucleic acid strand and is linked to a second portion that comprises a second polypeptide. In some embodiments, the second polypeptide can include a reporter enzyme or a processivity-enhancing domain. Optionally, the polymerase can possess 5' exonuclease activity or terminal transferase activity. In some embodiments, the polymerase can be

optionally reactivated, for example through the use of heat, chemicals or re-addition of new amounts of polymerase into a reaction mixture. In some embodiments, the polymerase can include a hot-start polymerase or an aptamer based polymerase that optionally can be reactivated.

[0044] As used herein, the term “nucleotide” and its variants comprises any compound, including without limitation any naturally occurring nucleotide or analog thereof, which can bind selectively to, or can be polymerized by, a polymerase. Typically, but not necessarily, selective binding of the nucleotide to the polymerase is followed by polymerization of the nucleotide into a nucleic acid strand by the polymerase; occasionally however the nucleotide may dissociate from the polymerase without becoming incorporated into the nucleic acid strand, an event referred to herein as a “non-productive” event. Such nucleotides include not only naturally occurring nucleotides but also any analogs, regardless of their structure, that can bind selectively to, or can be polymerized by, a polymerase. While naturally occurring nucleotides typically comprise base, sugar and phosphate moieties, the nucleotides of the present disclosure can include compounds lacking any one, some or all of such moieties. In some embodiments, the nucleotide can optionally include a chain of phosphorus atoms comprising three, four, five, six, seven, eight, nine, ten or more phosphorus atoms. In some embodiments, the phosphorus chain can be attached to any carbon of a sugar ring, such as the 5' carbon. The phosphorus chain can be linked to the sugar with an intervening O or S. In one embodiment, one or more phosphorus atoms in the chain can be part of a phosphate group having P and O. In another embodiment, the phosphorus atoms in the chain can be linked together with intervening O, NH, S, methylene, substituted methylene, ethylene, substituted ethylene, CNH_2 , $C(O)$, $C(CH_3)$, CH_2CH_2 , or $C(OH)CH_2R$ (where R can be a 4-pyridine or 1-imidazole). In one embodiment, the phosphorus atoms in the chain can have side groups having O, BH_2 , or S. In the phosphorus chain, a phosphorus atom with a side group other than O can be a substituted phosphate group. In the phosphorus chain, phosphorus atoms with an intervening atom other than O can be a substituted phosphate group. Some examples of nucleotide analogs are described in Xu, U.S. Patent No. 7,405,281. In some embodiments, the nucleotide comprises a label and referred to herein as a “labeled nucleotide”; the label of the labeled nucleotide is referred to herein as a “nucleotide label”. In some embodiments, the label can be in the form of a fluorescent dye attached to the terminal phosphate group, i.e., the phosphate group most distal from the sugar. Some examples of nucleotides that can be used in the disclosed methods and compositions include, but are not limited to, ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, ribonucleotide polyphosphates, deoxyribonucleotide polyphosphates, modified ribonucleotide

polyphosphates, modified deoxyribonucleotide polyphosphates, peptide nucleotides, modified peptide nucleotides, metallonucleosides, phosphonate nucleosides, and modified phosphate-sugar backbone nucleotides, analogs, derivatives, or variants of the foregoing compounds, and the like. In some embodiments, the nucleotide can comprise non-oxygen moieties such as, for example, thio- or borano- moieties, in place of the oxygen moiety bridging the alpha phosphate and the sugar of the nucleotide, or the alpha and beta phosphates of the nucleotide, or the beta and gamma phosphates of the nucleotide, or between any other two phosphates of the nucleotide, or any combination thereof. "Nucleotide 5'-triphosphate" refers to a nucleotide with a triphosphate ester group at the 5' position, and are sometimes denoted as "NTP", or "dNTP" and "ddNTP" to particularly point out the structural features of the ribose sugar. The triphosphate ester group can include sulfur substitutions for the various oxygens, e.g. alpha-thio-nucleotide 5'-triphosphates. For a review of nucleic acid chemistry, see: Shabarova, Z. and Bogdanov, A. *Advanced Organic Chemistry of Nucleic Acids*, VCH, New York, 1994.

[0045] The term "extension" and its variants, as used herein, when used in reference to a given primer, comprises any in vivo or in vitro enzymatic activity characteristic of a given polymerase that relates to polymerization of one or more nucleotides onto an end of an existing nucleic acid molecule. Typically but not necessarily such primer extension occurs in a template-dependent fashion; during template-dependent extension, the order and selection of bases is driven by established base pairing rules, which can include Watson-Crick type base pairing rules or alternatively (and especially in the case of extension reactions involving nucleotide analogs) by some other type of base pairing paradigm. In one non-limiting example, extension occurs via polymerization of nucleotides on the 3'OH end of the nucleic acid molecule by the polymerase.

[0046] The term "portion" and its variants, as used herein, when used in reference to a given nucleic acid molecule, for example a primer or a template nucleic acid molecule, comprises any number of contiguous nucleotides within the length of the nucleic acid molecule, including the partial or entire length of the nucleic acid molecule.

[0047] The terms "identity" and "identical" and their variants, as used herein, when used in reference to two or more nucleic acid sequences, refer to similarity in sequence of the two or more sequences (e.g., nucleotide or polypeptide sequences). In the context of two or more homologous sequences, the percent identity or homology of the sequences or subsequences thereof indicates the percentage of all monomeric units (e.g., nucleotides or amino acids) that are the same (i.e., about 70% identity, preferably 75%, 80%, 85%, 90%, 95%, 98% or 99% identity). The percent identity

can be over a specified region, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection. Sequences are said to be "substantially identical" when there is at least 85% identity at the amino acid level or at the nucleotide level. Preferably, the identity exists over a region that is at least about 25, 50, or 100 residues in length, or across the entire length of at least one compared sequence. A typical algorithm for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al, *Nuc. Acids Res.* 25:3389-3402 (1977). Other methods include the algorithms of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), and Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), etc. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent hybridization conditions.

[0048] The terms "complementary" and "complement" and their variants, as used herein, refer to any two or more nucleic acid sequences (e.g., portions or entireties of template nucleic acid molecules, target sequences and/or primers) that can undergo cumulative base pairing at two or more individual corresponding positions in antiparallel orientation, as in a hybridized duplex. Such base pairing can proceed according to any set of established rules, for example according to Watson-Crick base pairing rules or according to some other base pairing paradigm. Optionally there can be "complete" or "total" complementarity between a first and second nucleic acid sequence where each nucleotide in the first nucleic acid sequence can undergo a stabilizing base pairing interaction with a nucleotide in the corresponding antiparallel position on the second nucleic acid sequence. "Partial" complementarity describes nucleic acid sequences in which at least 20%, but less than 100%, of the residues of one nucleic acid sequence are complementary to residues in the other nucleic acid sequence. In some embodiments, at least 50%, but less than 100%, of the residues of one nucleic acid sequence are complementary to residues in the other nucleic acid sequence. In some embodiments, at least 70%, 80%, 90%, 95% or 98%, but less than 100%, of the residues of one nucleic acid sequence are complementary to residues in the other nucleic acid sequence. Sequences are said to be "substantially complementary" when at least 85% of the residues of one nucleic acid sequence are complementary to residues in the other nucleic acid sequence. In some embodiments, two complementary or substantially complementary sequences are capable of hybridizing to each other under standard or stringent hybridization conditions. "Non-complementary" describes nucleic acid sequences in which less than 20% of the residues of one nucleic acid sequence are

complementary to residues in the other nucleic acid sequence. Sequences are said to be "substantially non-complementary" when less than 15% of the residues of one nucleic acid sequence are complementary to residues in the other nucleic acid sequence. In some embodiments, two non-complementary or substantially non-complementary sequences cannot hybridize to each other under standard or stringent hybridization conditions. A "mismatch" is present at any position in the two opposed nucleotides are not complementary. Complementary nucleotides include nucleotides that are efficiently incorporated by DNA polymerases opposite each other during DNA replication under physiological conditions. In a typical embodiment, complementary nucleotides can form base pairs with each other, such as the A-T/U and G-C base pairs formed through specific Watson-Crick type hydrogen bonding, or base pairs formed through some other type of base pairing paradigm, between the nucleobases of nucleotides and/or polynucleotides in positions antiparallel to each other. The complementarity of other artificial base pairs can be based on other types of hydrogen bonding and/or hydrophobicity of bases and/or shape complementarity between bases.

[0049] As used herein, "amplified target sequences" and its derivatives, refers generally to a nucleic acid sequence produced by the amplification of/amplifying the target sequences using target-specific primers and the methods provided herein. The amplified target sequences may be either of the same sense (the positive strand produced in the second round and subsequent even-numbered rounds of amplification) or antisense (i.e., the negative strand produced during the first and subsequent odd-numbered rounds of amplification) with respect to the target sequences. For the purposes of this disclosure, the amplified target sequences are typically less than 50% complementary to any portion of another amplified target sequence in the reaction.

[0050] As used herein, the terms "ligating", "ligation" and their derivatives refer generally to the act or process for covalently linking two or more molecules together, for example, covalently linking two or more nucleic acid molecules to each other. In some embodiments, ligation includes joining nicks between adjacent nucleotides of nucleic acids. In some embodiments, ligation includes forming a covalent bond between an end of a first and an end of a second nucleic acid molecule. In some embodiments, for example embodiments wherein the nucleic acid molecules to be ligated include conventional nucleotide residues, the ligation can include forming a covalent bond between a 5' phosphate group of one nucleic acid and a 3' hydroxyl group of a second nucleic acid thereby forming a ligated nucleic acid molecule. In some embodiments, any means for joining nicks or bonding a 5' phosphate to a 3' hydroxyl between adjacent nucleotides can be employed. In an exemplary embodiment, an enzyme such as a ligase can be used. Generally for the purposes of this

disclosure, an amplified target sequence can be ligated to an adapter to generate an adapter-ligated amplified target sequence.

[0051] As used herein, “ligase” and its derivatives, refers generally to any agent capable of catalyzing the ligation of two substrate molecules. In some embodiments, the ligase includes an enzyme capable of catalyzing the joining of nicks between adjacent nucleotides of a nucleic acid. In some embodiments, the ligase includes an enzyme capable of catalyzing the formation of a covalent bond between a 5' phosphate of one nucleic acid molecule to a 3' hydroxyl of another nucleic acid molecule thereby forming a ligated nucleic acid molecule. Suitable ligases may include, but not limited to, T4 DNA ligase, T4 RNA ligase, and *E. coli* DNA ligase.

[0052] As used herein, “ligation conditions” and its derivatives, generally refers to conditions suitable for ligating two molecules to each other. In some embodiments, the ligation conditions are suitable for sealing nicks or gaps between nucleic acids. As defined herein, a “nick” or “gap” refers to a nucleic acid molecule that lacks a directly bound 5' phosphate of a mononucleotide pentose ring to a 3' hydroxyl of a neighboring mononucleotide pentose ring within internal nucleotides of a nucleic acid sequence. As used herein, the term nick or gap is consistent with the use of the term in the art. Typically, a nick or gap can be ligated in the presence of an enzyme, such as ligase at an appropriate temperature and pH. In some embodiments, T4 DNA ligase can join a nick between nucleic acids at a temperature of about 70-72°C.

[0053] As used herein, “blunt-end ligation” and its derivatives, refers generally to ligation of two blunt-end double-stranded nucleic acid molecules to each other. A “blunt end” refers to an end of a double-stranded nucleic acid molecule wherein substantially all of the nucleotides in the end of one strand of the nucleic acid molecule are base paired with opposing nucleotides in the other strand of the same nucleic acid molecule. A nucleic acid molecule is not blunt ended if it has an end that includes a single-stranded portion greater than two nucleotides in length, referred to herein as an “overhang”. In some embodiments, the end of nucleic acid molecule does not include any single stranded portion, such that every nucleotide in one strand of the end is based paired with opposing nucleotides in the other strand of the same nucleic acid molecule. In some embodiments, the ends of the two blunt ended nucleic acid molecules that become ligated to each other do not include any overlapping, shared or complementary sequence. Typically, blunted-end ligation excludes the use of additional oligonucleotide adapters to assist in the ligation of the double-stranded amplified target sequence to the double-stranded adapter, such as patch oligonucleotides as described in Mitra and

Varley, US2010/0129874, published May 27, 2010. In some embodiments, blunt-ended ligation includes a nick translation reaction to seal a nick created during the ligation process.

[0054] As used herein, the terms “adapter” or “adapter and its complements” and their derivatives, refers generally to any linear oligonucleotide which can be ligated to a nucleic acid molecule of the disclosure. Optionally, the adapter includes a nucleic acid sequence that is not substantially complementary to the 3' end or the 5' end of at least one target sequences within the sample. In some embodiments, the adapter is substantially non-complementary to the 3' end or the 5' end of any target sequence present in the sample. In some embodiments, the adapter includes any single stranded or double-stranded linear oligonucleotide that is not substantially complementary to an amplified target sequence. In some embodiments, the adapter is substantially non-complementary to at least one, some or all of the nucleic acid molecules of the sample. In some embodiments, suitable adapter lengths are in the range of about 10-100 nucleotides, about 12-60 nucleotides and about 15-50 nucleotides in length. Generally, the adapter can include any combination of nucleotides and/or nucleic acids. In some aspects, the adapter can include one or more cleavable groups at one or more locations. In another aspect, the adapter can include a sequence that is substantially identical, or substantially complementary, to at least a portion of a primer, for example a universal primer. In some embodiments, the adapter can include a barcode or tag to assist with downstream cataloguing, identification or sequencing. In some embodiments, a single-stranded adapter can act as a substrate for amplification when ligated to an amplified target sequence, particularly in the presence of a polymerase and dNTPs under suitable temperature and pH.

[0055] As used herein, “reamplifying” or “reamplification” and their derivatives refer generally to any process whereby at least a portion of an amplified nucleic acid molecule is further amplified via any suitable amplification process (referred to in some embodiments as a “secondary” amplification or “reamplification”, thereby producing a reamplified nucleic acid molecule. The secondary amplification need not be identical to the original amplification process whereby the amplified nucleic acid molecule was produced; nor need the reamplified nucleic acid molecule be completely identical or completely complementary to the amplified nucleic acid molecule; all that is required is that the reamplified nucleic acid molecule include at least a portion of the amplified nucleic acid molecule or its complement. For example, the reamplification can involve the use of different amplification conditions and/or different primers, including different target-specific primers than the primary amplification.

[0056] As defined herein, a “cleavable group” generally refers to any moiety that once incorporated into a nucleic acid can be cleaved under appropriate conditions. For example, a cleavable group can be incorporated into a target-specific primer, an amplified sequence, an adapter or a nucleic acid molecule of the sample. In an exemplary embodiment, a target-specific primer can include a cleavable group that becomes incorporated into the amplified product and is subsequently cleaved after amplification, thereby removing a portion, or all, of the target-specific primer from the amplified product. The cleavable group can be cleaved or otherwise removed from a target-specific primer, an amplified sequence, an adapter or a nucleic acid molecule of the sample by any acceptable means. For example, a cleavable group can be removed from a target-specific primer, an amplified sequence, an adapter or a nucleic acid molecule of the sample by enzymatic, thermal, photo-oxidative or chemical treatment. In one aspect, a cleavable group can include a nucleobase that is not naturally occurring. For example, an oligodeoxyribonucleotide can include one or more RNA nucleobases, such as uracil that can be removed by a uracil glycosylase. In some embodiments, a cleavable group can include one or more modified nucleobases (such as 7-methylguanine, 8-oxo-guanine, xanthine, hypoxanthine, 5,6-dihydrouracil or 5-methylcytosine) or one or more modified nucleosides (i.e., 7-methylguanosine, 8-oxo-deoxyguanosine, xanthosine, inosine, dihydrouridine or 5-methylcytidine). The modified nucleobases or nucleotides can be removed from the nucleic acid by enzymatic, chemical or thermal means. In one embodiment, a cleavable group can include a moiety that can be removed from a primer after amplification (or synthesis) upon exposure to ultraviolet light (i.e., bromodeoxyuridine). In another embodiment, a cleavable group can include methylated cytosine. Typically, methylated cytosine can be cleaved from a primer for example, after induction of amplification (or synthesis), upon sodium bisulfite treatment. In some embodiments, a cleavable moiety can include a restriction site. For example, a primer or target sequence can include a nucleic acid sequence that is specific to one or more restriction enzymes, and following amplification (or synthesis), the primer or target sequence can be treated with the one or more restriction enzymes such that the cleavable group is removed. Typically, one or more cleavable groups can be included at one or more locations with a target-specific primer, an amplified sequence, an adapter or a nucleic acid molecule of the sample.

[0057] As used herein, “cleavage step” and its derivatives, generally refers to any process by which a cleavable group is cleaved or otherwise removed from a target-specific primer, an amplified sequence, an adapter or a nucleic acid molecule of the sample. In some embodiments, the cleavage steps involves a chemical, thermal, photo-oxidative or digestive process.

[0058] As used herein, the term “hybridization” is consistent with its use in the art, and generally refers to the process whereby two nucleic acid molecules undergo base pairing interactions. Two nucleic acid molecule molecules are said to be hybridized when any portion of one nucleic acid molecule is base paired with any portion of the other nucleic acid molecule; it is not necessarily required that the two nucleic acid molecules be hybridized across their entire respective lengths and in some embodiments, at least one of the nucleic acid molecules can include portions that are not hybridized to the other nucleic acid molecule. The phrase “hybridizing under stringent conditions” and its variants refers generally to conditions under which hybridization of a target-specific primer to a target sequence occurs in the presence of high hybridization temperature and low ionic strength. In one exemplary embodiment, stringent hybridization conditions include an aqueous environment containing about 30 mM magnesium sulfate, about 300 mM Tris-sulfate at pH 8.9, and about 90 mM ammonium sulfate at about 60-68°C., or equivalents thereof. As used herein, the phrase “standard hybridization conditions” and its variants refers generally to conditions under which hybridization of a primer to an oligonucleotide (i.e., a target sequence), occurs in the presence of low hybridization temperature and high ionic strength. In one exemplary embodiment, standard hybridization conditions include an aqueous environment containing about 100 mM magnesium sulfate, about 500 mM Tris-sulfate at pH 8.9, and about 200 mM ammonium sulfate at about 50-55°C., or equivalents thereof.

[0059] As used herein, “triple nucleotide motif” and its derivatives, refers generally to any nucleotide sequence that is repeated contiguously over three nucleotides e.g., AAA or CCC. Generally, a triple nucleotide motif is not repeated more than five times in a target-specific primer (or adapter) of the disclosure.

[0060] As used herein, “an ACA nucleotide motif” and its derivatives, refers generally to the nucleotide sequence “ACA”. Generally, this motif is not repeated three or more times in a target-specific primer (or adapter) of the disclosure.

[0061] As used herein, “homopolymer” and its derivatives, refers generally to any repeating nucleotide sequence that is eight nucleotides or greater in length e.g., AAAAAAAAAA or CCCCCCCC. Generally, a homopolymer as defined herein is not present in a target-specific primer (or adapter) of the disclosure.

[0062] As used herein, “GC content” and its derivatives, refers generally to the cytosine and guanine content of a nucleic acid molecule. Generally, the GC content of a target-specific primer (or adapter)

of the disclosure is 85% or lower. More typically, the GC content of a target-specific primer or adapter of the disclosure is between 15-85%.

[0063] As used herein, the term "end" and its variants, when used in reference to a nucleic acid molecule, for example a target sequence or amplified target sequence, can include the terminal 30 nucleotides, the terminal 20 and even more typically the terminal 15 nucleotides of the nucleic acid molecule. A linear nucleic acid molecule comprised of linked series of contiguous nucleotides typically includes at least two ends. In some embodiments, one end of the nucleic acid molecule can include a 3' hydroxyl group or its equivalent, and can be referred to as the "3' end" and its derivatives. Optionally, the 3' end includes a 3' hydroxyl group that is not linked to a 5' phosphate group of a mononucleotide pentose ring. Typically, the 3' end includes one or more 5' linked nucleotides located adjacent to the nucleotide including the unlinked 3' hydroxyl group, typically the 30 nucleotides located adjacent to the 3' hydroxyl, typically the terminal 20 and even more typically the terminal 15 nucleotides. Generally, the one or more linked nucleotides can be represented as a percentage of the nucleotides present in the oligonucleotide or can be provided as a number of linked nucleotides adjacent to the unlinked 3' hydroxyl. For example, the 3' end can include less than 50% of the nucleotide length of the oligonucleotide. In some embodiments, the 3' end does not include any unlinked 3' hydroxyl group but can include any moiety capable of serving as a site for attachment of nucleotides via primer extension and/or nucleotide polymerization. In some embodiments, the term "3' end" for example when referring to a target-specific primer, can include the terminal 10 nucleotides, the terminal 5 nucleotides, the terminal 4, 3, 2 or fewer nucleotides at the 3' end. In some embodiments, the term "3' end" when referring to a target-specific primer can include nucleotides located at nucleotide positions 10 or fewer from the 3' terminus.

[0064] As used herein, "5' end", and its derivatives, generally refers to an end of a nucleic acid molecule, for example a target sequence or amplified target sequence, which includes a free 5' phosphate group or its equivalent. In some embodiments, the 5' end includes a 5' phosphate group that is not linked to a 3' hydroxyl of a neighboring mononucleotide pentose ring. Typically, the 5' end includes one or more linked nucleotides located adjacent to the 5' phosphate, typically the 30 nucleotides located adjacent to the nucleotide including the 5' phosphate group, typically the terminal 20 and even more typically the terminal 15 nucleotides. Generally, the one or more linked nucleotides can be represented as a percentage of the nucleotides present in the oligonucleotide or can be provided as a number of linked nucleotides adjacent to the 5' phosphate. For example, the 5' end can be less than 50% of the nucleotide length of an oligonucleotide. In another exemplary

embodiment, the 5' end can include about 15 nucleotides adjacent to the nucleotide including the terminal 5' phosphate. In some embodiments, the 5' end does not include any unlinked 5' phosphate group but can include any moiety capable of serving as a site of attachment to a 3' hydroxyl group, or to the 3' end of another nucleic acid molecule. In some embodiments, the term "5' end" for example when referring to a target-specific primer, can include the terminal 10 nucleotides, the terminal 5 nucleotides, the terminal 4, 3, 2 or fewer nucleotides at the 5' end. In some embodiments, the term "5' end" when referring to a target-specific primer can include nucleotides located at positions 10 or fewer from the 5' terminus. In some embodiments, the 5' end of a target-specific primer can include only non-cleavable nucleotides, for example nucleotides that do not contain one or more cleavable groups as disclosed herein, or a cleavable nucleotide as would be readily determined by one of ordinary skill in the art.

[0065] As used herein, "protecting group" and its derivatives, refers generally to any moiety that can be incorporated into an adapter or target-specific primer that imparts chemical selectivity or protects the target-specific primer or adapter from digestion or chemical degradation. Typically, but not necessarily, a protecting group can include modification of an existing functional group in the target-specific primer or adapter to achieve chemical selectivity. Suitable types of protecting groups include alcohol, amine, phosphate, carbonyl, or carboxylic acid protecting groups. In an exemplary embodiment, the protecting group can include a spacer compound having a chain of carbon atoms.

[0066] As used herein, "DNA barcode" or "DNA tagging sequence" and its derivatives, refers generally to a unique short (6-14 nucleotide) nucleic acid sequence within an adapter that can act as a 'key' to distinguish or separate a plurality of amplified target sequences in a sample. For the purposes of this disclosure, a DNA barcode or DNA tagging sequence can be incorporated into the nucleotide sequence of an adapter.

[0067] As used herein, the phrases "two rounds of target-specific hybridization" or "two rounds of target-specific selection" and their derivatives refers generally to any process whereby the same target sequence is subjected to two consecutive rounds of hybridization-based target-specific selection, wherein a target sequence is hybridized to a target-specific sequence. Each round of hybridization based target-specific selection can include multiple target-specific hybridizations to at least some portion of a target-specific sequence. In one exemplary embodiment, a round of target-specific selection includes a first target-specific hybridization involving a first region of the target sequence and a second target-specific hybridization involving a second region of the target sequence. The first and second regions can be the same or different. In some embodiments, each round of

hybridization-based target-specific selection can include use of two target specific oligonucleotides (e.g., a forward target-specific primer and a reverse target-specific primer), such that each round of selection includes two target-specific hybridizations.

[0068] As used herein, “comparable maximal minimum melting temperatures” and its derivatives, refers generally to the melting temperature (T_m) of each nucleic acid fragment for a single adapter or target-specific primer after cleavage of the cleavable groups. The hybridization temperature of each nucleic acid fragment generated by a single adapter or target-specific primer is compared to determine the maximal minimum temperature required preventing hybridization of any nucleic acid fragment from the target-specific primer or adapter to the target sequence. Once the maximal hybridization temperature is known, it is possible to manipulate the adapter or target-specific primer, for example by moving the location of the cleavable group along the length of the primer, to achieve a comparable maximal minimum melting temperature with respect to each nucleic acid fragment.

[0069] As used herein, “addition only” and its derivatives, refers generally to a series of steps in which reagents and components are added to a first or single reaction mixture. Typically, the series of steps excludes the removal of the reaction mixture from a first vessel to a second vessel in order to complete the series of steps. Generally, an addition only process excludes the manipulation of the reaction mixture outside the vessel containing the reaction mixture. Typically, an addition-only process is amenable to automation and high-throughput.

[0070] As used herein, “synthesizing” and its derivatives, refers generally to a reaction involving nucleotide polymerization by a polymerase, optionally in a template-dependent fashion. Polymerases synthesize an oligonucleotide via transfer of a nucleoside monophosphate from a nucleoside triphosphate (NTP), deoxynucleoside triphosphate (dNTP) or dideoxynucleoside triphosphate (ddNTP) to the 3' hydroxyl of an extending oligonucleotide chain. For the purposes of this disclosure, synthesizing includes to the serial extension of a hybridized adapter or a target-specific primer via transfer of a nucleoside monophosphate from a deoxynucleoside triphosphate.

[0071] As used herein, “polymerizing conditions” and its derivatives, refers generally to conditions suitable for nucleotide polymerization. In typical embodiments, such nucleotide polymerization is catalyzed by a polymerase. In some embodiments, polymerizing conditions include conditions for primer extension, optionally in a template-dependent manner, resulting in the generation of a synthesized nucleic acid sequence. In some embodiments, the polymerizing conditions include polymerase chain reaction (PCR). Typically, the polymerizing conditions include use of a reaction mixture that is sufficient to synthesize nucleic acids and includes a polymerase and nucleotides. The

polymerizing conditions can include conditions for annealing of a target-specific primer to a target sequence and extension of the primer in a template dependent manner in the presence of a polymerase. In some embodiments, polymerizing conditions can be practiced using thermocycling. Additionally, polymerizing conditions can include a plurality of cycles where the steps of annealing, extending, and separating the two nucleic strands are repeated. Typically, the polymerizing conditions include a cation such as $MgCl_2$. Generally, polymerization of one or more nucleotides to form a nucleic acid strand includes that the nucleotides be linked to each other via phosphodiester bonds, however, alternative linkages may be possible in the context of particular nucleotide analogs.

[0072] As used herein, the term "nucleic acid" refers to natural nucleic acids, artificial nucleic acids, analogs thereof, or combinations thereof, including polynucleotides and oligonucleotides. As used herein, the terms "polynucleotide" and "oligonucleotide" are used interchangeably and mean single-stranded and double-stranded polymers of nucleotides including, but not limited to, 2'-deoxyribonucleotides (nucleic acid) and ribonucleotides (RNA) linked by internucleotide phosphodiester bond linkages, *e.g.* 3'-5' and 2'-5', inverted linkages, *e.g.* 3'-3' and 5'-5', branched structures, or analog nucleic acids. Polynucleotides have associated counter ions, such as H^+ , NH_4^+ , trialkylammonium, Mg^{2+} , Na^+ and the like. An oligonucleotide can be composed entirely of deoxyribonucleotides, entirely of ribonucleotides, or chimeric mixtures thereof. Oligonucleotides can be comprised of nucleobase and sugar analogs. Polynucleotides typically range in size from a few monomeric units, *e.g.* 5-40, when they are more commonly frequently referred to in the art as oligonucleotides, to several thousands of monomeric nucleotide units, when they are more commonly referred to in the art as polynucleotides; for purposes of this disclosure, however, both oligonucleotides and polynucleotides may be of any suitable length. Unless denoted otherwise, whenever a oligonucleotide sequence is represented, it will be understood that the nucleotides are in 5' to 3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, "T" denotes thymidine, and "U" denotes deoxyuridine. Oligonucleotides are said to have "5' ends" and "3' ends" because mononucleotides are typically reacted to form oligonucleotides via attachment of the 5' phosphate or equivalent group of one nucleotide to the 3' hydroxyl or equivalent group of its neighboring nucleotide, optionally via a phosphodiester or other suitable linkage.

[0073] As defined herein, the term "nick translation" and its variants comprise the translocation of one or more nicks or gaps within a nucleic acid strand to a new position along the nucleic acid strand. In some embodiments, a nick can be formed when a double stranded adapter is ligated to a double

stranded amplified target sequence. In one example, the primer can include at its 5' end, a phosphate group that can ligate to the double stranded amplified target sequence, leaving a nick between the adapter and the amplified target sequence in the complementary strand. In some embodiments, nick translation results in the movement of the nick to the 3' end of the nucleic acid strand. In some embodiments, moving the nick can include performing a nick translation reaction on the adapter-ligated amplified target sequence. In some embodiments, the nick translation reaction can be a coupled 5' to 3' DNA polymerization/degradation reaction, or coupled to a 5' to 3' DNA polymerization/strand displacement reaction. In some embodiments, moving the nick can include performing a DNA strand extension reaction at the nick site. In some embodiments, moving the nick can include performing a single strand exonuclease reaction on the nick to form a single stranded portion of the adapter-ligated amplified target sequence and performing a DNA strand extension reaction on the single stranded portion of the adapter-ligated amplified target sequence to a new position. In some embodiments, a nick is formed in the nucleic acid strand opposite the site of ligation.

[0074] As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K. B. Mullis U.S. Pat. Nos. 4,683,195 and 4,683,202, hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a polynucleotide of interest in a mixture of genomic DNA without cloning or purification. This process for amplifying the polynucleotide of interest consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired polynucleotide of interest, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded polynucleotide of interest. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the polynucleotide of interest molecule. Following annealing, the primers are extended with a polymerase to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired polynucleotide of interest. The length of the amplified segment of the desired polynucleotide of interest (amplicon) is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of repeating the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the polynucleotide of interest become the predominant nucleic acid sequences (in terms

of concentration) in the mixture, they are said to be "PCR amplified". As defined herein, target nucleic acid molecules within a sample including a plurality of target nucleic acid molecules are amplified via PCR. In a modification to the method discussed above, the target nucleic acid molecules can be PCR amplified using a plurality of different primer pairs, in some cases, one or more primer pairs per target nucleic acid molecule of interest, thereby forming a multiplex PCR reaction. Using multiplex PCR, it is possible to simultaneously amplify multiple nucleic acid molecules of interest from a sample to form amplified target sequences. It is also possible to detect the amplified target sequences by several different methodologies (e.g., quantitation with a bioanalyzer or qPCR, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ³²P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified target sequence). Any oligonucleotide sequence can be amplified with the appropriate set of primers, thereby allowing for the amplification of target nucleic acid molecules from genomic DNA, cDNA, formalin-fixed paraffin-embedded DNA, fine-needle biopsies and various other sources. In particular, the amplified target sequences created by the multiplex PCR process as disclosed herein, are themselves efficient substrates for subsequent PCR amplification or various downstream assays or manipulations.

[0075] As defined herein "multiplex amplification" refers to selective and non-random amplification of two or more target sequences within a sample using at least one target-specific primer. In some embodiments, multiplex amplification is performed such that some or all of the target sequences are amplified within a single reaction vessel. The "plexy" or "plex" of a given multiplex amplification refers generally to the number of different target-specific sequences that are amplified during that single multiplex amplification. In some embodiments, the plexy can be about 12-plex, 24-plex, 48-plex, 96-plex, 100-plex, 130-plex, 192-plex, 384-plex, 768-plex, 1536-plex, 3072-plex, 6144-plex or higher.

[0076] **SNP Panels for Human Identification.** Panels of Single Nucleotide Polymorphism (SNP) regions are herein disclosed and are particularly useful for specifically identifying a human as each set demonstrates high average heterozygosity and globally low population variability (R_{∞}). Using any of these sets to identify a human provides match probabilities for individual identification without significant dependence on ancestry. The sets can provide coverage of autosomal, Y-, X-chromosome, and phenotypic SNPs simultaneously, particularly when analyzed in highly multiplexed next generation sequencing applications such as Ion

Torrent PGM™ or Ion Torrent Proton™ (Life Technologies). As described in the following sections, a plurality of primers may be designed to provide a primer pair adapted to selectively hybridize to each of the SNP regions in the panel of SNPs as well as incorporating design features required for the workflow of these next generation sequencers.

[0077] In some embodiments, the disclosure relates generally to methods, compositions, systems, apparatuses and kits for avoiding or reducing the formation of amplification artifacts (for example primer-dimers) during selective amplification of one or more target nucleic acid molecules in a population of nucleic acid molecules.

[0078] In some embodiments, the disclosure relates generally to the amplification of multiple target-specific sequences from a population of nucleic acid molecules. In some embodiments, the method comprises hybridizing one or more target-specific primer pairs to the target sequence, extending a first primer of the primer pair, denaturing the extended first primer product from the population of nucleic acid molecules, hybridizing to the extended first primer product the second primer of the primer pair, extending the second primer to form a double stranded product, and digesting the target-specific primer pair away from the double stranded product to generate a plurality of amplified target sequences. In some embodiments, the digesting includes partial digesting of one or more of the target-specific primers from the amplified target sequence. In some embodiments, the amplified target sequences can be ligated to one or more adapters. In some embodiments, the adapters can include one or more DNA barcodes or tagging sequences. In some embodiments, the amplified target sequences once ligated to an adapter can undergo a nick translation reaction and/or further amplification to generate a library of adapter-ligated amplified target sequences.

[0079] In some embodiments, the disclosure relates generally to the preparation and formation of multiple target-specific amplicons. In some embodiments, the method comprises hybridizing one or more target-specific primer pairs to a nucleic acid molecule, extending a first primer of the primer pair, denaturing the extended first primer from the nucleic acid molecule, hybridizing to the extended first primer product, a second primer of the primer pair and extending the second primer, digesting the target-specific primer pairs to generate a plurality of target-specific amplicons. In some embodiments, adapters can be ligated to the ends of the target-specific amplicons prior to performing a nick translation reaction to generate a plurality of target-specific amplicons suitable for nucleic acid sequencing. In some embodiments, the one or more target specific amplicons can be amplified using bridge amplification or emPCR to generate a plurality of clonal templates suitable for nucleic acid sequencing. In some embodiments, the disclosure generally relates to methods for preparing a target-

specific amplicon library, for use in a variety of downstream processes or assays such as nucleic acid sequencing or clonal amplification. In one embodiment, the disclosure relates to a method of performing target-specific multiplex PCR on a nucleic acid sample having a plurality of target sequences using primers having a cleavable group.

[0080] In one embodiment, nucleic acid templates to be sequenced using the Ion Torrent PGM™ or Ion Torrent Proton™ system can be prepared from a population of nucleic acid molecules using the target-specific amplification techniques as outlined herein. Optionally, following target-specific amplification a secondary and/or tertiary amplification process including, but not limited to, a library amplification step and/or a clonal amplification step such as emPCR can be performed.

[0081] In some embodiments, the disclosure relates to a composition comprising a plurality of target-specific primer pairs, each containing a forward primer and a reverse primer having at least one cleavable group located at either a) the 3' end or the 5' end, and/or b) at about the central nucleotide position of the target-specific primer, and wherein the target-specific primer pairs can be substantially non-complementary to other primer pairs in the composition. In some embodiments, the composition comprises at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130 or more target-specific primer pairs. In some embodiments, the target-specific primer pairs comprise about 15 nucleotides to about 40 nucleotides in length, wherein at least one nucleotide is replaced with a cleavable group. In some embodiments the cleavable group can be a uridine nucleotide. In some embodiments, the target specific primer sets are designed to amplify more than one single nucleotide polymorphism (SNP), and in some embodiments, the target specific primer sets are designed to amplify a panel of SNPs. In some embodiments, the target-specific primer pairs when hybridized to a target sequence and amplified as outlined herein can generate a library of adapter-ligated amplified target sequences that are about 100 to about 500 base pairs in length. In some embodiments, no one adapter-ligated amplified target sequence is overexpressed in the library by more than 30% as compared to the remainder of the adapter-ligated amplified target sequences in the library. In some embodiments, the adapter-ligated amplified target sequence library is substantially homogenous with respect to GC content, amplified target sequence length or melting temperature (T_m).

[0082] In some embodiments, the disclosure relates generally to a kit for performing multiplex PCR comprising a plurality of target-specific primers having a cleavable group, a DNA polymerase, an adapter, dATP, dCTP, dGTP and dTTP. In some embodiments, the cleavable group can be a uracil nucleotide. The kit can further include one or more antibodies, nucleic acid barcodes, purification solutions or columns.

[0083] In some embodiments, the disclosure relates to a kit for generating a target-specific amplicon library comprising a plurality of target-specific primers having a cleavable group, a DNA polymerase, an adapter, dATP, dCTP, dGTP, dTTP, and a cleaving reagent. In some embodiments, the kit further comprises one or more antibodies, nucleic acid barcodes, purification solutions or columns.

[0084] In one embodiment, the disclosure generally relates to the amplification of multiple target-specific sequences from a single nucleic acid source or sample. In another embodiment, the disclosure relates generally to the target-specific amplification of two or more target sequences from two or more nucleic acid sources, samples or species. For example, it is envisioned by the disclosure that a single nucleic acid sample can include genomic DNA or fixed-formalin paraffin-embedded (FFPE) DNA. It is also envisioned that the sample can be from a single individual, a collection of nucleic acid samples from genetically related members, multiple nucleic acid samples from genetically unrelated members, multiple nucleic acid samples (matched) from a single individual such as a tumor sample and normal tissue sample, or genetic material from a single source that contains two distinct forms of genetic material such as maternal and fetal DNA obtained from a maternal subject, or the presence of contaminating bacteria DNA in a sample that contains plant or animal DNA. In some embodiments, the source of nucleic acid material can include nucleic acids obtained from a newborn, for example as typically procured as a blood sample for newborn screening.

[0085] The nucleic acid sample can include high molecular weight material such as genomic DNA or cDNA. The sample can include low molecular weight material such as nucleic acid molecules obtained from FFPE or archived DNA samples. In another embodiment, low molecular weight material includes enzymatically or mechanically sheared DNA. The sample can include cell-free circulating DNA such as material obtained from a maternal subject. In some embodiments, the sample can include nucleic acid molecules obtained from biopsies, tumors, scrapings, swabs, blood, mucus, urine, plasma, semen, hair, laser capture micro-dissections, surgical resections, and other clinical or laboratory obtained samples. In some embodiments, the sample can be a forensic sample.

[0086] In some embodiments, the sample can include nucleic acid molecules obtained a human source. In other embodiments, the sample may have nucleic acid molecules obtained from a human and also contain contaminating nucleic acid from a plant, bacteria, virus, fungus or a non-human animal. In some embodiments, the source of the nucleic acid molecules may be an archived or extinct sample or species.

[0087] In some embodiments, the disclosure relates generally to the selective amplification of at least one target sequence from a tissue, biopsy, core, tumor, fluid, forensic or other biological sample. In some embodiments, the disclosure generally relates to the selective amplification of at least one target sequence and the detection and/or identification of mutations in the sample. In some embodiments, the sample can include whole genomic DNA, formalin-fixed paraffin-embedded tissue (FFPE), sheared or enzymatically treated DNA. In some embodiments, the disclosure is directed to the selective amplification of at least one target sequence and detection and/or identification of clinically actionable mutations. In some embodiments, the disclosure is directed to the detection and/or identification of mutations associated with drug resistance or drug susceptibility. In some embodiments, the disclosure is generally directed to the identification and/or quantitation of genetic markers associated with organ transplantation or organ rejection.

[0088] In some embodiments, the disclosure relates generally to the selective amplification of at least one target sequence in cell-free circulating DNA. In some embodiments, the selective amplification of at least one target sequence in a sample includes a mixture of different nucleic acid molecules. The selective amplification can optionally be accompanied by detection and/or identification of mutations observed in circulating DNA. In some embodiments, the selective amplification can optionally be accompanied by detection and/or identification of mutations associated with cancer or an inherited disease such as metabolic, neuromuscular, developmental, cardiovascular, autoimmune or other inherited disorder.

[0089] In some embodiments, the target-specific primers and primer pairs are target-specific sequences that can amplify specific regions of a nucleic acid molecule. In some embodiments, the target-specific primers can amplify genomic DNA or cDNA. In some embodiments, the target-specific primers can amplify mammalian DNA, such as human DNA. In some embodiments, the amount of DNA required for selective amplification can be from about 1 ng to 1 microgram. In some embodiments, the amount of DNA required for selective amplification of one or more target sequences can be about 1 ng, about 5 ng or about 10 ng. In some embodiments, the amount of DNA required for selective amplification of target sequence is about 10 ng to about 200 ng.

[0090] In some embodiments, selective amplification of at least one target sequence further includes nucleic acid sequencing of the amplified target sequence. Optionally, the method further includes detecting and/or identifying mutations present in the sample identified through nucleic acid sequencing of the amplified target sequence. In one embodiment, the mutations can include substitutions, insertions, inversions, point mutations, deletions, mismatches and translocations. In

one embodiment, the mutations can include variation in copy number. In one embodiment, the mutations can include germline or somatic mutations.

[0091] In some embodiments, target-specific primers are designed using the primer criteria disclosed herein.

[0092] In some embodiments, target sequences or amplified target sequences are directed to nucleic acids obtained from a forensic sample. In one embodiment, forensic samples can include nucleic acids obtained from a crime scene, nucleic acids obtained from a missing persons DNA database, nucleic acids obtained from a laboratory associated with a forensic investigation or include forensic samples obtained by law enforcement agencies, one or more military services or any such personnel. In some embodiments, target sequences can be present in one or more bodily fluids including but not limited to, blood, sputum, plasma, semen, urine and serum. In some embodiments, target sequences can be obtained from hair, skin, tissue samples, autopsy or remains of a victim. In some embodiments, nucleic acids including one or more target sequences can be obtained from a deceased animal or human. In some embodiments, target sequences can include nucleic acids obtained from non-human DNA such a microbial, plant or entomological DNA. In some embodiments, target sequences or amplified target sequences are directed to purposes of human identification. In some embodiments, the disclosure relates generally to methods for identifying a nucleic acid sample from an animal, including a human. In some embodiments, the disclosure relates generally to methods for identifying characteristics of a forensic sample. In some embodiments, the disclosure relates generally to human identification methods using one or more target-specific primers disclosed herein or one or more target-specific primers prepared using the primer criteria outlined herein.

[0093] In one embodiment, a forensic or human identification sample containing at least one target sequence can be amplified using any one or more target-specific primers designed using the primer criteria outlined herein. In some embodiments, a forensic or human identification sample containing one or more target sequences can be identified by amplifying the at least one or more target sequences with any one or more target-specific primers designed to amplify one or more region of a chromosome associated with single nucleotide polymorphisms as shown in Table 1. Please see Table A for the reference SNP cluster record (rs#) for each disclosed SNP, the NCBI reference sequence or submitter's referenced number (GenBank Accession) and the chromosome location of the SNP (world wide web.ncbi.nlm.nih.gov/projects/SNP/).

[0094] **Table 1. Regions containing SNPs useful for human identification.**

	Rs-id	Chr	SNP-Start	SNP-End
1.	rs1004357	chr17	41691525	41691526
2.	rs10092491	chr8	28411071	28411072
3.	rs1019029	chr7	13894275	13894276
4.	rs10500617	chr11	5099392	5099393
5.	rs1058083	chr13	100038232	100038233
6.	rs10768550	chr11	5098713	5098714
7.	rs10773760	chr12	130761695	130761696
8.	rs10776839	chr9	137417307	137417308
9.	rs12480506	chr20	16241415	16241416
10.	rs12997453	chr2	182413258	182413259
11.	rs13134862	chr4	76425895	76425896
12.	rs13182883	chr5	136633337	136633338
13.	rs13218440	chr6	12059953	12059954
14.	rs1358856	chr6	123894977	123894978
15.	rs1410059	chr10	97172594	97172595
16.	rs1478829	chr6	120560693	120560694
17.	rs1490413	chr1	4367322	4367323
18.	rs1498553	chr11	5709027	5709028
19.	rs1523537	chr20	51296161	51296162
20.	rs1554472	chr4	157489905	157489906
21.	rs159606	chr5	17374897	17374898
22.	rs1736442	chr18	55225776	55225777
23.	rs1821380	chr15	39313401	39313402
24.	rs1872575	chr3	113804978	113804979
25.	rs2073383	chr22	23802170	23802171
26.	rs214955	chr6	152697705	152697706
27.	rs2175957	chr17	41286821	41286822
28.	rs2255301	chr12	6909441	6909442
29.	rs2269355	chr12	6945913	6945914
30.	rs2270529	chr9	14747132	14747133
31.	rs2272998	chr6	148761455	148761456
32.	rs2291395	chr17	80526138	80526139
33.	rs2292972	chr17	80765787	80765788
34.	rs2342747	chr16	5868699	5868700
35.	rs2567608	chr20	23017081	23017082
36.	rs2811231	chr6	55155703	55155704
37.	rs2833736	chr21	33582721	33582722
38.	rs321198	chr7	137029837	137029838
39.	rs338882	chr5	178690724	178690725
40.	rs3744163	chr17	80739858	80739859
41.	rs3780962	chr10	17193345	17193346
42.	rs4288409	chr8	136839228	136839229
43.	rs430046	chr16	78017050	78017051

44.	rs4530059	chr14	104769148	104769149
45.	rs4606077	chr8	144656753	144656754
46.	rs464663	chr21	28023369	28023370
47.	rs4789798	chr17	80531642	80531643
48.	rs521861	chr18	47371013	47371014
49.	rs560681	chr1	160786669	160786670
50.	rs576261	chr19	39559806	39559807
51.	rs590162	chr11	122195988	122195989
52.	rs6444724	chr3	193207379	193207380
53.	rs6591147	chr11	105912983	105912984
54.	rs6811238	chr4	169663614	169663615
55.	rs689512	chr17	80715701	80715702
56.	rs6955448	chr7	4310364	4310365
57.	rs7041158	chr9	27985937	27985938
58.	rs7229946	chr18	22739000	22739001
59.	rs740598	chr10	118506898	118506899
60.	rs7520386	chr1	14155401	14155402
61.	rs7704770	chr5	159487952	159487953
62.	rs8070085	chr17	41341983	41341984
63.	rs891700	chr1	239881925	239881926
64.	rs901398	chr11	11096220	11096221
65.	rs9546538	chr13	84456734	84456735
66.	rs9606186	chr22	19920358	19920359
67.	rs985492	chr18	29311033	29311034
68.	rs9866013	chr3	59488339	59488340
69.	rs987640	chr22	33559507	33559508
70.	rs9951171	chr18	9749878	9749879
71.	rs1005533	chr20	39487109	39487110
72.	rs1024116	chr18	75432385	75432386
73.	rs1028528	chr22	48362289	48362290
74.	rs1029047	chr6	1135938	1135939
75.	rs10495407	chr1	238439307	238439308
76.	rs1355366	chr3	190806107	190806108
77.	rs1357617	chr3	961781	961782
78.	rs1382387	chr16	80106360	80106361
79.	rs1413212	chr1	242806796	242806797
80.	rs1454361	chr14	25850831	25850832
81.	rs1463729	chr9	126881447	126881448
82.	rs1493232	chr18	1127985	1127986
83.	rs1886510	chr13	22374699	22374700
84.	rs1979255	chr4	190318079	190318080
85.	rs2040411	chr22	47836411	47836412
86.	rs2056277	chr8	139399115	139399116
87.	rs2107612	chr12	888319	888320
88.	rs2111980	chr12	106328253	106328254

89.	rs251934	chr5	174778677	174778678
90.	rs354439	chr13	106938410	106938411
91.	rs717302	chr5	2879394	2879395
92.	rs719366	chr19	28463336	28463337
93.	rs722098	chr21	16685597	16685598
94.	rs727811	chr6	165045333	165045334
95.	rs729172	chr16	5606196	5606197
96.	rs733164	chr22	27816783	27816784
97.	rs735155	chr10	3374177	3374178
98.	rs737681	chr7	155990812	155990813
99.	rs873196	chr14	98845530	98845531
100.	rs876724	chr2	114973	114974
101.	rs914165	chr21	42415928	42415929
102.	rs917118	chr7	4457002	4457003
103.	rs964681	chr10	132698418	132698419
104.	rs1276034	chrY	23984055	23984056
105.	rs1276035	chrY	23979898	23979899
106.	rs1515817	chrY	8550110	8550111
107.	rs1558843	chrY	22750582	22750583
108.	rs1800865	chrY	2658270	2658271
109.	rs1864258	chrY	16628930	16628931
110.	rs1865680	chrY	6868117	6868118
111.	rs2020857	chrY	15030751	15030752
112.	rs2032595	chrY	14813990	14813991
113.	rs2032598	chrY	14850340	14850341
114.	rs2032599	chrY	14851553	14851554
115.	rs2032600	chrY	14888782	14888783
116.	rs2032601	chrY	14869075	14869076
117.	rs2032604	chrY	14969633	14969634
118.	rs2032607	chrY	14904858	14904859
119.	rs2032611	chrY	21866423	21866424
120.	rs2032624	chrY	15026423	15026424
121.	rs2032626	chrY	21903382	21903383
122.	rs2032631	chrY	21867786	21867787
123.	rs2032653	chrY	15591536	15591537
124.	rs2032658	chrY	15581982	15581983
125.	rs2032668	chrY	15437332	15437333
126.	rs2032673	chrY	21894057	21894058
127.	rs2072422	chrY	15590673	15590674
128.	rs2075181	chrY	7546725	7546726
129.	rs2075182	chrY	7527957	7527958
130.	rs2075183	chrY	7527956	7527957
131.	rs2075640	chrY	2722505	2722506
132.	rs2267801	chrY	2828195	2828196
133.	rs2299942	chrY	2731886	2731887

134.	rs3897	chrY	18571025	18571026
135.	rs3900	chrY	21730256	21730257
136.	rs891407	chrY	21843089	21843090

[0095] One method of designing primers which can function in either of the Ion Torrent PGM™ or Ion Torrent Proton™ workflow is by use of the AmpliSeq™ Designer, described in further detail below and in U.S. Application Serial No. 13/458,739.

[0096] In some embodiments, target sequences from all of the SNP regions disclosed in Table I are amplified (entries 1-136). In other embodiments, target sequences from a subset of the SNP regions disclosed in Table I are amplified. In some embodiments the set of SNP regions included in the multiplex amplification are SNP regions at the following locations: rs1004357, rs10092491, rs1019029, rs10500617, rs1058083, rs10768550, rs10773760, rs10776839, rs12480506, rs12997453, rs13134862, rs13182883, rs13218440, rs1358856, rs1410059, rs1478829, rs1490413, rs1498553, rs1523537, rs1554472, rs159606, rs1736442, rs1821380, rs1872575, rs2073383, rs214955, rs2175957, rs2255301, rs2269355, rs2270529, rs2272998, rs2291395, rs2292972, rs2342747, rs2567608, rs2811231, rs2833736, rs321198, rs338882, rs3744163, rs3780962, rs4288409, rs430046, rs4530059, rs4606077, rs464663, rs4789798, rs521861, rs560681, rs576261, rs590162, rs6444724, rs6591147, rs6811238, rs689512, rs6955448, rs7041158, rs7229946, rs740598, rs7520386, rs7704770, rs8070085, rs891700, rs901398, rs9546538, rs9606186, rs985492, rs9866013, rs987640, rs9951171, rs1005533, rs1024116, rs1028528, rs1029047, rs10495407, rs1355366, rs1357617, rs1382387, rs1413212, rs1454361, rs1463729, rs1493232, rs1886510, rs1979255, rs2040411, rs2056277, rs2107612, rs2111980, rs251934, rs354439, rs717302, rs719366, rs722098, rs727811, rs729172, rs733164, rs735155, rs737681, rs873196, rs876724, rs914165, rs917118, and rs964681 (entries 1-103 in Table 1). In yet another embodiment, a set of SNP regions is entries 1-70. A further embodiment provides a set of SNP regions which is entries 71-103.

[0097] In one embodiment, a sample containing one or more target sequences can be amplified using any one or more of the target-specific primers directed to SNP bearing regions of specific chromosomes as disclosed herein. In another embodiment, amplified target sequences obtained using the methods (and associated compositions, systems, apparatuses and kits) disclosed herein, can be coupled to a downstream process, such as but not limited to, nucleic acid sequencing. For example, once the nucleic acid sequence of an amplified target sequence is known, the nucleic acid sequence can be compared to one or more reference samples such as Hg19 genome. The Hg19

genome is commonly used in the genomics field as a reference genome sample for humans. In some embodiments, a sample suspected of containing one or more SNPs can be identified by amplifying the sample suspected of containing the SNP with any one or more of the target-specific primers directed to SNP bearing regions of specific chromosomes. Consequently, the output from the amplification procedure can be optionally analyzed for example by nucleic acid sequencing to determine if the expected amplification product based on the target-specific primers is present in the amplification output. The identification of an appropriate SNP or STR amplification product can in some instances provide additional information regarding the source of the sample or its characteristics (e.g., a male or female sample or a sample of particular ancestral origin).

[0098] It is envisaged that one of ordinary skill in the art can readily prepare one or more target-specific primers using the primer criteria disclosed herein without undue experimentation. In some embodiments, at least one of the target-specific primers associated with human identification includes a non-cleavable nucleotide at the 3' end. In some embodiments, the non-cleavable nucleotide at the 3' end includes the terminal 3' nucleotide.

[0099] In some embodiments, the disclosure relates generally to methods (and associated compositions, systems, apparatuses and kits) for reducing the formation of amplification artifacts in a multiplex PCR. In some embodiments, primer-dimers or non-specific amplification products are obtained in lower number or yield as compared to standard multiplex PCR of the prior art. In some embodiments, the reduction in amplification artifacts is in part, governed by the use of target-specific primer pairs in the multiplex PCR reaction. In one embodiment, the number of target-specific primer pairs in the multiplex PCR reaction can be greater than 1000, 3000, 5000, 10000, 12000, or more. In some embodiments, the disclosure relates generally to methods (and associated compositions, systems, apparatuses and kits) for performing multiplex PCR using target-specific primers that contain a cleavable group. In one embodiment, target-specific primers containing a cleavable group can include one or more cleavable moieties per primer of each primer pair. In some embodiments, a target-specific primer containing a cleavable group includes a nucleotide neither normally present in a non-diseased sample nor native to the population of nucleic acids undergoing multiplex PCR. For example, a target-specific primer can include one or more non-native nucleic acid molecules such as, but not limited to thymine dimers, 8-oxo-2'-deoxyguanosine, inosine, deoxyuridine, bromodeoxyuridine, apurinic nucleotides, and the like.

[0100] In some embodiments, the disclosed methods (and associated compositions, systems, etc.) involve performing a primary amplification of target sequences from a population of nucleic

acids, optionally using target-specific primers. In some embodiments, the disclosed methods involve amplifying target sequences using target-specific forward and reverse primer pairs. The target-specific forward and reverse primer pairs can optionally include one or more intron-specific and/or exon specific forward and reverse primer pairs. In some embodiments, each primer pair is directed to a single or discrete exon. In some embodiments, the disclosed methods involve amplifying target sequences using exon-specific forward and reverse primer pairs containing at least one cleavable group. In some embodiments, the target-specific forward and reverse primer pairs contain a uracil nucleotide as the one or more cleavable groups. In one embodiment, a target-specific primer pair can include a uracil nucleotide in each of the forward and reverse primers of each primer pair. In one embodiment, a target-specific forward or reverse primer contains one, two, three or more uracil nucleotides. In some embodiments, the disclosed methods involve amplifying at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130 or more, target sequences from a population of nucleic acids having a plurality of target sequences using target-specific forward and reverse primer pairs containing at least two uracil nucleotides.

[00101] In some embodiments, target-specific primers (including but not limited to intron-specific and exon-specific primers, which can be forward and/or reverse primers) can be designed *de novo* using algorithms that generate oligonucleotide sequences according to specified design criteria. For example, the primers may be selected according to any one or more of criteria specified herein. In some embodiments, one or more of the target-specific primers are selected or designed to satisfy any one or more of the following criteria: (1) inclusion of two or more modified nucleotides within the primer sequence, at least one of which is included near or at the termini of the primer and at least one of which is included at, or about the center nucleotide position of the primer sequence; (2) primer length of about 15 to about 40 bases in length; (3) T_m of from about 60°C to about 70°C; (4) low cross-reactivity with non-target sequences present in the target genome or sample of interest; (5) for each primer in a given reaction, the sequence of at least the first four nucleotides (going from 3' to 5' direction) are not complementary to any sequence within any other primer present in the same reaction; and (6) no amplicon includes any consecutive stretch of at least 5 nucleotides that is complementary to any sequence within any other amplicon.

[00102] In some embodiments, the target-specific primers include one or more primer pairs designed to amplify target sequences from the sample that are about 100 base pairs to about 500 base pairs in length. In some embodiments, the target-specific primers include a plurality of primer pairs designed to amplify target sequences, where the amplified target sequences are predicted to vary in

length from each other by no more than 50%, typically no more than 25%, even more typically by no more than 10%, or 5%. For example, if one target-specific primer pair is selected or predicted to amplify a product that is 100 nucleotides in length, then other primer pairs are selected or predicted to amplify products that are between 50-150 nucleotides in length, typically between 75-125 nucleotides in length, even more typically between 90-110 nucleotides, or 95-105 nucleotides, or 99-101 nucleotides in length.

[00103] In some embodiments, at least one primer pair in the amplification reaction is not designed *de novo* according to any predetermined selection criteria. For example, at least one primer pair can be an oligonucleotide sequence selected or generated at random, or previously selected or generated for other applications. In one exemplary embodiment, the amplification reaction can include at least one primer pair selected from the TaqMan® probe reagents (Roche Molecular Systems). The TaqMan® reagents include labeled probes and can be useful, *inter alia*, for measuring the amount of target sequence present in the sample, optionally in real time. Some examples of TaqMan technology are disclosed in U.S. Pat. Nos. 5,210,015, 5,487,972, 5,804,375, 6,214,979, 7,141,377 and 7,445,900, hereby incorporated by reference in their entireties.

[00104] In some embodiments, at least one primer within the amplification reaction can be labeled, for example with an optically detectable label, to facilitate a particular application of interest. For example, labeling may facilitate quantification of target template and/or amplification product, isolation of the target template and/or amplification product, and the like.

[00105] In some embodiments, the target-specific primers can be provided as a set of target-specific primer pairs in a single amplification vessel. In some embodiments, the target-specific primers can be provided in one or more aliquots of target-specific primer pairs that can be pooled prior to performing the multiplex PCR reaction in a single amplification vessel or reaction chamber. In one embodiment, the target-specific primers can be provided as a pool of target-specific forward primers and a separate pool of target-specific reverse primers. In another embodiment, target-specific primer pairs can be pooled into subsets such as non-overlapping target-specific primer pairs. In some embodiments, the pool of target-specific primer pairs can be provided in a single reaction chamber or microwell, for example on a PCR plate to perform multiplex PCR using a thermocycler. In some embodiments, the target-specific forward and reverse primer pairs can be substantially complementary to the target sequences.

[00106] In some embodiments, the method of performing multiplex PCR amplification includes contacting a plurality of target-specific primer pairs having a forward and reverse primer,

with a population of target sequences to form a plurality of template/primer duplexes; adding a DNA polymerase and a mixture of dNTPs to the plurality of template/primer duplexes for sufficient time and at sufficient temperature to extend either (or both) the forward or reverse primer in each target-specific primer pair via template-dependent synthesis thereby generating a plurality of extended primer product/template duplexes; denaturing the extended primer product/template duplexes; annealing to the extended primer product the complementary primer from the target-specific primer pair; and extending the annealed primer in the presence of a DNA polymerase and dNTPs to form a plurality of target-specific double-stranded nucleic acid molecules. In some embodiments, the steps of the amplification PCR method can be performed in any order. In some instances, the methods disclosed herein can be further optimized to remove one or more steps and still obtain sufficient amplified target sequences to be used in a variety of downstream processes. For example, the number of purification or clean-up steps can be modified to include more or less steps than disclose herein, providing the amplified target sequences are generated in sufficient yield.

[00107] In some embodiments, the target-specific primer pairs do not contain a common extension (tail) at the 3' or 5' end of the primer. In another embodiment, the target-specific primers do not contain a Tag or universal sequence. In some embodiments, the target-specific primer pairs are designed to eliminate or reduce interactions that promote the formation of non-specific amplification.

[00108] In one embodiment, the target-specific primer pairs comprise at least one cleavable group per forward and reverse target-specific primer. In one embodiment, the cleavable group can be a uracil nucleotide. In one embodiment, the target-specific primer pairs are partially or substantially removed after generation of the amplified target sequence. In one embodiment, the removal can include enzymatic, heat or alkali treatment of the target-specific primer pairs as part of the amplified target sequence. In some embodiments, the amplified target sequences are further treated to form blunt-ended amplification products, referred to herein as, blunt-ended amplified target sequences.

[00109] In some embodiments, any one or more of the target-specific primers disclosed in the methods, compositions, kits, systems and apparatuses may be designed using the following primer selection criteria.

[00110] In accordance with the teachings and principles embodied in this application, new methods, computer readable media, and systems are provided that identify or design products or kits that use PCR to enrich one or more genomic regions or targets of interest for subsequent sequencing and/or that include primers or assays that maximize coverage of one or more genomic regions or

targets of interest while minimizing one or more of off-target hybridization, a number of primers, and a number of primer pools.

[00111] FIG. 3 illustrates a system for designing primers or assays according to an exemplary embodiment. The system includes a data receiving module 1701, a primer providing module 1702, a scoring (in silico PCR) module 1703, a scoring (SNP overlap) module 1704, a filtering module 1705, a pooling module 1706, and a reporting module 1707. The system also includes a database 1708, which may include data regarding genetic annotations, SNP-related data, or other genetic data such as identification of a repeat, chromosome, position, direction, etc., for example, or any other type of information that could be related to a genomic region or target of interest, and a database 1709, which may include primer-related data such as a melting temperature (T_m), a chromosome, a position, a direction, and SNP overlap information, etc., for example, or any other type of information that could be related to primers. The system may be implemented in or using one or more computers and/or servers using one or more software components, which may not be accessible or released to customers who may be ordering custom primers or assays that may be designed using such a system. Customers may order custom primers or assays at least in part through a web-accessible data portal by providing one or more genomic regions or targets of interest in any suitable format. In an exemplary embodiment, there is provided a method performing steps including the general steps associated with modules 1701-1707 and databases 1708 and 1709 (e.g., receiving data, providing primers, scoring primers and/or amplicons, filtering primers and/or amplicons, pooling primers and/or amplicons, reporting results, and querying databases).

[00112] FIG. 4 illustrates a system for designing primers or assays according to an exemplary embodiment. The system includes a target generator module, which may generate one or more coordinate-based genomic regions or targets of interest and which may query and/or receive information from an annotation database (which may include data regarding genetic annotations, SNP-related data, or other genetic data such as identification of a repeat, chromosome, position, direction, etc., for example, as well as information regarding primers or any other type of information that could be related to a genomic region or target of interest); a designing module, which may design one or more primers or assays and determine and/or apply various scoring and filtering procedures for the primers or assays and which may perform various quality control procedures; a loader module, which may load the primers or assays and/or related information (such as quality control results, for example) to a primer database (which may be in communication with or comprised within the annotation database and which may include primer-related data such as a

melting temperature (T_m), a chromosome, a position, a direction, and SNP overlap information, etc., for example, or any other type of information that could be related to primers); a SNP overlap/repeat overlap module; a driver module; a tiler module, which may determine a subset of amplicons or tiles maximizing a coverage of a genomic region or target of interest; a pooler module, which may determine a pooling of the amplicons or tiles into one or more pools of amplicons; and a report generator module. The system may be implemented in or using one or more computers and/or servers using one or more software components, which may not be accessible or released to customers who may be ordering custom primers or assays that may be designed using such a system. Customers may order custom primers or assays at least in part through a web-accessible data portal by providing one or more genomic regions or targets of interest in any suitable format. In an exemplary embodiment, there is provided a method performing steps including the general steps associated with these modules and databases.

[00113] FIG. 5 illustrates an amplicon sequence including an insert sequence surrounded by a pair of primers designed according to an exemplary embodiment. The amplicon may include a forward primer and a reverse primer surrounding the insert sequence. The two primers may together form an assay, which may be customized and ordered. The primer component of an amplicon may be a copy of a spiked-in primer, rather than the underlying sample, and one or more inserts may be selected to cover the target.

[00114] FIG. 6 illustrates PCR amplification of an amplicon sequence (which may be referred to as "tile" herein) including an insert surrounded by a pair of primers designed according to an exemplary embodiment. Shown are denaturation, annealing, and elongation steps ultimately leading to exponential growth of the amplicon.

[00115] FIG. 7 illustrates a set of candidate amplicons for a given target region, each including an insert surrounded by a pair of primers, for tiling and pooling according to an exemplary embodiment. The dotted lines indicate the boundaries of a target region (on chromosome 19 in this example). There are 112 candidate amplicons for covering the target region in this example, but the number of candidate amplicons could of course be different, including much lower or much higher, and may be selected by taking into account computational resources, the length of the target region, and any other relevant factor.

[00116] According to various exemplary embodiments, there are provided methods for designing primers using a design pipeline that allows design of oligonucleotide primers across genomic areas of interest while incorporating various design criteria and considerations including

amplicon size, primer composition, potential off-target hybridization, and SNP overlap of the primers. In an embodiment, the design pipeline includes several functional modules that may be sequentially executed as discussed next.

[00117] In an embodiment, a sequence retrieval module may be configured to retrieve sequences based on instructions of an operator regarding a final product desired by a customer. The operator may request a design of primer pairs for genomic regions which may be specified by chromosome and genome coordinates or by a gene symbol designator. In the latter case, the sequence retrieval module may retrieve the sequence based on the exon coordinates. The operator may also specify whether to include a 5' UTR sequence (untranslated sequence).

[00118] The invention may also provide an assay design module configured to design primer pairs using a design engine, which may be a public tool such as Primer3 or another primer design software that can generate primer pairs across the entire sequence regions retrieved by the sequence retrieval module, for example. The primers pairs may be selected to tile densely across the nucleotide sequence. The primer design may be based on various parameters, including: (1) the melting temperature of the primer (which may be calculated using the nearest neighbor algorithm set forth in John Santalucia, Jr., "A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics," Proc. Natl. Acad. Sci. USA, vol. 95, 1460-1465 (1998), the contents of which is incorporated by reference herein in its entirety), (2) the primer composition (e.g., nucleotide composition such as GC content may be determined and filtered and penalized by the software, as may be primer hairpin formation, composition of the GC content in the 3' end of primer, and specific parameters that may be evaluated are stretches of homopolymeric nucleotides, hairpin formation, GC content, and amplicon size), (3) scores of forward primer, reverse primer and amplicon (the scores may be added up to obtain a probe set score, and the score may reflect how close the amplicon confirms with the intended parameters), and (4) conversion of some of the T's to U's (T's may be placed such that the predicted T_m of the T delimited fragments of a primer have a minimum average T_m.)

[00119] In an embodiment, a primer mapping module may be configured to use a mapping software (e.g., e-PCR (NCBI), see Rotmistrovsky et al., "A web server for performing electronic PCR," Nucleic Acids Research, vol. 32, W108-W112 (2004), and Schuler, "Sequence Mapping by Electronic PCR," Genome Research, vol. 7, 541-550 (1997), which are both incorporated by reference herein in their entirety, or other similar software) to map primers to a genome. The primers mapping may be scored using a mismatch matrix. In an embodiment, a perfect match may receive a

score of 0, and mismatched primers may receive a score of greater than 0. The mismatch matrix takes the position of the mismatch and the nature of the mismatch into account. For example, the mismatch matrix may assign a mismatch score to every combination of a particular motif (e.g., AA, AC, AG, CA, CC, CT, GA, GG, GT, TC, TG, TT, A-, C-, G-, T-, -A, -C, -G, and -T, where "-" denotes an ambiguous base or gap) with a particular position (e.g., base at 3' end, second base from 3' end, third base from 3' end, third base from 5' end, second base from 5' end, base at 5' end, and positions therebetween), which may be derived empirically and may be selected to reflect that mismatches closer to the 3' end tend to weaker PCR reactions more than mismatches closer to the 5' end and may therefore be generally larger. The mismatch scores for motifs with an ambiguous base or gap may be assigned an average of scores of other motifs consistent therewith (e.g., A- may be assigned an average of the scores of AA, AC, and AG). Based on the number of hits with a certain score threshold, an amplicon cost may be calculated.

[00120] In another embodiment, a SNP module may be configured to determine underlying SNPs and repeat regions: SNPs may be mapped to the primers and based on the distance of a SNP from the 3' end, primers may be filtered as potential candidates. Similarly, if a primer overlaps to a certain percentage with a repeat region, the primer might be filtered.

[00121] Additionally, in an embodiment, a pooler module may be configured to use a pooling algorithm that prevents amplicon overlaps, and ensures that the average number of primers in a pool does not deviate by more than a preset value.

[00122] FIG. 8 illustrates a method according to an exemplary embodiment. In step 2201, a module or other hardware and/or software component receives one or more genomic regions or sequences of interest. In step 2202, a module or other hardware and/or software component determines one or more target sequences for the received one or more genomic regions or sequences of interest. In step 2203, a module or other hardware and/or software component provides one or more primer pairs for each of the determined one or more target sequences. In step 2204, a module or other hardware and/or software component scores the one or more primer pairs, wherein the scoring comprises a penalty based on the performance of in silico PCR for the one or more primer pairs, and wherein the scoring further comprises an analysis of SNP overlap for the one or more primer pairs. In step 2205, a module or other hardware and/or software component filters the one or more primer pairs based on a plurality of factors, including at least the penalty and the analysis of SNP overlap, to identify a filtered set of primer pairs corresponding to one or more candidate amplicon sequences for the one or more genomic regions or sequences of interest.

[00123] According to an exemplary embodiment, there is provided a method, comprising: (1) receiving one or more genomic regions or sequences of interest; (2) determining one or more target sequences for the received one or more genomic regions or sequences of interest; (3) providing one or more primer pairs for each of the determined one or more target sequences; (4) scoring the one or more primer pairs, wherein the scoring comprises a penalty based on the performance of in silico PCR for the one or more primer pairs, and wherein the scoring further comprises an analysis of SNP overlap for the one or more primer pairs; and (5) filtering the one or more primer pairs based on a plurality of factors, including at least the penalty and the analysis of SNP overlap, to identify a filtered set of primer pairs corresponding to one or more candidate amplicon sequences for the one or more genomic regions or sequences of interest.

[00124] In various embodiments, receiving one or more genomic regions or sequences of interest may comprise receiving a list of one or more gene symbols or identifiers. Receiving one or more genomic regions or sequences of interest may comprise receiving a list of one or more genomic coordinates or other genomic location identifiers. Receiving one or more genomic regions or sequences of interest may comprise receiving a list of one or more BED coordinates.

[00125] In various embodiments, determining one or more target sequences may comprise determining one or more exons or coding regions that correspond to each of the one or more genomic regions or sequences of interest. Determining one or more target sequences may comprise querying an amplicon or other genomic sequence database for a presence therein of the one or more genomic regions or sequences of interest and information related thereto.

[00126] In various embodiments, providing one or more primer pairs may comprise designing one or more primer pairs. Providing one or more primer pairs may comprise querying an amplicon or other genomic sequence database for a presence therein of the one or more genomic regions or sequences of interest or of the one or more primer pairs and information related thereto.

[00127] In various embodiments, the performance of in silico PCR may comprise performing in silico PCR against a reference or previously sequenced genome of any species. The performance of in silico PCR may comprise performing in silico PCR against an hg19 reference genome. The performance of in silico PCR against a reference genome may comprise determining a number of off-target hybridizations for each of the one or more primer pairs. The performance of in silico PCR against a reference genome may comprise determining a worst case attribute or score for each of the one or more primer pairs. The performance of in silico PCR may comprise determining one or more genomic coordinates for each of the one or more primer pairs. The performance of in silico PCR

may comprise determining one or more predicted amplicon sequences for each of the one or more primer pairs. The performance of in silico PCR may comprise querying an amplicon or other genomic sequence database for a presence therein of the one or more genomic regions or sequences of interest or of in silico PCR results for the one or more primer pairs and information related thereto.

[00128] In various embodiments, the analysis of SNP overlap may comprise determining a SNP class for each of the one or more primer pairs. The analysis of SNP overlap may comprise querying an amplicon or other genomic sequence database for a presence therein of the one or more genomic regions or sequences of interest or of SNP overlap results for the one or more primer pairs and information related thereto.

[00129] In various embodiments, the plurality of factors may include one or more of an indication of forward SNP overlap, an indication of a reverse SNP overlap, an indication of a frequency of forward repeats, an indication of a frequency of reverse repeats, an indication of an off-target hybridization of each of the one or more primer pairs, and a composition of each of the one or more primer pairs. The plurality of factors may include one or more of a forward triplet factor, a reverse triplet factor, a forward A run factor, a reverse A run factor, a forward C run factor, a reverse C run factor, a forward G run factor, a reverse G run factor, a forward T run factor, and a reverse T run factor. The plurality of factors may include one or more of an indication of an extent to which each of the one or more primer pairs includes one or more homopolymers. The plurality of factors may include an indication of an extent to which each of the one or more primer pairs includes one or more repeating sequences. The plurality of factors may include a length of the one or more primer pairs, wherein a score for the one or more primer pairs decreases as the length gets shorter than a minimal length threshold and decreases as the length gets longer than a maximal length threshold. The plurality of factors may include a maximal number of a given base in the one or more primer pairs, wherein a score for the one or more primer pairs decreases as the number of instances of the given base exceeds a maximal base inclusion threshold. The plurality of factors may include a maximal number of contiguous instances of a given base, wherein a score for the one or more primer pairs decreases as the number of contiguous instances of the given base exceeds a maximal contiguous base inclusion threshold.

[00130] The plurality of factors may include a maximal percentage of a set of two given bases, wherein a score for the one or more primer pairs decreases as the percentage of the two given bases increases. The plurality of factors may include a maximal percentage of G and C bases, wherein a score for the one or more primer pairs decreases as the percentage of G and C bases

increases. The plurality of factors may include a deviation of a predicted melting temperature for the one or more primer pairs relative to minimal and maximal melting temperature thresholds. The plurality of factors may include a number of primer-dimer inclusions for the one or more primer pairs. The plurality of factors may include a level of local complementarity for the one or more primer pairs. The plurality of factors may include an indication of a complexity level of each of the one or more primer pairs. The plurality of factors may include an indication of SNP overlap of each of the one or more primer

[00131] In various embodiments, the method may comprise selecting a subset of the one or more candidate amplicon sequences that substantially covers the one or more genomic regions or sequences of interest while minimizing a cost function associated with the candidate amplicon sequences. Minimizing the cost function may include generating an overlap graph comprising a source vertex, one or more amplicon vertices, and a sink vertex.

[00132] In various embodiments, the method may comprise assembling the primer pairs in the filtered set of primer pairs that correspond with the selected subset of the one or more candidate amplicon sequences into a plurality of separate pools of primer pairs. Assembling the primer pairs may include limiting an inclusion of one or more primer pairs in the filtered set of primer pairs that correspond with the selected subset of the one or more candidate amplicon sequences into a given pool based at least on a minimal threshold distance between amplicon sequences in the given pool. The minimal threshold distance may be between about 5 base pairs and about 100 base pairs, or between about 15 base pairs and about 90 base pairs, or between about 25 base pairs and about 75 base pairs, or between about 40 base pairs and about 60 base pairs, for example. In some embodiments, the minimum threshold distance between amplicons may include any integer, including a negative one. For example, a value of 0 can mean that any two amplicons are allowed to "touch," and a value of -8 can mean that any two amplicons can overlap by up to 8 bases.

[00133] In various embodiments, assembling the filtered set of primer pairs into a plurality of separate pools of primer pairs may comprise splitting the primer pairs between tubes so as to prevent amplicon overlap within any given tube. Assembling the primer pairs may include limiting an inclusion of one or more primer pairs in the filtered set of primer pairs that correspond with the selected subset of the one or more candidate amplicon sequences into a given pool based at least on a pre-determined amplicon capacity of the given pool. Assembling the primer pairs may include limiting an inclusion of one or more primer pairs in the filtered set of primer pairs that correspond with the selected subset of the one or more candidate amplicon sequences into a given pool based at

least on an inequality relating a size of the given pool with a product between a balance factor and a maximum value of the sizes of the separate pools.

[00134] In various embodiments, the method may comprise providing a report reporting on any one or more element of information of data used or generated by any one or more of the receiving, providing, scoring, filtering, selecting, and assembling steps.

[00135] According to an exemplary embodiment, there is provided a non-transitory machine-readable storage medium comprising instructions which, when executed by a processor, cause the processor to perform a method comprising: (1) receiving one or more genomic regions or sequences of interest; (2) determining one or more target sequences for the received one or more genomic regions or sequences of interest; (3) providing one or more primer pairs for each of the determined one or more target sequences; (4) scoring the one or more primer pairs, wherein the scoring comprises a penalty based on the performance of *in silico* PCR for the one or more primer pairs, and wherein the scoring further comprises an analysis of SNP overlap for the one or more primer pairs; and (5) filtering the one or more primer pairs based on a plurality of factors, including at least the penalty and the analysis of SNP overlap, to identify a filtered set of primer pairs corresponding to one or more candidate amplicon sequences for the one or more genomic regions or sequences of interest.

[00136] In various embodiments, such a non-transitory machine-readable storage medium may comprise instructions which, when executed by a processor, cause the processor to perform a method further comprising: (6) selecting a subset of the one or more candidate amplicon sequences that substantially covers the one or more genomic regions or sequences of interest while minimizing a cost function associated with the candidate amplicon sequences; and (7) assembling the primer pairs in the filtered set of primer pairs that correspond with the selected subset of the one or more candidate amplicon sequences into a plurality of separate pools of primer pairs.

[00137] According to an exemplary embodiment, there is provided a system, comprising: (1) a machine-readable memory; and (2) a processor configured to execute machine-readable instructions, which, when executed by the processor, cause the system to perform steps including: (a) receiving one or more genomic regions or sequences of interest; (b) determining one or more target sequences for the received one or more genomic regions or sequences of interest; (c) providing one or more primer pairs for each of the determined one or more target sequences; (d) scoring the one or more primer pairs, wherein the scoring comprises a penalty based on the performance of *in silico* PCR for the one or more primer pairs, and wherein the scoring further comprises an analysis of SNP

overlap for the one or more primer pairs; and (e) filtering the one or more primer pairs based on a plurality of factors, including at least the penalty and the analysis of SNP overlap, to identify a filtered set of primer pairs corresponding to one or more candidate amplicon sequences for the one or more genomic regions or sequences of interest.

[00138] In various embodiments, the processor of such a system may further be configured to execute machine-readable instructions, which, when executed by the processor, cause the system to perform steps including: (f) selecting a subset of the one or more candidate amplicon sequences that substantially covers the one or more genomic regions or sequences of interest while minimizing a cost function associated with the candidate amplicon sequences; and (g) assembling the primer pairs in the filtered set of primer pairs that correspond with the selected subset of the one or more candidate amplicon sequences into a plurality of separate pools of primer pairs.

[00139] According to various exemplary embodiment, various parameters or criteria may be used to select primers and/or amplicons.

[00140] In an embodiment, a forward SNP score may be used and may be given a numerical attribute/score of 1 if there is no SNP within a given length of base pairs of the forward primer (such as 4, for example) or a numerical attribute of 0 if there is one or more SNPs within a length of 4 base pairs. In one embodiment, the forward SNP score may be given a numerical attribute/score of 1 if there is no SNP within a given length of base pairs from the 3' end of the forward primer. In some embodiments, a SNP can include one or more SNPs found on UCSC's Genome Browser Web Page including but not limited to, the SNP reference table referred to as "dbSNP132 common". An attribute/score of 1 may be a minimal attribute/score such that failure to achieve that attribute/score would result in disqualification. The base length threshold for the attribute/score determination could be lower or higher than 4, and could be 5, 6, 7, 8, 9, 10, 15, 20, for example, or more generally any positive integer larger than 4. The attribute/score could be other than binary and could be a more complex linear or non-linear function of the number of SNPs within the given length of base pairs.

[00141] In an embodiment, a reverse SNP score may be used and may be given a numerical attribute/score of 1 if there is no SNP within a given length of base pairs of the reverse primer (such as 4, for example) or a numerical attribute of 0 if there is one or more SNPs within a length of 4 base pairs. In one embodiment, the reverse SNP score may be given a numerical attribute/score of 1 if there is no SNP within a given length of base pairs from the 3' end of the reverse primer. In some embodiments, a SNP can include one or more SNPs found on UCSC's Genome Browser Web Page including but not limited to, the SNP reference table referred to as "dbSNP132 common". An

attribute/score of 1 may be a minimal attribute/score such that failure to achieve that attribute/score would result in disqualification. The base length threshold for the attribute/score determination could be lower or higher than 4, and could be 5, 6, 7, 8, 9, 10, 15, 20, for example, or more generally any positive integer larger than 4. The attribute/score could be other than binary and could be a more complex linear or non-linear function of the number of SNPs within the given length of base pairs.

[00142] In an embodiment, a forward repeat score may be used and may be given a numerical attribute/score of 1 if there is no repeat within a given length of base pairs of the forward primer (such as 4, for example) or a numerical attribute of 0 if there is one or more repeats within a length of 4 base pairs. In one embodiment, the forward repeat score may be given a numerical attribute/score of 1 if there is less than 30% overlap of the forward primer with known repeats. In some embodiments, known repeats may include one or more repeats reported by UCSC's Genome Browser, for example repeat regions as provided by the repeat masked hg19 genome from UCSC. An attribute/score of 1 may be a minimal attribute/score such that failure to achieve that attribute/score would result in disqualification. The base length threshold for the attribute/score determination could be lower or higher than 4, and could be 5, 6, 7, 8, 9, 10, 15, 20, for example, or more generally any positive integer larger than 4. The attribute/score could be other than binary and could be a more complex linear or non-linear function of the number of repeats within the given length of base pairs.

[00143] In an embodiment, a reverse repeat score may be used and may be given a numerical attribute/score of 1 if there is no repeat within a given length of base pairs of the reverse primer (such as 4, for example) or a numerical attribute of 0 if there is one or more repeats within a length of 4 base pairs. In one embodiment, the reverse repeat score may be given a numerical attribute/score of 1 if there is less than 30% overlap of the reverse primer with known repeats. In some embodiments, known repeats may include one or more repeats reported by UCSC's Genome Browser, for example repeat regions as provided by the repeat masked hg19 genome from UCSC. An attribute/score of 1 may be a minimal attribute/score such that failure to achieve that attribute/score would result in disqualification. The base length threshold for the attribute/score determination could be lower or higher than 4, and could be 5, 6, 7, 8, 9, 10, 15, 20, for example, or more generally any positive integer larger than 4. The attribute/score could be other than binary and could be a more complex linear or non-linear function of the number of repeats within the given length of base pairs.

[00144] In various embodiments, one or more of a forward triplet score, a reverse triplet score, a forward A run score, a reverse A run score, a forward C run score, a reverse C run score, a

forward G run score, a reverse G run score, a forward T run score, and a reverse T run score, may be used and may be given a numerical attribute/score equal to the number of forward triplets, reverse triplets, forward A runs, reverse A runs, forward C runs, reverse C runs, forward G runs, reverse G runs, forward T runs, and reverse T runs within the entire primer. An attribute/score of 3 may be a maximal attribute/score for the triplets such that failure to remain at or below that attribute/score would result in disqualification. An attribute/score of 5 may be a maximal attribute/score for the runs such that failure to remain at or below that attribute/score would result in disqualification. The attribute/score could be other than binary and could be a more complex linear or non-linear function of the number of triplets/runs.

[00145] In an embodiment, a length of the primers may be limited by a minimum primer length threshold and a maximum primer length, and a length score for the primers may be set so as to decrease as the length gets shorter than the minimum primer length threshold and to decrease as the length gets longer than the maximum primer length threshold. In an embodiment, the minimum primer length threshold may be 16. In other embodiments, the minimum primer length threshold may be 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5, for example, and may also be 17, 18, 19, 20, 21, 22, 23, and 24, for example. In an embodiment, the maximum primer length threshold may be 28. In other embodiments, the maximum primer length threshold may be 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, and 40, for example, and may also be 27, 26, 25, 24, 23, 22, 21, and 20, for example. In an embodiment, the primer length criterion may be given a score of 1.0 if the length thresholds are satisfied, for example, and that score may go down to 0.0 as the primer length diverges from the minimum or maximum length threshold. For example, if the maximum primer length threshold were set to 28, then the score could be set to 1.0 if the length does not exceed 28, to 0.7 if the length is 29, to 0.6 if the length is 30, to 0.5 if the length is 31, to 0.3 if the length is 32, to 0.1 if the length is 33, and to 0.0 if the length is 34 or more. The attribute/score could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the score varies with an increase difference relative to the threshold could be any other or more complex linear or non-linear function that does not lead to increases in score for primer that further diverge from length thresholds.

[00146] In an embodiment, a number of G bases (or of A, C, or T bases) in the primers may be limited by a maximum threshold, and corresponding score for the primers may be set so as to decrease as the number of G bases (or of A, C, or T bases) exceeds the maximum threshold. In an embodiment, the maximum threshold may be 3. In other embodiments, the maximum threshold may be 2, 4, 5, 6, 7, 8, 9, and 10, for example. In an embodiment, the number of G bases (or of A, C, or T

bases) criterion may be given a score of 1.0 if the maximum threshold is satisfied, for example, and that score may go down to 0.0 as the number of G bases (or of A, C, or T bases) diverges from the maximum threshold. For example, if the maximum threshold were set to 4, then the score could be set to 1.0 if the number of G bases (or of A, C, or T bases) does not exceed 4, to 0.9 if the number is 5, to 0.8 if the number is 6, to 0.6 if the number is 7, to 0.4 if the number is 8, to 0.2 if the number is 9, and to 0.0 if the number is 10 or more. The score could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the score varies with an increased difference between the number of G bases (or of A, C, or T bases) and the maximum threshold could be any other or more complex linear or non-linear function that does not lead to increases in score for primer that further diverge from the maximum threshold.

[00147] In an embodiment, the numbers of contiguous and total matches in a loop (e.g., hairpin) in the primers may be limited by a maximum threshold, and corresponding scores for the primers may be set so as to decrease as the numbers of contiguous and total matches in a loop exceed the maximum threshold. In an embodiment, the maximum threshold for contiguous matches may be 3 and the maximum threshold for total matches may be 5. In other embodiments, the maximum threshold for contiguous matches may be 2, 4, 5, 6, 7, 8, 9, and 10, for example, and the maximum threshold for total matches may be 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15, for example. In an embodiment, the numbers of contiguous and total matches in a loop criteria may be given a score of 1.0 if the maximum threshold is satisfied, for example, and that score may go down to 0.0 as the number of the numbers of contiguous and total matches in a loop diverge from the corresponding maximum threshold. For example, if the maximum threshold for contiguous matches were set to 3, then the score could be set to 1.0 if the number of contiguous matches does not exceed 3, to 0.9 if the number is 4, to 0.7 if the number is 5, to 0.4 if the number is 6, to 0.2 if the number is 7, to 0.1 if the number is 8, and to 0.0 if the number is 9 or more. For example, if the maximum threshold for total matches were set to 5, then the score could be set to 1.0 if the number of total matches does not exceed 5, to 0.9 if the number is 6, to 0.8 if the number is 7, to 0.6 if the number is 8, to 0.4 if the number is 9, to 0.2 if the number is 10, to 0.1 if the number is 11, and to 0.0 if the number is 12 or more. The scores could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the scores vary with an increased difference between the number of contiguous/total matches and the corresponding maximum thresholds could be any other or more complex linear or non-linear function that does not lead to increases in score for primer that further diverge from the maximum threshold.

[00148] In an embodiment, a number of G and C bases (or any two of the A, C, G, and T bases) in the last five bases of the primers may be limited by a maximum threshold, and corresponding score for the primers may be set so as to decrease as the number of G and C bases (or any two of the A, C, G, and T bases) exceeds the maximum threshold. In an embodiment, the maximum threshold may be 2. In other embodiments, the maximum threshold may be 3, 4, and 5, for example. In an embodiment, the number of G and C bases (or any two of the A, C, G, and T bases) criterion may be given a score of 1.0 if the maximum threshold is satisfied, for example, and that score may go down to 0.0 as the number of G and C bases (or any two of the A, C, G, and T bases) diverges from the maximum threshold. For example, if the maximum threshold were set to 2, then the score could be set to 1.0 if the number of G and C bases (or any two of the A, C, G, and T bases) does not exceed 2, to 0.8 if the number is 3, to 0.4 if the number is 4, and to 0.1 if the number is 5. The score could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the score varies with an increased difference between the number of G and C bases (or any two of the A, C, G, and T bases) and the maximum threshold could be any other or more complex linear or non-linear function that does not lead to increases in score for primer that further diverge from the maximum threshold. In other embodiments, this criterion could consider the number of G and C bases (or any two of the A, C, G, and T bases) in a larger window of bases, such as in the last six bases, the last seven bases, the last eight bases, etc., for example.

[00149] In an embodiment, a percentage of G and C bases (or any two of the A, C, G, and T bases) in the primers may be limited by minimum and maximum thresholds, and corresponding score for the primers may be set so as to decrease as the percentage of G and C bases (or any two of the A, C, G, and T bases) diverges from the minimum or maximum threshold. In an embodiment, the minimum threshold may be 0.2 (20%) and the maximum threshold may be 0.8 (80%). In other embodiments, the minimum threshold may be any percentage between about 0.2 (20%) and about 0.5 (50%) and the maximum threshold may be any percentage between about 0.8 (80%) and 0.5 (50%), for example. In an embodiment, the percentage of G and C bases (or any two of the A, C, G, and T bases) criterion may be given a score of 1.0 if the minimum and maximum thresholds are satisfied, for example, and that score may go down to 0.0 if either of the thresholds is not satisfied. The score could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the score varies with an increased difference between the percentage of G and C bases (or any two of the A, C, G, and T bases) and the minimum or maximum threshold could be any other or more complex

linear or non-linear function that does not lead to increases in score for primer that further diverge from the minimum or maximum threshold.

[00150] In an embodiment, a melting temperature (T_m) of the primers may be limited by minimum and maximum thresholds, and corresponding score for the primers may be set so as to decrease as the melting temperature diverges from the minimum or maximum threshold. In an embodiment, the minimum threshold may be 60 and the maximum threshold may be 67 with a target melting temperature of 62. In other embodiments, the minimum threshold may be a value between about 55 and about 65 and the maximum threshold may be a value between about 62 and about 72, for example. In an embodiment, the melting temperature criterion may be given a score of 1.0 if the minimum and maximum thresholds are satisfied, for example, and that score may go down to 0.0 if either of the thresholds is not satisfied. The score could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the score varies with an increased difference between the melting temperature and the minimum or maximum threshold could be any other or more complex linear or non-linear function that does not lead to increases in score for primer that further diverge from the minimum or maximum threshold. The melting temperature of a primer may be calculated using the teachings set forth in John Santalucia, Jr., "A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics," *Proc. Natl. Acad. Sci. USA*, vol. 95, 1460-1465 (1998), the contents of which is incorporated by reference herein in its entirety.

[00151] In an embodiment, a primer-dimer propensity in the primers may be limited by a maximum threshold of contiguous primer-dimers at the 3' end and a maximum threshold of total contiguous matches over the full length, and corresponding score for the primers may be set so as to decrease as the primer-dimer propensity diverges from the maximum thresholds. In an embodiment, the maximum threshold of contiguous primer-dimers at the 3' end may be 4 and the maximum threshold of total contiguous matches over the full length may be 8. In other embodiments, the maximum threshold of contiguous primer-dimers at the 3' end may be a value between about 2 and about 6 and the maximum threshold of total contiguous matches over the full length may be a value between about 4 and 10, for example. In an embodiment, the primer-dimer propensity criteria may be given a score of 1.0 if the threshold is satisfied, for example, and that score may go down to 0.0 if the threshold is not satisfied. The score could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the score varies with an increased difference between the primer-dimer propensity and the maximum threshold could be any other or more complex linear or

non-linear function that does not lead to increases in score for primer that further diverge from the maximum threshold.

[00152] In an embodiment, a percentage of G and C bases (or any two of the A, C, G, and T bases) in an amplicon sequence may be limited by minimum and maximum thresholds, and corresponding score for the amplicons may be set so as to decrease as the percentage of G and C bases (or any two of the A, C, G, and T bases) diverges from the minimum or maximum threshold. In an embodiment, the minimum threshold may be 0.0 (0%) and the maximum threshold may be 1.0 (100%). In other embodiments, the minimum threshold may be any percentage between about 0.1 (10%) and about 0.25 (25%) and the maximum threshold may be any percentage between about 0.75 (75%) and 0.9 (90%), for example. In an embodiment, the percentage of G and C bases (or any two of the A, C, G, and T bases) criterion may be given a score of 1.0 if the minimum and maximum thresholds are satisfied, for example, and that score may go down to 0.0 if either of the thresholds is not satisfied. The score could be scaled between values other than 0.0 and 1.0, of course, and the function defining how the score varies with an increased difference between the percentage of G and C bases (or any two of the A, C, G, and T bases) and the minimum or maximum threshold could be any other or more complex linear or non-linear function that does not lead to increases in score for amplicons that further diverge from the minimum or maximum threshold.

[00153] In an embodiment, a length of the amplicons may be limited by a minimum amplicon length threshold and a maximum amplicon length, and a length score for the amplicons may be set so as to decrease as the length gets shorter than the minimum amplicon length threshold and to decrease as the length gets longer than the maximum amplicon length threshold. In an embodiment, the minimum amplicon length threshold may be 110. In other embodiments, the minimum primer length threshold may be a value between about 80 and about 140, for example. In an embodiment, the maximum amplicon length threshold may be 240. In other embodiments, the maximum amplicon length threshold may be a value between about 200 and about 280, for example. In an embodiment, the amplicon length criterion may be given a score of 1.0 if the length thresholds are satisfied and of 0.0 if either is not satisfied. In another embodiment, that score may go down to 0.0 as the amplicon length diverges from the minimum or maximum length threshold. For example, if the maximum amplicon length threshold were set to 240, then the score could be set to 1.0 if the length does not exceed 240, to 0.8 if the length is at least 250, to 0.6 if the length is at least 260, to 0.4 if the length is at least 270, to 0.1 if the length is at least 280, and to 0.0 if the length is at least 290. The attribute/score could be scaled between values other than 0.0 and 1.0, of course, and the function

defining how the score varies with an increase difference relative to the threshold could be any other or more complex linear or non-linear function that does not lead to increases in score for amplicons that further diverge from length thresholds.

[00154] According to an exemplary embodiment, there is provided a method for selecting a subset (which may be referred to as “tiling” herein) of amplicons (which may be referred to as “tiles” herein) from a plurality of candidate amplicons for covering one or more specific desired (e.g., customized) genomic regions or targets using one or more pools of amplicons. The method for tiling and pooling is described in further detail in U.S. Application Serial No. No. 13/458,739, entitled “Methods and Composition for Multiplex PCR”, inventors John Leamon, Mark Andersen, and Michael Thornton, filed on April 27, 2012, and herein incorporated by reference in its entirety.

[00155] According to various exemplary embodiments, one or more features of any one or more of the above-discussed teachings and/or exemplary embodiments may be performed or implemented using appropriately configured and/or programmed hardware and/or software elements. Determining whether an embodiment is implemented using hardware and/or software elements may be based on any number of factors, such as desired computational rate, power levels, heat tolerances, processing cycle budget, input data rates, output data rates, memory resources, data bus speeds, etc., and other design or performance constraints.

[00156] Examples of hardware elements may include processors, microprocessors, input(s) and/or output(s) (I/O) device(s) (or peripherals) that are communicatively coupled via a local interface circuit, circuit elements (e.g., transistors, resistors, capacitors, inductors, and so forth), integrated circuits, application specific integrated circuits (ASIC), programmable logic devices (PLD), digital signal processors (DSP), field programmable gate array (FPGA), logic gates, registers, semiconductor device, chips, microchips, chip sets, and so forth. The local interface may include, for example, one or more buses or other wired or wireless connections, controllers, buffers (caches), drivers, repeaters and receivers, etc., to allow appropriate communications between hardware components. A processor is a hardware device for executing software, particularly software stored in memory. The processor can be any custom made or commercially available processor, a central processing unit (CPU), an auxiliary processor among several processors associated with the computer, a semiconductor based microprocessor (e.g., in the form of a microchip or chip set), a macroprocessor, or generally any device for executing software instructions. A processor can also represent a distributed processing architecture. The I/O devices can include input devices, for example, a keyboard, a mouse, a scanner, a microphone, a touch screen, an interface for various

medical devices and/or laboratory instruments, a bar code reader, a stylus, a laser reader, a radio-frequency device reader, etc. Furthermore, the I/O devices also can include output devices, for example, a printer, a bar code printer, a display, etc. Finally, the I/O devices further can include devices that communicate as both inputs and outputs, for example, a modulator/demodulator (modem; for accessing another device, system, or network), a radio frequency (RF) or other transceiver, a telephonic interface, a bridge, a router, etc.

[00157] Examples of software may include software components, programs, applications, computer programs, application programs, system programs, machine programs, operating system software, middleware, firmware, software modules, routines, subroutines, functions, methods, procedures, software interfaces, application program interfaces (API), instruction sets, computing code, computer code, code segments, computer code segments, words, values, symbols, or any combination thereof. A software in memory may include one or more separate programs, which may include ordered listings of executable instructions for implementing logical functions. The software in memory may include a system for identifying data streams in accordance with the present teachings and any suitable custom made or commercially available operating system (O/S), which may control the execution of other computer programs such as the system, and provides scheduling, input-output control, file and data management, memory management, communication control, etc.

[00158] According to various exemplary embodiments, one or more features of any one or more of the above-discussed teachings and/or exemplary embodiments may be performed or implemented using appropriately configured and/or programmed non-transitory machine-readable medium or article that may store an instruction or a set of instructions that, if executed by a machine, may cause the machine to perform a method and/or operations in accordance with the exemplary embodiments. Such a machine may include, for example, any suitable processing platform, computing platform, computing device, processing device, computing system, processing system, computer, processor, scientific or laboratory instrument, etc., and may be implemented using any suitable combination of hardware and/or software. The machine-readable medium or article may include, for example, any suitable type of memory unit, memory device, memory article, memory medium, storage device, storage article, storage medium and/or storage unit, for example, memory, removable or non-removable media, erasable or non-erasable media, writeable or re-writable media, digital or analog media, hard disk, floppy disk, read-only memory compact disc (CD-ROM), recordable compact disc (CD-R), rewriteable compact disc (CD-RW), optical disk, magnetic media, magneto-optical media, removable memory cards or disks, various types of Digital Versatile Disc

(DVD), a tape, a cassette, etc., including any medium suitable for use in a computer. Memory can include any one or a combination of volatile memory elements (e.g., random access memory (RAM, such as DRAM, SRAM, SDRAM, etc.)) and nonvolatile memory elements (e.g., ROM, EPROM, EEROM, Flash memory, hard drive, tape, CDROM, etc.). Moreover, memory can incorporate electronic, magnetic, optical, and/or other types of storage media. Memory can have a distributed architecture where various components are situated remote from one another, but are still accessed by the processor. The instructions may include any suitable type of code, such as source code, compiled code, interpreted code, executable code, static code, dynamic code, encrypted code, etc., implemented using any suitable high-level, low-level, object-oriented, visual, compiled and/or interpreted programming language.

[00159] According to various exemplary embodiments, one or more features of any one or more of the above-discussed teachings and/or exemplary embodiments may be performed or implemented at least partly using a distributed, clustered, remote, or cloud computing resource.

[00160] According to various exemplary embodiments, one or more features of any one or more of the above-discussed teachings and/or exemplary embodiments may be performed or implemented using a source program, executable program (object code), script, or any other entity comprising a set of instructions to be performed. When a source program, the program can be translated via a compiler, assembler, interpreter, etc., which may or may not be included within the memory, so as to operate properly in connection with the O/S. The instructions may be written using (a) an object oriented programming language, which has classes of data and methods, or (b) a procedural programming language, which has routines, subroutines, and/or functions, which may include, for example, C, C++, Pascal, Basic, Fortran, Cobol, Perl, Java, and Ada.

[00161] According to various exemplary embodiments, one or more of the above-discussed exemplary embodiments may include transmitting, displaying, storing, printing or outputting to a user interface device, a computer readable storage medium, a local computer system or a remote computer system, information related to any information, signal, data, and/or intermediate or final results that may have been generated, accessed, or used by such exemplary embodiments. Such transmitted, displayed, stored, printed or outputted information can take the form of searchable and/or filterable lists of runs and reports, pictures, tables, charts, graphs, spreadsheets, correlations, sequences, and combinations thereof, for example.

[00162] Various additional exemplary embodiments may be derived by repeating, adding, or substituting any generically or specifically described features and/or components and/or substances

and/or steps and/or operating conditions set forth in one or more of the above-described exemplary embodiments. Further, it should be understood that an order of steps or order for performing certain actions is immaterial so long as the objective of the steps or action remains achievable, unless specifically stated otherwise. Furthermore, two or more steps or actions can be conducted simultaneously so long as the objective of the steps or action remains achievable, unless specifically stated otherwise. Moreover, any one or more feature, component, aspect, step, or other characteristic mentioned in one of the above-discussed exemplary embodiments may be considered to be a potential optional feature, component, aspect, step, or other characteristic of any other of the above-discussed exemplary embodiments so long as the objective of such any other of the above-discussed exemplary embodiments remains achievable, unless specifically stated otherwise.

[00163] Various additional exemplary embodiments may be derived by incorporating in the above described exemplary embodiments one or more of the features described in U.S. Pat. Appl. Publ. No. 2008/0228589 A1, published September 18, 2008, and U.S. Pat. Appl. Publ. No. 2004/0175733 A1, published September 9, 2004, the contents of both of which are incorporated by reference herein in their entireties.

[00164] In some embodiments, the amplified target sequences of the disclosed methods can be used in various downstream analysis or assays with, or without, further purification or manipulation. For example, the amplified target sequences can be clonally amplified by techniques known in the art, such a bridge amplification or emPCR to generate a template library that can be used in next generation sequencing. In some embodiments, the amplified target sequences of the disclosed methods or the resulting template libraries can be used for single nucleotide polymorphism (SNP) analysis, genotyping or epigenetic analysis, copy number variation analysis, gene expression analysis, analysis of gene mutations including but not limited to detection, prognosis and/or diagnosis, detection and analysis of rare or low frequency allele mutations, nucleic acid sequencing including but not limited to *de novo* sequencing, targeted resequencing and synthetic assembly analysis. In one embodiment, amplified target sequences can be used to detect mutations at less than 5% allele frequency. In some embodiments, the methods disclosed herein can be used to detect mutations in a population of nucleic acids at less than 4%, 3%, 2% or at about 1% allele frequency. In another embodiment, amplified target sequences prepared as described herein can be sequenced to detect and/or identify germline or somatic mutations from a population of nucleic acid molecules.

[00165] In some embodiments, the forward and/or reverse target-specific primers in the target-specific primer pairs can be “complementary” or “substantially complementary” to the

population of nucleic acid molecules. As termed herein "substantially complementary to the population of nucleic acid molecules" refers to percentage complementarity between the primer and the nucleic acid molecule to which the primer will hybridize. Generally, the term "substantially complementary" as used herein refers to at least 70% complementarity. Therefore, substantially complementary refers to a range of complementarity of at least 70% but less than 100% complementarity between the primer and the nucleic acid molecule. A complementary primer is one that possesses 100% complementarity to the nucleic acid molecule. In one embodiment, each target-specific primer pair is designed to minimize cross-hybridization to another primer (or primer pair) in the same multiple PCR reaction (i.e., reduce the prevalence of primer-dimers). In another embodiment, each target-specific primer pair is designed to minimize cross-hybridization to non-specific nucleic acid sequences in the population of nucleic acid molecules (i.e., minimize off-target hybridization). In one embodiment, each target-specific primer is designed to minimize self-complementarity, formation of hairpin structures or other secondary structures.

[00166] In some embodiments, the amplified target sequences are formed via polymerase chain reaction. Extension of target-specific primers can be accomplished using one or more DNA polymerases. In one embodiment, the polymerase can be any Family A DNA polymerase (also known as pol I family) or any Family B DNA polymerase. In some embodiments, the DNA polymerase can be a recombinant form capable of extending target-specific primers with superior accuracy and yield as compared to a non-recombinant DNA polymerase. For example, the polymerase can include a high-fidelity polymerase or thermostable polymerase. In some embodiments, conditions for extension of target-specific primers can include 'Hot Start' conditions, for example Hot Start polymerases, such as Amplitaq Gold® DNA polymerase (Applied Biosciences), Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) or KOD Hot Start DNA polymerase (EMD Biosciences). Generally, a 'Hot Start' polymerase includes a thermostable polymerase and one or more antibodies that inhibit DNA polymerase and 3'-5' exonuclease activities at ambient temperature. In some instances, 'Hot Start' conditions can include an aptamer.

[00167] In some embodiments, the polymerase can be an enzyme such as Taq polymerase (from *Thermus aquaticus*), Tfi polymerase (from *Thermus filiformis*), Bst polymerase (from *Bacillus stearothermophilus*), Pfu polymerase (from *Pyrococcus furiosus*), Tth polymerase (from *Thermus thermophilus*), Pow polymerase (from *Pyrococcus woesei*), Tli polymerase (from *Thermococcus litoralis*), Ulüma polymerase (from *Thermotoga maritima*), KOD polymerase (from *Thermococcus kodakaraensis*), Pol I and II polymerases (from *Pyrococcus abyssi*) and Pab (from *Pyrococcus*

abyssi). In some embodiments, the DNA polymerase can include at least one polymerase such as Amplitaq Gold® DNA polymerase (Applied Biosciences), Stoffel fragment of Amplitaq® DNA Polymerase (Roche), KOD polymerase (EMD Biosciences), KOD Hot Start polymerase (EMD Biosciences), Deep Vent™ DNA polymerase (New England Biolabs), Phusion polymerase (New England Biolabs), Klentaq1 polymerase (DNA Polymerase Technology, Inc), Klentaq Long Accuracy polymerase (DNA Polymerase Technology, Inc), Omni KlenTaq™ DNA polymerase (DNA Polymerase Technology, Inc), Omni KlenTaq™ LA DNA polymerase (DNA Polymerase Technology, Inc), Platinum® Taq DNA Polymerase (Invitrogen), Hemo Klentaq™ (New England Biolabs), Platinum® Taq DNA Polymerase High Fidelity (Invitrogen), Platinum® Pfx (Invitrogen), Accuprime™ Pfx (Invitrogen), or Accuprime™ Taq DNA Polymerase High Fidelity (Invitrogen).

[00168] In some embodiments, the DNA polymerase can be a thermostable DNA polymerase. In some embodiments, the mixture of dNTPs can be applied concurrently, or sequentially, in a random or defined order. In some embodiments, the amount of DNA polymerase present in the multiplex reaction is significantly higher than the amount of DNA polymerase used in a corresponding single plex PCR reaction. As defined herein, the term “significantly higher” refers to an at least 3-fold greater concentration of DNA polymerase present in the multiplex PCR reaction as compared to a corresponding single plex PCR reaction.

[00169] In some embodiments, the amplification reaction does not include a circularization of amplification product, for example as disclosed by rolling circle amplification.

[00170] In some embodiments, the methods of the disclosure include selectively amplifying target sequences in a sample containing a plurality of nucleic acid molecules and ligating the amplified target sequences to at least one adapter and/or barcode. Adapters and barcodes for use in molecular biology library preparation techniques are well known to those of skill in the art. The definitions of adapters and barcodes as used herein are consistent with the terms used in the art. For example, the use of barcodes allows for the detection and analysis of multiple samples, sources, tissues or populations of nucleic acid molecules per multiplex reaction. A barcoded and amplified target sequence contains a unique nucleic acid sequence, typically a short 6-15 nucleotide sequence, that identifies and distinguishes one amplified nucleic acid molecule from another amplified nucleic acid molecule, even when both nucleic acid molecules minus the barcode contain the same nucleic acid sequence. The use of adapters allows for the amplification of each amplified nucleic acid molecule in a uniformed manner and helps reduce strand bias. Adapters can include universal adapters or propriety adapters both of which can be used downstream to perform one or more distinct

functions. For example, amplified target sequences prepared by the methods disclosed herein can be ligated to an adapter that may be used downstream as a platform for clonal amplification. The adapter can function as a template strand for subsequent amplification using a second set of primers and therefore allows universal amplification of the adapter-ligated amplified target sequence. In some embodiments, selective amplification of target nucleic acids to generate a pool of amplicons can further comprise ligating one or more barcodes and/or adapters to an amplified target sequence. The ability to incorporate barcodes enhances sample throughput and allows for analysis of multiple samples or sources of material concurrently. In one example, amplified target nucleic acid molecules prepared by the disclosed methods can be ligated to Ion Torrent™ Sequencing Adapters (A and P1 Adapters, sold as a component of the Ion Fragment Library Kit, Life Technologies, Part No. 4466464) or Ion Torrent™ DNA Barcodes (Life Technologies, Part No. 4468654).

[00171] The methods disclosed herein are directed to the amplification of multiple target sequences via polymerase chain reaction (PCR). In some embodiments the multiplex PCR comprises hybridizing one or more target-specific primer pairs to a nucleic acid molecule, extending the primers of the target-specific primer pairs via template dependent synthesis in the presence of a DNA polymerase and dNTPs; repeating the hybridization and extension steps for sufficient time and sufficient temperature there generating a plurality of amplified target sequences. In some embodiments, the steps of the multiplex amplification reaction method can be performed in any order.

[00172] The amount of nucleic acid material required for successful multiplex amplification can be about 1 ng. In some embodiments, the amount of nucleic acid material can be about 10 ng to about 50 ng, about 10 ng to about 100 ng, or about 1 ng to about 200 ng of nucleic acid material. Higher amounts of input material can be used, however one aspect of the disclosure is to selectively amplify a plurality of target sequence from a low (ng) amount of starting material.

[00173] The multiplex PCR amplification reactions disclosed herein can include a plurality of "cycles" typically performed on a thermocycler. Generally, each cycle includes at least one annealing step and at least one extension step. In one embodiment, a multiplex PCR amplification reaction is performed wherein target-specific primer pairs are hybridized to a target sequence; the hybridized primers are extended generating an extended primer product/nucleic acid duplex; the extended primer product/nucleic acid duplex is denatured allowing the complementary primer to hybridize to the extended primer product, wherein the complementary primer is extended to generate a plurality of amplified target sequences. In one embodiment, the methods disclosed herein have

about 5 to about 18 cycles per preamplification reaction. The annealing temperature and/or annealing duration per cycle can be identical; can include incremental increases or decreases, or a combination of both. The extension temperature and/or extension duration per cycle can be identical; can include incremental increases or decreases, or a combination of both. For example, the annealing temperature or extension temperature can remain constant per cycle. In some embodiments, the annealing temperature can remain constant each cycle and the extension duration can incrementally increase per cycle. In some embodiments, increases or decreases in duration can occur in 15 second, 30 second, 1 minute, 2 minute or 4 minute increments. In some embodiments, increases or decrease in temperature can occur as 0.5, 1, 2, 3, or 4 Celsius deviations. In some embodiments, the amplification reaction can be conducted using hot-start PCR techniques. These techniques include the use of a heating step (>60°C) before polymerization begins to reduce the formation of undesired PCR products. Other techniques such as the reversible inactivation or physical separation of one or more critical reagents of the reaction, for example the magnesium or DNA polymerase can be sequestered in a wax bead, which melts as the reaction is heated during the denaturation step, releasing the reagent only at higher temperatures. The DNA polymerase can also be kept in an active state by binding to an aptamer or an antibody. This binding is disrupted at higher temperatures, releasing the functional DNA polymerase that can proceed with the PCR unhindered.

[00174] In some embodiments, the disclosed methods can optionally include destroying one or more primer-containing amplification artifacts, e.g., primer-dimers, dimer-dimers or superamplicons. In some embodiments, the destroying can optionally include treating the primer and/or amplification product so as to cleave specific cleavable groups present in the primer and/or amplification product. In some embodiments, the treating can include partial or complete digestion of one or more target-specific primers. In one embodiment, the treating can include removing at least 40% of the target specific primer from the amplification product. The cleavable treatment can include enzymatic, acid, alkali, thermal, photo or chemical activity. The cleavable treatment can result in the cleavage or other destruction of the linkages between one or more nucleotides of the primer, or between one or more nucleotides of the amplification product. The primer and/or the amplification product can optionally include one or more modified nucleotides or nucleobases. In some embodiments, the cleavage can selectively occur at these sites, or adjacent to the modified nucleotides or nucleobases. In some embodiments, the cleavage or treatment of the amplified target sequence can result in the formation of a phosphorylated amplified target sequence. In some embodiments, the amplified target sequence is phosphorylated at the 5' terminus.

[00175] In some embodiments, the template, primer and/or amplification product includes nucleotides or nucleobases that can be recognized by specific enzymes. In some embodiments, the nucleotides or nucleobases can be bound by specific enzymes. Optionally, the specific enzymes can also cleave the template, primer and/or amplification product at one or more sites. In some embodiments, such cleavage can occur at specific nucleotides within the template, primer and/or amplification product. For example, the template, primer and/or amplification product can include one or more nucleotides or nucleobases including uracil, which can be recognized and/or cleaved by enzymes such as uracil DNA glycosylase (UDG, also referred to as UNG) or formamidopyrimidine DNA glycosylase (Fpg). The template, primer and/or amplification product can include one or more nucleotides or nucleobases including RNA-specific bases, which can be recognized and/or cleaved by enzymes such as RNAseH. In some embodiments, the template, primer and/or amplification product can include one or more abasic sites, which can be recognized and/or cleaved using various proofreading polymerases or apyrase treatments. In some embodiments, the template, primer and/or amplification product can include 7,8-dihydro-8-oxoguanine (8-oxoG) nucleobases, which can be recognized or cleaved by enzymes such as Fpg. In some embodiments, one or more amplified target sequences can be partially digested by a FuPa reagent.

[00176] In some embodiments, the primer and/or amplification product includes one or more modified nucleotides including bases that bind, e.g., base pair, with other nucleotides, for example nucleotides in a complementary nucleic acid strand, via chemical linkages. In some embodiments, the chemical linkages are subject to specific chemical attack that selectively cleaves the modified nucleotides (or selectively cleaves one or more covalent linkages between the modified nucleotides and adjacent nucleotides within the primer and/or amplification product) but leaves the other nucleotides unaffected. For example, in some embodiments modified nucleotides can form disulfite linkages with other nucleotides in a complementary strand. Such disulfite linkages can be oxidized via suitable treatments. Similarly, certain modified nucleotides can base pair with other nucleotides in a complementary nucleic acid strand through linkages that can be selectively disrupted via alkali treatment. In some embodiments, the primer and/or amplification product includes one or more modified nucleotides that bind, e.g., base pair, with other nucleotides in a complementary nucleic acid strand through linkages exhibiting decreased thermal stability relative to typical base pairing linkages formed between natural bases. Such reduced-thermal stability linkages can be selectively disrupted through exposure of the primer and/or amplification product to elevated temperatures following amplification.

[00177] An exemplary embodiment is depicted in Figure 1, which depicts a schematic of degradable amplification primers. The amplification primers are bisulfite in design, with either a 5' universal forward amplification sequence linked to a 3' target-specific forward primer, or a 5' universal reverse amplification sequence linked to a 3' target-specific reverse primer. Both primers contain modified nucleotides.

[00178] In some embodiments, primers are synthesized that are complementary to, and can hybridize with, discrete segments of a nucleic acid template strand, including: a primer that can hybridize to the 5' region of the template, which encompasses a sequence that is complementary to either the forward or reverse amplification primer. In some embodiments, the forward primers, reverse primers, or both, share no common nucleic acid sequence, such that they hybridize to distinct nucleic acid sequences. For example, target-specific forward and reverse primers can be prepared that do not compete with other primer pairs within the primer pool to amplify the same nucleic acid sequence. In this example, primer pairs that do not compete with other primer pairs in the primer pool assist in the reduction of non-specific or spurious amplification products. In some embodiments, the forward and reverse primers of each primer pair are unique, in that the nucleotide sequence for each primer is non-complementary and non-identical to the other primer in the primer pair. In some embodiments, the primer pair can differ by at least 10 %, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% nucleotide identity. In some embodiments, the forward and reverse primers in each primer pair are non-complementary or non-identical to other primer pairs in the primer pool or multiplex reaction. For example, the primer pairs within a primer pool or multiplex reaction can differ by at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, or at least 70% nucleotide identity to other primer pairs within the primer pool or multiplex reaction. Generally, primers are designed to minimize the formation of primer-dimers, dimer-dimers or other non-specific amplification products. Typically, primers are optimized to reduce GC bias and low melting temperatures (T_m) during the amplification reaction. In some embodiments, the primers are designed to possess a T_m of about 55°C to about 72°C. In some embodiments, the primers of a primer pool can possess a T_m of about 59°C to about 70°C, 60°C to about 68°C, or 60°C to about 65°C. In some embodiments, the primer pool can possess a T_m that does not deviate by more than 5°C.

[00179] In some embodiments, the target-specific primers do not contain a carbon-spacer or terminal linker. In some embodiments, the target-specific primers or amplified target sequences do not contain an enzymatic, magnetic, optical or fluorescent label.

[00180] The template can include a 3' region that contains the sequence for either the upstream or downstream regions surrounding a particular gene or region of interest, such that the region of interest is bracketed by a forward amplification/upstream gene-specific fusion, and a reverse amplification/downstream region of interest fusion primer. In some embodiments, an internal separator sequence can separate the template regions that can hybridize to the amplification and gene-specific primers, and this may act as a key or barcode for subsequent downstream applications such as sequencing, etc. In some embodiments, a barcode or key can be incorporated into each of the amplification products to assist with data analysis and for example, cataloging. In some embodiments, the barcodes can be Ion Torrent™ DNA barcodes (Life Technologies).

[00181] In some embodiments, the primer includes a sufficient number of modified nucleotides to allow functionally complete degradation of the primer by the cleavage treatment, but not so many as to interfere with the primer's specificity or functionality prior to such cleavage treatment, for example in the amplification reaction. In some embodiments, the primer includes at least one modified nucleotide, but no greater than 75% of nucleotides of the primer are modified.

[00182] In some embodiments, multiple different primers including at least one modified nucleotide can be used in a single amplification reaction. For example, multiplexed primers including modified nucleotides can be added to the amplification reaction mixture, where each primer (or set of primers) selectively hybridizes to, and promotes amplification of different target nucleic acid molecules within the nucleic acid population. In some embodiments, different primer combinations can be added to the amplification reaction at plexy of at least about 24, 96, 384, 768, 1000, 2000, 3000, 6000 or 10000, or more (where "plexy" indicates the total number of different targets that can theoretically be amplified in a sequence-specific manner in the amplification reaction). In some embodiments, the modified primers contain at least one modified nucleotide near or at the termini of the primer. In some embodiments, the modified primers contain two or more modified nucleotides within the primer sequence. In an exemplary embodiment, the primer sequence contains a uracil near, or at, the termini of the primer sequence. For the purposes of this disclosure "near" or "at the termini" of the primer sequences refers up to 10 nucleotides from the termini of the primer sequence. In some embodiments, the primer sequence contains a uracil located at, or about, the center nucleotide position of the primer sequence. For the purposes of this disclosure "at, or

about the center nucleotide position of the primer sequence” refers to the incorporation of a uracil moiety at the center nucleotide of the primer sequence or within eight nucleotides, in either a 3’ or 5’ direction flanking the center nucleotide. In one embodiment, the target-specific primer sequence can contain a modified nucleobase at or about the center nucleotide position and contain a modified nucleobase at the 3’ and/or 5’ terminus. In some embodiments, the length of the forward or reverse primer sequence can be about 15 to about 40 bases in length. In some embodiments, the T_m of the primer sequence used in the multiplex reaction can be about 55°C to about 72°C. In some embodiments, the primer pairs are designed to amplify sequences from the target nucleic acid molecules or amplicons that are about 100 base pairs to about 500 base pairs in length.

[00183] In some embodiments, the amplification reactions are conducted in parallel within a single reaction phase (for example, within the same amplification reaction mixture within a single tube). See Figure 2. In some instances, an amplification reaction can generate a mixture of products including both the intended amplicon product as well as unintended, unwanted, nonspecific amplification artifacts such as primer-dimers. Post amplification, the reactions are then treated with any suitable agent that will selectively cleave or otherwise selectively destroy the nucleotide linkages of the modified nucleotides within the excess unincorporated primers and the amplification artifacts without cleaving or destroying the specification amplification products. For example, the primers can include uracil-containing nucleobases that can be selectively cleaved using UNG/UDG (optionally with heat and/or alkali). In some embodiments, the primers can include uracil-containing nucleotides that can be selectively cleaved using UNG and Fpg. In some embodiments, the cleavage treatment includes exposure to oxidizing conditions for selective cleavage of dithiols, treatment with RNaseH for selective cleavage of modified nucleotides including RNA-specific moieties (e.g., ribose sugars, etc.), and the like. This cleavage treatment can effectively fragment the original amplification primers and non-specific amplification products into small nucleic acid fragments that include relatively few nucleotides each. Such fragments are typically incapable of promoting further amplification at elevated temperatures. Such fragments can also be removed relatively easily from the reaction pool through the various post-amplification cleanup procedures known in the art (e.g., spin columns, EtOH precipitation, etc).

[00184] In some embodiments, amplification products following cleavage or other selective destruction of the nucleotide linkages of the modified nucleotides are optionally treated to generate amplification products that possess a phosphate at the 5’ termini. In some embodiments, the phosphorylation treatment includes enzymatic manipulation to produce 5’ phosphorylated

amplification products. In one embodiment, enzymes such as polymerases can be used to generate 5' phosphorylated amplification products. For example, T4 polymerase can be used to prepare 5' phosphorylated amplicon products. Klenow can be used in conjunction with one or more other enzymes to produce amplification products with a 5' phosphate. In some embodiments, other enzymes known in the art can be used to prepare amplification products with a 5' phosphate group. For example, incubation of uracil nucleotide containing amplification products with the enzyme UDG, Fpg and T4 polymerase can be used to generate amplification products with a phosphate at the 5' termini. It will be apparent to one of skill in the art that other techniques, other than those specifically described herein, can be applied to generate phosphorylated amplicons. It is understood that such variations and modifications that are applied to practice the methods, systems, kits, compositions and apparatuses disclosed herein, without resorting to undue experimentation are considered within the scope of the disclosure.

[00185] In some embodiments, primers that are incorporated in the intended (specific) amplification products, these primers are similarly cleaved or destroyed, resulting in the formation of "sticky ends" (e.g., 5' or 3' overhangs) within the specific amplification products. Such "sticky ends" can be addressed in several ways. For example, if the specific amplification products are to be cloned, the overhang regions can be designed to complement overhangs introduced into the cloning vector, thereby enabling sticky ended ligations that are more rapid and efficient than blunt ended ligations. Alternatively, the overhangs may need to be repaired (as with several next-generation sequencing methods). Such repair can be accomplished either through secondary amplification reactions using only forward and reverse amplification primers (in the embodiment shown in Figure 1, this corresponds to A and P1 primers) comprised of only natural bases. In this manner, subsequent rounds of amplification rebuild the double-stranded templates, with nascent copies of the amplicon possessing the complete sequence of the original strands prior to primer destruction. Alternatively, the sticky ends can be removed using some forms of fill-in and ligation processing, wherein the forward and reverse primers are annealed to the templates. A polymerase can then be employed to extend the primers, and then a ligase, optionally a thermostable ligase, can be utilized to connect the resulting nucleic acid strands. This could obviously be also accomplished through various other reaction pathways, such as cyclical extend-ligation, etc. In some embodiments, the ligation step can be performed using one or more DNA ligases.

[00186] The amplification reaction can include any reaction that increases the copy number of a nucleic acid molecule, optionally in a cyclical fashion, and can include without limitation

isothermal amplification (for example, rolling circle amplification or isothermal amplification as described in US Provisional Application Nos. 61/424,599, 61/445,324 and 61/451,919, hereby incorporated by reference in their entireties), amplification using thermocycling, and the like.

[00187] In some embodiments, the disclosure generally relates to methods for single-tube multiplex PCR. In some embodiments, the method for single-tube multiplex PCR can include target-specific or exon-specific primers. In some embodiments, the exon-specific or target-specific primers can include at least one uracil nucleotide. In some embodiments, single-tube multiplex PCR can include selective amplification of at least 1000, 2000, 3000, 4000, 5000, 6000 or more target nucleic acid molecules using target-specific or exon-specific uracil based primers.

[00188] In some embodiments, the disclosure relates generally to methods for generating a target-specific or exon-specific amplicon library. In some embodiments, the amplicon library generated using target-specific or exon-specific primers can be associated with mutations of human cancers. In some embodiments, the mutations can be in the KRAS, BRAF and/or EGFR genes. In some embodiments, the amplicon library can be generated from genomic DNA or formalin-fixed, paraffin-embedded (FFPE) tissue. In some embodiments, the amplicons of the amplicon library prepared using the methods disclosed herein can be about 100 to about 300 base pairs in length, about 100 to about 250 base pairs in length, about 120 to about 220 base pairs in length or about 135 to about 205 base pairs in length. In some embodiments, the amplicon library can be prepared using primer pairs that are targeted to cancer specific mutations. In some embodiments, the primer pairs can be directed to non-cancer related mutations, such as inherited diseases, e.g., cystic fibrosis and the like. In some embodiments, the primer pairs can be used to generate amplicons that once sequenced by any sequencing platform, including semi-conductor sequencing technology can be used to detect genetic mutations such as inversion, deletions, point mutations and variations in copy number.

[00189] In some embodiments, the primer pairs used to produce an amplicon library can result in the amplification of target-specific nucleic acid molecules possessing one or more of the following metrics: greater than 97% target coverage at 20x if normalized to 100x average coverage depth; greater than 97% of bases with greater than 0.2x mean; greater than 90% base without strand bias; greater than 95% of all reads on target; greater than 99% of bases with greater than 0.01x mean; and greater than 99.5% per base accuracy.

[00190] In some embodiments, the amplicon library can be used to detect and/or identify known mutations or *de novo* mutations in a sample.

[00191] In some embodiments, the amplicon library prepared using target-specific primer pairs can be used in downstream enrichment applications such as emulsion PCR or bridge PCR. In some embodiments, the amplicon library can be used in an enrichment application and a sequencing application. For example, an amplicon library can be sequenced using any suitable DNA sequencing platform. In some embodiments, an amplicon library can be sequenced using an Ion Torrent PGM Sequencer (Life Technologies). In some embodiments, a PGM sequencer can be coupled to server that applies parameters or software to determine the sequence of the amplified target nucleic acid molecules. In some embodiments, the amplicon library can be prepared, enriched and sequenced in less than 24 hours. In some embodiments, the amplicon library can be prepared, enriched and sequenced in approximately 9 hours. In some embodiments, an amplicon library can be a paired library, that is, a library that contains amplicons from a tumor sample and amplicons from a non-diseased sample. Each pair can be aligned, to detect and/or identify mutations present in the target nucleic acid molecules.

[00192] In some embodiments, methods for generating an amplicon library can include: amplifying genomic DNA targets using exon-specific or target-specific primers to generate amplicons; purifying the amplicons from the input DNA and primers; phosphorylating the amplicons; ligating adapters to the phosphorylated amplicons (steps shown in Figure 2); purifying the ligated amplicons; nick-translating the amplified amplicons; and purifying the nick-translated amplicons to generate the amplicon library. In some embodiments, additional amplicon library manipulations can be conducted following the step of amplification of genomic DNA targets to generate the amplicons. In some embodiments, any combination of additional reactions can be conducted in any order, and can include: purifying; phosphorylating; ligating adapters; nick-translating; amplification and/or sequencing. In some embodiments, any of these reactions can be omitted or can be repeated. It will be readily apparent to one of skill in the art that the method can repeat or omit any one or more of the above steps. It will also be apparent to one of skill in the art that the order and combination of steps may be modified to generate the required amplicon library, and is not therefore limited to the exemplary methods provided.

[00193] A phosphorylated amplicon can be joined to an adapter to conduct a nick translation reaction, subsequent downstream amplification (e.g., template preparation), or for attachment to particles (e.g., beads), or both. For example, an adapter that is joined to a phosphorylated amplicon can anneal to an oligonucleotide capture primer which is attached to a particle, and a primer extension reaction can be conducted to generate a complementary copy of the amplicon attached to

the particle or surface, thereby attaching an amplicon to a surface or particle. Adapters/Adapters can have one or more amplification primer hybridization sites, sequencing primer hybridization sites, barcode sequences, and combinations thereof. In some embodiments, amplicons prepared by the methods disclosed herein can be joined to one or more Ion Torrent™ compatible Adapters/adapters to construct an amplicon library. Amplicons generated by such methods can be joined to one or more Adapters/adapters for library construction to be compatible with a next generation sequencing platform. For example, the amplicons produced by the teachings of the present disclosure can be attached to adapters provided in the Ion Fragment Library Kit (Life Technologies, Catalog No. 4466464).

[00194] In some embodiments, amplification of genomic DNA targets (such as high molecular weight DNA) or FFPE samples can be conducted using a 2x AmpliSeq Hi Fi Master Mix. In some embodiments, the AmpliSeq Hi Fi Master Mix can include glycerol, dNTPs, and a DNA polymerase, such as Platinum® Taq DNA polymerase High Fidelity. In some embodiments, the 2x AmpliSeq Hi Fi Master Mix can further include at least one of the following: a preservative, magnesium sulfate, tris-sulfate and/or ammonium sulfate.

[00195] In some embodiments, amplification of genomic DNA targets (such as high molecular weight DNA) or FFPE samples can be conducted using a 5x Ion AmpliSeq Hi Fi Master Mix. In some embodiments, the 5x Ion AmpliSeq Hi Fi Master Mix can include glycerol, dNTPs, and a DNA polymerase such as Platinum® Taq DNA polymerase High Fidelity. In some embodiments, the 5x Ion AmpliSeq Hi Fi Master Mix can further include at least one of the following: a preservative, magnesium chloride, magnesium sulfate, tris-sulfate and/or ammonium sulfate.

[00196] In some embodiments, phosphorylation of the amplicons can be conducted using a FuP reagent. In some embodiments, the FuP reagent can include a DNA polymerase, a DNA ligase, at least one uracil cleaving or modifying enzyme, and/or a storage buffer. In some embodiments, the FuP reagent can further include at least one of the following: a preservative and/or a detergent.

[00197] In some embodiments, phosphorylation of the amplicons can be conducted using a FuPa reagent. In some embodiments, the FuPa reagent can include a DNA polymerase, at least one uracil cleaving or modifying enzyme, an antibody and/or a storage buffer. In some embodiments, the FuPa reagent can further include at least one of the following: a preservative and/or a detergent. In some embodiments, the antibody is provided to inhibit the DNA polymerase and 3'-5' exonuclease activities at ambient temperature.

[00198] In some embodiments, the amplicon library produced by the teachings of the present disclosure are sufficient in yield to be used in a variety of downstream applications including the Ion Xpress™ Template Kit using an Ion Torrent™ PGM system (e.g., PCR-mediated addition of the nucleic acid fragment library onto Ion Sphere™ Particles)(Life Technologies, Part No. 4467389). For example, instructions to prepare a template library from the amplicon library can be found in the Ion Xpress Template Kit User Guide (Life Technologies, Part No. 4465884), hereby incorporated by reference in its entirety. Instructions for loading the subsequent template library onto the Ion Torrent™ Chip for nucleic acid sequencing are described in the Ion Sequencing User Guide (Part No. 4467391), hereby incorporated by reference in its entirety. In some embodiments, the amplicon library produced by the teachings of the present disclosure can be used in paired end sequencing (e.g., paired-end sequencing on the Ion Torrent™ PGM system (Life Technologies, Part No. MAN0006191), hereby incorporated by reference in its entirety.

[00199] It will be apparent to one of ordinary skill in the art that numerous other techniques, platforms or methods for clonal amplification such as wildfire PCR and bridge amplification can be used in conjunction with the amplified target sequences of the present disclosure. It is also envisaged that one of ordinary skill in art upon further refinement or optimization of the conditions provided herein can proceed directly to nucleic acid sequencing (for example using the Ion Torrent PGM™ or Proton™ sequencers, Life Technologies) without performing a clonal amplification step.

[00200] In some embodiments, at least one of the amplified targets sequences to be clonally amplified can be attached to a support or particle. The support can be comprised of any suitable material and have any suitable shape, including, for example, planar, spheroid or particulate. In some embodiments, the support is a scaffolded polymer particle as described in U.S. Published App. No. 20100304982, hereby incorporated by reference in its entirety.

[00201] In some embodiments, nucleic acid sequencing of the amplified target sequences produced by the teachings of this disclosure include *de novo* sequencing or targeted resequencing. In some embodiments, nucleic acid sequencing further includes comparing the nucleic acid sequencing results of the amplified target sequences against a reference nucleic acid sample. In some embodiments, the reference sample can be normal tissue or well documented tumor sample. In some embodiments, nucleic acid sequencing of the amplified target sequences further includes determining the presence or absence of a mutation within the nucleic acid sequence. In some embodiments, the method further includes correlating the presence of a mutation with drug susceptibility, prognosis of treatment and/or organ rejection. In some embodiments, nucleic acid sequencing includes the

identification of genetic markers associated with cancer and/or inherited diseases. In some embodiments, nucleic acid sequencing includes the identification of copy number variation in a sample under investigation.

[00202] In some embodiments, a kit is provided for amplifying multiple target sequences from a population of nucleic acid molecules in a single reaction. In some embodiments, the kit includes a plurality of target-specific primer pairs containing one or more cleavable groups, one or more DNA polymerases, a mixture of dNTPs and at least one cleaving reagent. In one embodiment, the cleavable group can be 8-oxo-deoxyguanosine, deoxyuridine or bromodeoxyuridine. In some embodiments, the at least one cleaving reagent includes RNaseH, uracil DNA glycosylase, Fpg or alkali. In one embodiment, the cleaving reagent can be uracil DNA glycosylase. In some embodiments, the kit is provided to perform multiplex PCR in a single reaction chamber or vessel. In some embodiments, the kit includes at least one DNA polymerase, which can be a thermostable DNA polymerase. In some embodiments, the concentration of the one or more DNA polymerases is present in a 3-fold excess as compared to a single PCR reaction. In some embodiments, the final concentration of each target-specific primer pair is present at about 25 nM to about 50 nM. In one embodiment, the final concentration of each target-specific primer pair can be present at a concentration that is 50% lower than conventional single plex PCR reactions. In some embodiments, the kit provides amplification of at least 100, 500, 1000, 3000, 6000, 10000, 12000, or more, target sequences from a population of nucleic acid molecules in a single reaction chamber.

[00203] In some embodiments, the kit further comprises one or more adapters, barcodes, and/or antibodies.

[00204] The following description of various exemplary embodiments is exemplary and explanatory only and is not to be construed as limiting or restrictive in any way. Other embodiments, features, objects, and advantages of the present teachings will be apparent from the description and accompanying drawings, and from the claims.

[00205] Although the present description described in detail certain exemplary embodiments, other embodiments are also possible and within the scope of the present invention. Variations and modifications will be apparent to those skilled in the art from consideration of the specification and figures and practice of the teachings described in the specification and figures, and the claims.

Examples

[00206] **Example 1. Library Preparation**

[00207] **PCR Amplify Genomic DNA Targets**

[00208] A multiplex polymerase chain reaction is performed to amplify 136 individual amplicons across a genomic DNA sample. A pool of forward and reverse primers are designed to amplify the target regions of Table 1. Each primer pair in the primer pool is designed to contain at least one uracil nucleotide near the terminus of each forward and reverse primer. Each primer pair is also designed to selectively hybridize to, and promote amplification of a specific target region of the SNP panel of Table 1.

[00209] To a single well of a 96-well PCR plate is added 5 microliters of the primer pool containing forward and reverse primers at a concentration of 15 μ M in TE, 10-50 ng genomic DNA and 10 microliters of an amplification reaction mixture (2x AmpliSeq HiFi Master Mix) that can include glycerol, dNTPs, and Platinum® Taq High Fidelity DNA Polymerase (Invitrogen, Catalog No. 11304) to a final volume of 20 microliters with DNase/RNase Free Water (Life Technologies, CA, Part No. 600004).

[00210] The PCR plate is sealed and loaded into a thermal cycler (GeneAmp® PCR system 9700 Dual 96-well thermal cycler (Life Technologies, CA, Part No. N8050200 and 4314445)) and is run on the following temperate profile to generate the preamplified amplicon library.

[00211] An initial holding stage is performed at 98°C for 2 minutes, followed by 16 cycles of denaturing at 98°C for 15 seconds and an annealing and extending stage at 60°C for 4 minutes. After cycling, the preamplified amplicon library is held at 4°C until proceeding to the purification step outlined below. A schematic of an exemplary library amplification process is shown in FIG. 2.

[00212] **Purify the Amplicons from input DNA and Primers**

[00213] Two rounds of Agencourt® AMPure® XP Reagent (Beckman Coulter, CA) binding, wash, and elution at 0.6x and 1.2x volume ratios is found to remove genomic DNA and unbound or excess primers. The amplification and purification step outlined herein produces amplicons of about 100 bp to about 600 bp in length.

[00214] In a 1.5ml LoBind tube (Eppendorf, Part No. 022431021), the preamplified amplicon library (20 microliters) is combined with 12 microliters (0.6x volumes) of Agencourt® AMPure® XP reagent (Beckman Coulter, CA). The bead suspension is pipetted up and down to thoroughly mix the bead suspension with the preamplified amplicon library. The sample is then pulse-spin and incubated for 5 minutes at room temperature.

[00215] The tube containing the sample is placed on a magnetic rack such as a DynaMag™-2 spin magnet (Life Technologies, CA, Part No. 123-21D) for 2 minutes to capture the beads. Once the solution cleared, the supernatant is transferred to a new tube, where 24 microliters (1.2x volume) of

Agencourt® AMPure® XP beads (Beckman Coulter, CA) are added to the supernatant. The mixture is pipetted to ensure the bead suspension mixed with the preamplified amplicon library. The sample is then pulse-spin and incubated at room temperature for 5 minutes. The tube containing the sample is placed on the magnetic rack for 2 minutes to capture the beads. Once the solution cleared, the supernatant is carefully discarded without disturbing the bead pellet. The desired preamplified amplicon library is now bound to the beads. Without removing the tube from the magnetic rack, 200 microliters of freshly prepared 70% ethanol is introduced into the sample. The sample is incubated for 30 seconds while gently rotating the tube on the magnetic rack. After the solution cleared, the supernatant is discarded without disturbing the pellet. A second ethanol wash is performed and the supernatant discarded. Any remaining ethanol is removed by pulse-spinning the tube and carefully removing residual ethanol while not disturbing the pellet. The pellet is air-dried for about 5 minutes at room temperature.

[00216] Once the tube is dry, the tube is removed from the magnetic rack and 20 microliters of DNase/RNase Free Water is added (Life Technologies, CA, Part No. 600004). The tube is vortexed and pipetted to ensure the sample was mixed thoroughly. The sample is pulse-spin and placed on the magnetic rack for two minutes. After the solution clears, the supernatant containing the eluted DNA is transferred to a new tube.

[00217] **Phosphorylate the Amplicons**

[00218] To the eluted DNA (~ 20 microliters), 3 microliters of DNA ligase buffer (Invitrogen, Catalog No. 15224041), 2 microliters dNTP mix, and 2 microliters of FuP reagent are added. The reaction mixture is mixed thoroughly to ensure uniformity and incubated at 37°C for 10 minutes.

[00219] **Ligate Adapters to the Amplicons and Purify the Ligated Amplicons**

[00220] After incubation, the reaction mixture proceeds directly to a ligation step. Here, the reaction mixture now containing the phosphorylated amplicon library is combined with 1 microliter of A/P1 Adapters (20 µm each) (sold as a component of the Ion Fragment Library Kit, Life Technologies, Part No. 4466464) and 1 microliter of DNA ligase (sold as a component of the Ion Fragment Library Kit, Life Technologies, Part No. 4466464), and is incubated at room temperature for 30 minutes.

[00221] After the incubation step, 52 microliters (1.8x sample volume) of Agencourt® AMPure® Reagent (Beckman Coulter, CA) is added to the ligated DNA. The mixture is pipetted thoroughly to mix the bead suspension with the ligated DNA. The mixture is pulse-spin and incubated at room temperature for 5 minutes. The samples undergoes another pulse-spin and were

placed on a magnetic rack such as a DynaMag™-2 spin magnet (Life Technologies, CA, Part No. 123-21D) for two minutes. After the solution clears, the supernatant is discarded. Without removing the tube from the magnetic rack, 200 microliters of freshly prepared 70% ethanol is introduced into the sample. The sample is incubated for 30 seconds while gently rotating the tube on the magnetic rack. After the solution clears, the supernatant is discarded without disturbing the pellet. A second ethanol wash is performed and the supernatant is discarded. Any remaining ethanol is removed by pulse-spinning the tube and carefully removing residual ethanol while not disturbing the pellet. The pellet is air-dried for about 5 minutes at room temperature.

[00222] The pellet is resuspended in 20 microliters of DNase/RNase Free Water (Life Technologies, CA, Part No. 600004) and is vortexed to ensure the sample is mixed thoroughly. The sample was pulse-spin and placed on the magnetic rack for two minutes. After the solution clears, the supernatant containing the ligated DNA is transferred to a new LoBind tube (Eppendorf, Part No. 022431021).

[00223] **Nick Translate and Amplify the Amplicon Library and Purify the Library**

[00224] The ligated DNA (~20 microliters) is combined with 76 microliters of Platinum® PCR SuperMix High Fidelity (Life Technologies, CA, Part No. 12532-016, sold as a component of the Ion Fragment Library Kit, Life Technologies, Part No. 4466464) and 4 microliters of Library Amplification Primer Mix (5 µM each)(Life Technologies, CA, Part No. 602-1068-01, sold as a component of the Ion Fragment Library Kit, Life Technologies, Part No. 4466464), the mixture is pipetted thoroughly to ensure a uniformed solution. The solution is applied to a single well of a 96-well PCR plate and sealed. The plate is loaded into a thermal cycler (GeneAmp® PCR system 9700 Dual 96-well thermal cycler (Life Technologies, CA, Part No. N8050200 and 4314445)) and is run on the following temperate profile to generate the final amplicon library.

[00225] A nick-translation is performed at 72°C for 1 minute, followed by an enzyme activation stage at 98°C for 2 minutes, followed by 5-10 cycles of denaturing at 98°C for 15 seconds and an annealing and extending stage at 60°C for 1 minute. After cycling, the final amplicon library is held at 4°C until proceeding to the final purification step outlined below.

[00226] In a 1.5ml LoBind tube (Eppendorf, Part No. 022431021), the final amplicon library (~100 microliters) is combined with 180 microliters (1.8x sample volume) of Agencourt® AMPure® XP reagent (Beckman Coulter, CA). The bead suspension is pipetted up and down to thoroughly mix the bead suspension with the final amplicon library. The sample is then pulse-spin and is incubated for 5 minutes at room temperature.

[00227] The tube containing the final amplicon library is placed on a magnetic rack such as a DynaMag™-2 spin magnet (Life Technologies, CA, Part No. 123-21D) for 2 minutes to capture the beads. Once the solution clears, the supernatant is carefully discarded without disturbing the bead pellet. Without removing the tube from the magnetic rack, 400 microliters of freshly prepared 70% ethanol is introduced into the sample. The sample is incubated for 30 seconds while gently rotating the tube on the magnetic rack. After the solution clears, the supernatant is discarded without disturbing the pellet. A second ethanol wash is performed and the supernatant is discarded. Any remaining ethanol is removed by pulse-spinning the tube and carefully removing residual ethanol while not disturbing the pellet. The pellet is air-dried for about 5 minutes at room temperature.

[00228] Once the tube is dry, the tube is removed from the magnetic rack and 20 microliters of Low TE is added (Life Technologies, CA, Part No. 602-1066-01). The tube is pipetted and vortexed to ensure the sample is mixed thoroughly. The sample is pulse-spin and is placed on the magnetic rack for two minutes. After the solution clears, the supernatant containing the final amplicon library is transferred to a new Lobind tube (Eppendorf, Part No. 022431021).

[00229] **Assess the Library Size Distribution and Determine the Template Dilution Factor**

[00230] The final amplicon library is quantitated to determine the library dilution (Template Dilution Factor) that results in a concentration within the optimized target range for Template Preparation (e.g., PCR-mediated addition of library molecules onto Ion Sphere™ Particles). The final amplicon library is typically quantitated for downstream Template Preparation procedure using an Ion Library Quantitation Kit (qPCR) (Life Technologies, Part No. 4468802) and/or a Bioanalyzer™ (Agilent Technologies, Agilent 2100 Bioanalyzer) to determine the molar concentration of the amplicon library, from which the Template Dilution Factor is calculated. For example, instructions to determine the Template Dilution Factor by quantitative real-time PCR (qPCR) can be found in the Ion Library Quantitation Kit User Guide (Life Technologies, Part No. 4468986), hereby incorporated by reference in its entirety.

[00231] In this example, 1 microliter of the final amplicon library preparation is analyzed on the 2100 Bioanalyzer™ with an Agilent High Sensitivity DNA Kit (Agilent Technologies, Part No. 5067-4626) to generate peaks in the 135-205 bp size range and at a concentration of about 5×10^9 copies per microliter.

[00232] **Proceed to Template Preparation**

[00233] An aliquot of the final library is used to prepare DNA templates that are clonally amplified on Ion Sphere™ Particles using emulsion PCR (emPCR). The preparation of template in the instant example is prepared according to the manufacturer's instructions using an Ion Xpress Template Kit (Life Technologies, Part No. 4466457), hereby incorporated by reference in its entirety. Once template-positive Ion Sphere Particles are enriched, an aliquot of the Ion Spheres are loaded onto an Ion 314™ Chip (Life Technologies, Part No. 4462923) as described in the Ion Sequencing User Guide (Part No. 4467391), hereby incorporated in its entirety, and is subjected to analysis and sequencing as described in the Ion Torrent PGM Sequencer User Guide (Life Technologies, Part No. 4462917), hereby incorporated in its entirety.

[00234] TABLE A

Reference SNP	GenBank Accession	Chromosome Position
rs1004357	NT_010783.15	41691526
rs1005533	NT_011362.10	39487110
rs10092491	NT_023666	28411072
rs1019029	NT_007819.17	13894276
rs1024116	NT_025028.14	75432386
rs1028528	NT_011520.12	48362290
rs1029047	NT_007592.15	1135939
rs10495407	NT_167186.1	238439308
rs10500617	NT_009237.18	5099393
rs1058083	NT_009952	1E 08
rs10768550	NT_009237.18	5098714
rs10773760	NT_009755.19	130761696
rs10776839	NT_019501.13	137417308
rs12480506	NT_12480506	16241416
rs1276034	NT_011903.12	23984056
rs1276035	NT_011903.12	23979899
rs12997453	NT_005403	1.82E 08
rs13134862	NT_016354	76425896
rs13182883	NT_034772	1.37E 08
rs13218440	NT_007592	12059954
rs1355366	NT_005612.16	190806108
rs1357617	NT_022517.18	961782
rs1358856	NT_025741	1.24E 08
rs1382387	NT_010498.15	80106351

rs1410059	NC_000010.8		97172595
rs1413212	NT_167186.1		242806797
rs1454361	NT_026437.12		25850832
rs1463729	NT_008470.19		126881448
rs1478829	NT_025741.15		120560694
rs1490413	NT_021937.19		4367323
rs1493232	NT_010859.14		1127986
rs1498553	NT_009237		5709028
rs1515817	NT_011896.9		8550111
rs1523537	NT_011362.10		51296162
rs1554472	NT_016354.19		157489906
rs1558843	NT_011875		22750583
rs159606	NT_006576.16		17374898
rs1736442	NT_025028		55225665
rs1800865	NT_011896.9		2658271
rs1821380	NT_010194.17		39313402
rs1864258	NT_011875.12		16628931
rs1865680	NT_011896.7		6868118
rs1872575	NT_005795.1	1.14E 08	
rs1886510	NT_024524.14		22374700
rs1979255	NT_016354.19		190318080
rs2020857	NC_000024.7		15030752
rs2032595			14813991
rs2032598	NT_011875.0		14850341
rs2032599	NC_000024.0		14851554
rs2032600	NC_000024.7		14888783
rs2032601	NC_000024.7		14869076
rs2032604	NC_000024.7		14969934
rs2032607			14904859
rs2032611	NT_011875.12		21866424
rs2032624	NT_011875		15026424
rs2032626	BV679069		21903383
rs2032631	NC_000024.7		21867787
rs2032653	AC006376.2		15591537
rs2032658	NT_011875.0		15581983
rs2032668			15437333
rs2032673	NC_000024		21894058
rs2040411	NT_011520.12		47836412
rs2056277	NT_028251	1.39E 08	
rs2072422	AC006376.2		15590674

rs2073383	NT_011520.12		23802171
rs2075181	NT_011896.9		7546726
rs2075182	NT_011896.9		7527958
rs2075183	NT_011896.9		7527957
rs2075640	NT_011896		2722506
rs2107612	NT_009759		888320
rs2111980	NT_029419.12		106328254
rs214955	NT_025741.14	1.53E 08	
rs2175957	NT_025741		41286822
rs2255301	U47924		6909442
rs2267801	NT_001609.2		2828196
rs2269355	U47924.1		6945914
rs2270529	NT_008413		14747133
rs2272998	NT_025741.14	1.49E 08	
rs2291395	NT_010663		80526139
rs2292972	NT_010663		80765788
rs2299942	NT_001609.2		2731887
rs2342747	NT_010393.16		5868700
rs251934	NT_023133.13		174778678
rs2567608	NT_011441.1		23017082
rs2811231	NT_007592.15		55155704
rs2833736	NT_011512.11		33582722
rs321198	NT_007933.15		137029838
rs338882	NT_077451	1.79E 08	
rs354439	NT_009952.14		106938411
rs3744163	NT_010663.1		80739859
rs3780962	NT_077569		17193346
rs3897	NT_011875.12		18571026
rs3900	NT_011875		21730257
rs4288409	NT_008046.16		136839229
rs430046	NT_010498.15		78017051
rs4530059	NT_026437.12		104769149
rs4606077	NT_008046.16		144656754
rs464663	NT_011512.11		28023370
rs4789798	NT_010663		80531643
rs521861	NT_010966		47371014
rs560681	NT_079484	1.61E 08	
rs576261	NT_011109.16		39559807
rs590162	NT_033899.8		122195989
rs6444724	NT_005962	1.93E 08	

rs6591147	NT_033899.8		105912984
rs6811238	NT_022792	1.7E 08	
rs689512	NT_010663		80715702
rs6955448	NT_007819.17		4310365
rs7041158	NT_008413		27985938
rs717302	NT_006576.16		2879395
rs719366	NT_011109.16		28463337
rs722098	NT_011512.11		16685598
rs7229946	NT_010966		22739001
rs727811	NT_007422	1.65E 08	
rs729172	NT_010552.10		5606197
rs733164	NT_011520.8		27816784
rs735155	NT_077567		3374178
rs737681	NT_007741	1.56E 08	
rs740598	NT_030059	1.19E 08	
rs7520386	NT_004610.19		14155402
rs7704770	NT_023133	1.59E 08	
rs8070085	NT_010755		411341984
rs873196	NT_026437.12		98845531
rs876724	NT_022221.13		114974
rs891407	NT_011875.12		21843090
rs891700	NT_004836	2.4E 08	
rs901398		11036221	11096221
rs914165	NT_011512.11		42415929
rs917118	NT_007819.17		4457003
rs9546538	NT_024524		84456735
rs9606186	NT_011519		19920359
rs964681	NT_008818.16		132698419
rs985492	NT_010966.14		29311034
rs9866013	NT_022517.18		59488340
rs987640	NT_011520.12		33559508
rs9951171	NT_010859		9749879

CLAIMS

We claim:

1. A method for amplifying a plurality of different target sequences within a sample, comprising:

amplifying within a single amplification reaction mixture a plurality of different target sequences from a sample including the plurality of different target sequences, wherein the amplifying includes contacting at least some portion of the sample with a plurality of target-specific primers, and a polymerase under amplification conditions, thereby producing an amplified plurality of target sequences, wherein at least two of the different amplified target sequences are less than 50% complementary to each other and wherein at least one of the plurality of target-specific primers and at least one of the amplified target sequences includes a cleavable group;

cleaving a cleavable group of at least one amplified target sequence of the amplified plurality of target sequences;

ligating at least one adapter to at least one amplified target sequence in a blunt-ended ligation reaction, thereby producing one or more adapter-ligated amplified target sequences, and reamplifying at least one of the adapter-ligated amplified target sequences.

2. The method of claim 1, wherein one or more of the at least one adapter is not substantially complementary to at least one amplified target sequence.

3. The method of claim 1, wherein the reamplifying includes contacting the at least one adapter-ligated amplified target sequence with one or more primers including a sequence that is complementary to at least one of the adapters or their complement, and a polymerase under amplification conditions, thereby producing at least one reamplified adapter-ligated amplified target sequence.

4. The method of claim 3, wherein at least one of the one or more adapters or their complements is not substantially complementary to at least one amplified target sequence.

5. The method of claim 1, wherein at least one target-specific primer is substantially complementary to at least a portion of a corresponding target sequence in the sample.

6. The method of claim 1, wherein an adapter that is ligated to at least one of the amplified target sequences is susceptible to exonuclease digestion.

7. The method of claim 1, wherein an adapter that is ligated to at least one of the amplified target sequences does not include a protecting group.

8. The method of claim 1, wherein the ligating includes contacting at least one amplified target sequence having a 3' end and a 5' end with a ligation reaction mixture including one or more adapters and a ligase under ligation conditions, wherein none of the adapters in the ligation reaction mixture includes, prior to the ligating, a target-specific sequence.

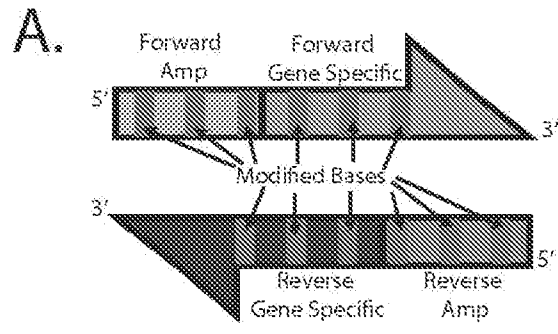
9. The method of claim 8, wherein the ligating includes contacting at least one amplified target sequence with a ligation reaction mixture including one or more adapters and a ligase under ligation conditions, wherein the ligation reaction mixture does not include one or more additional oligonucleotide adapters prior to ligating the one or more adapters to at least one amplified target sequence.

10. The method of claim 1, wherein the amplifying further includes a digestion step prior to the ligating, thereby producing a plurality of blunt-end amplified target sequences possessing a 5' phosphate group.

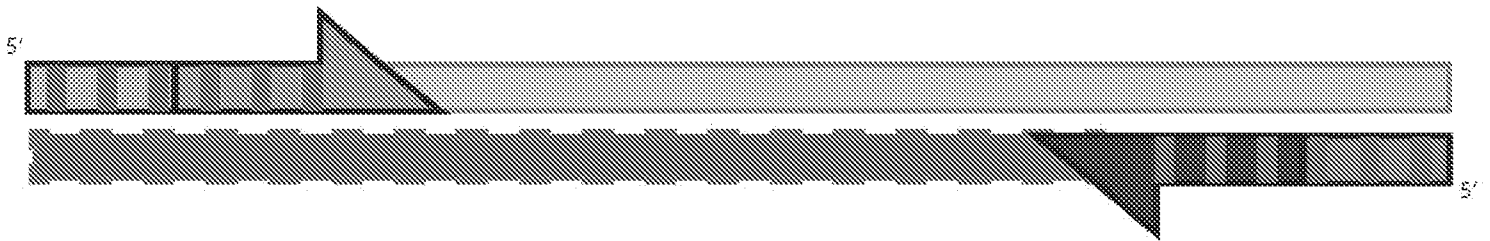
11. A plurality of primer pairs configured to specifically hybridize to a panel of SNP regions of a human genome, wherein the panel of SNP regions is selected from the SNP regions of Table 1, and wherein at least one primer of at least one primer pair comprises a cleavable nucleotide.

12. The plurality of primer pairs wherein the panel of SNPs comprises entries 1-136 of Table 1.

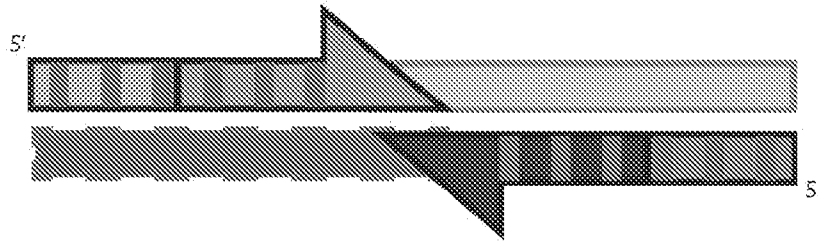
13. The plurality of primer pairs wherein the panel of SNPs comprises entries 1-103 of Table 1.



B1.



B2.



C.

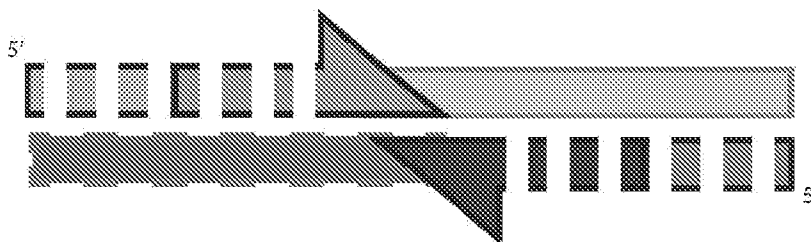
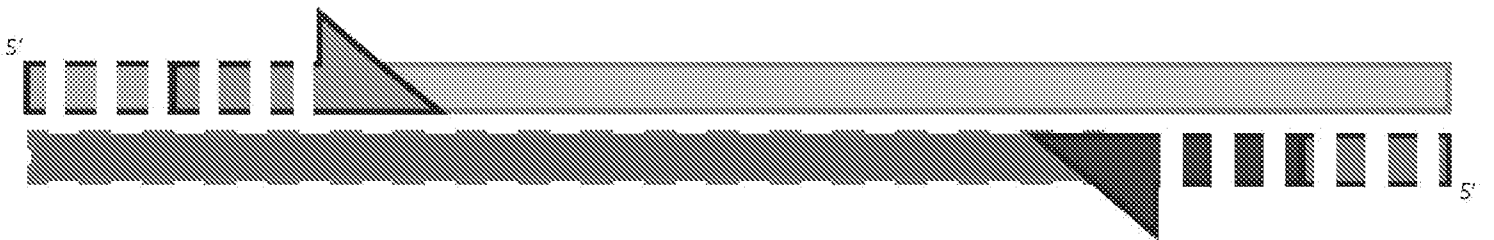
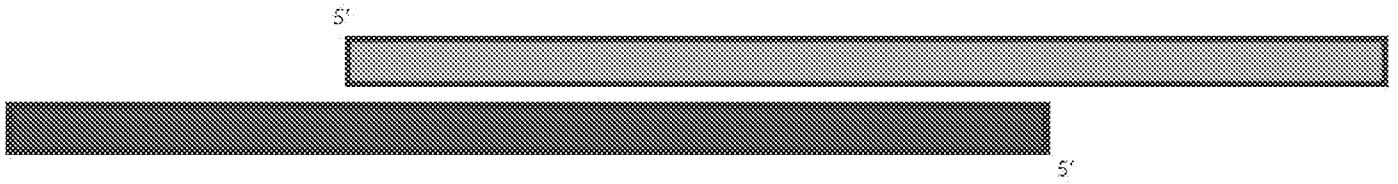
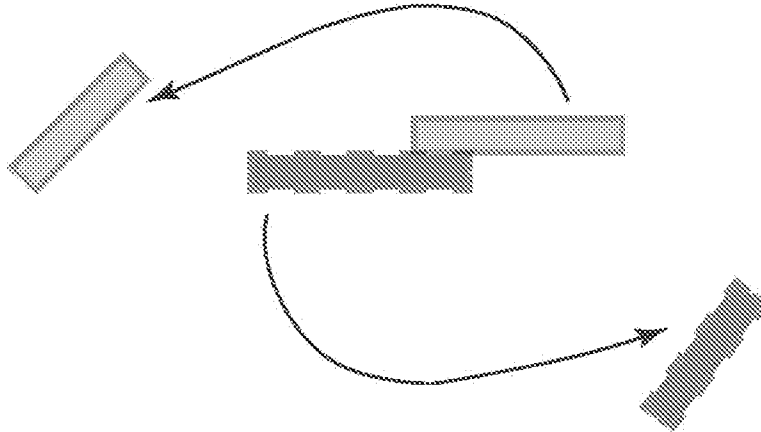


FIG. 1A

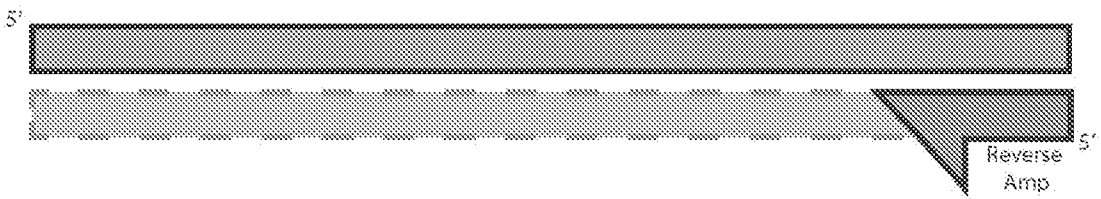
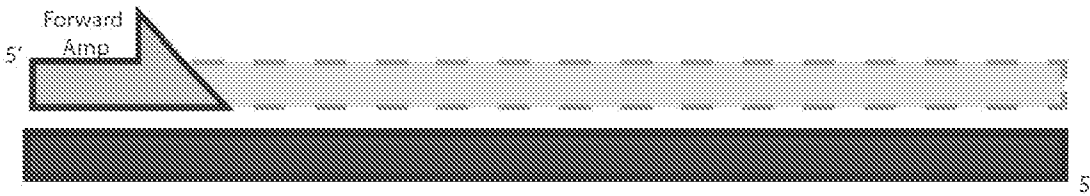
D1.



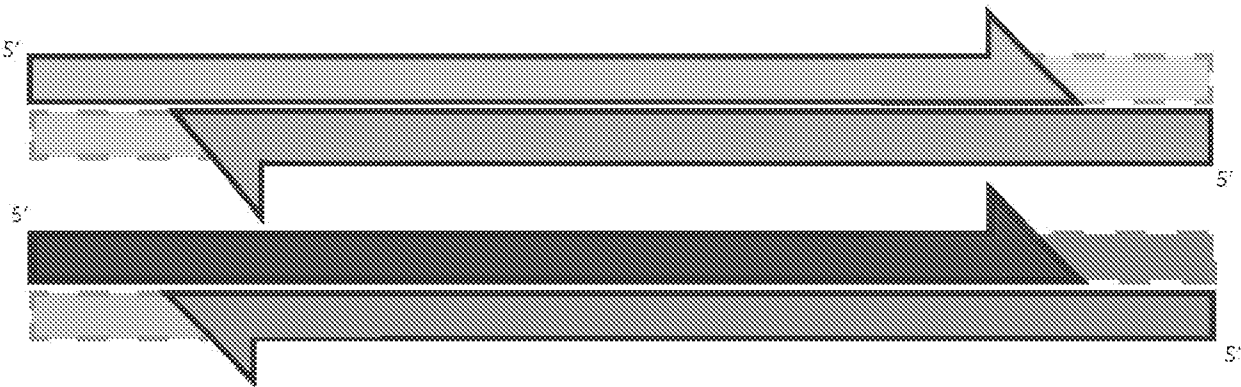
D2.



E1.



E1i.



E2.

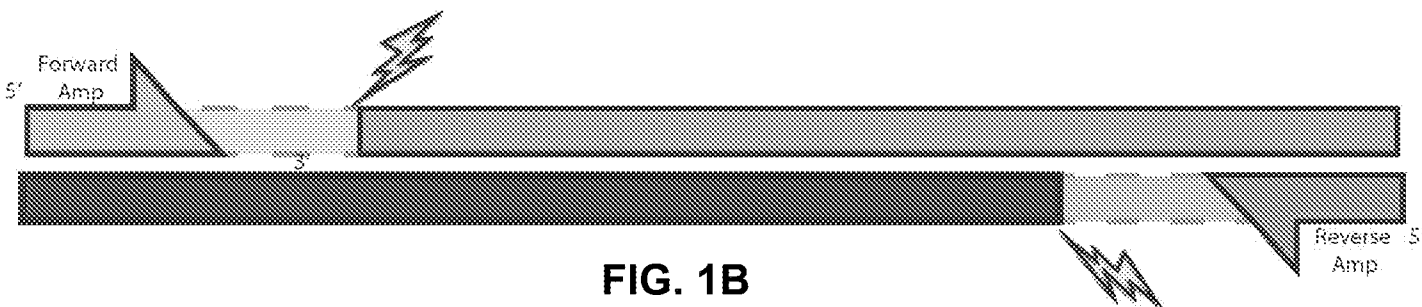


FIG. 1B

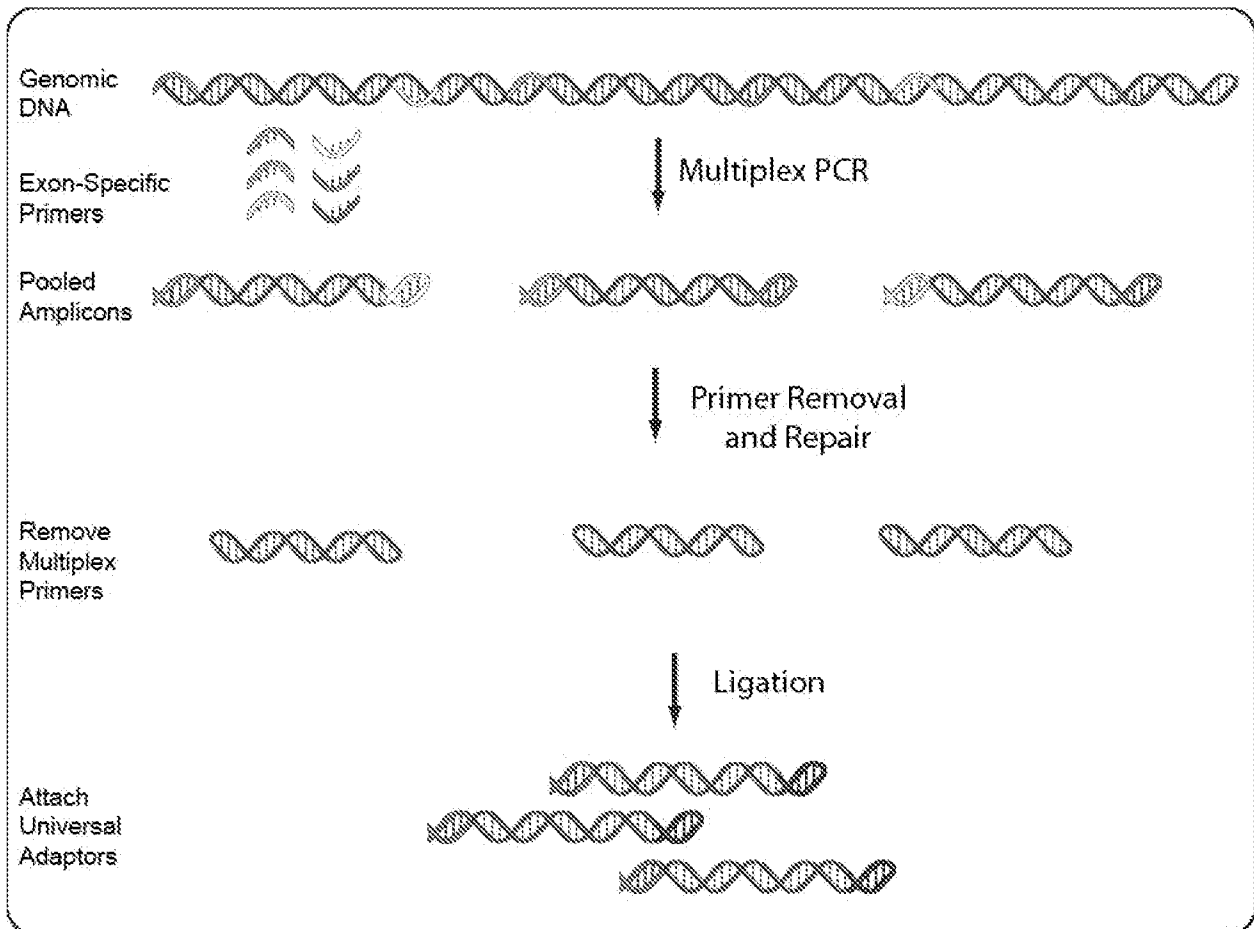


FIG. 2

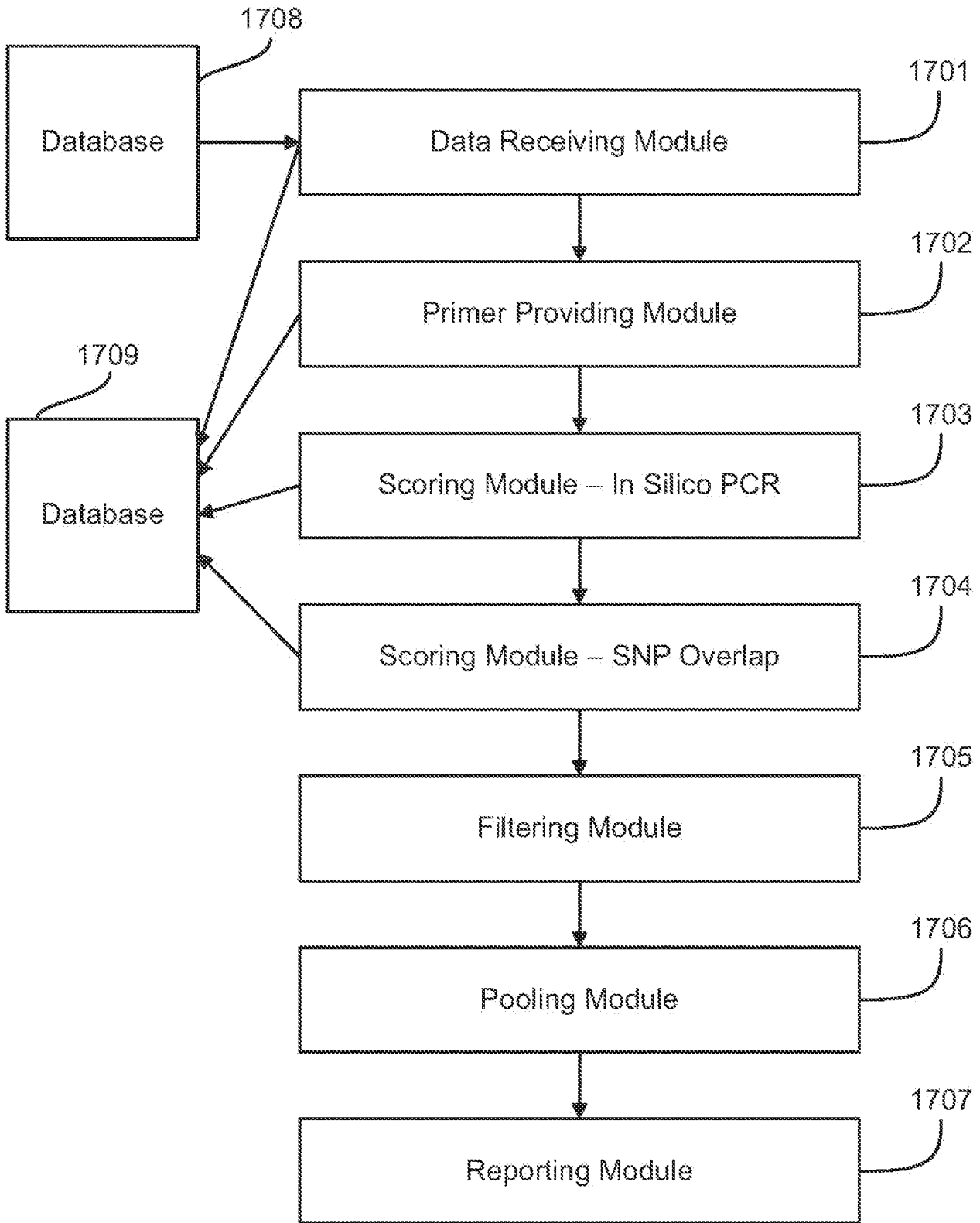


FIG. 3

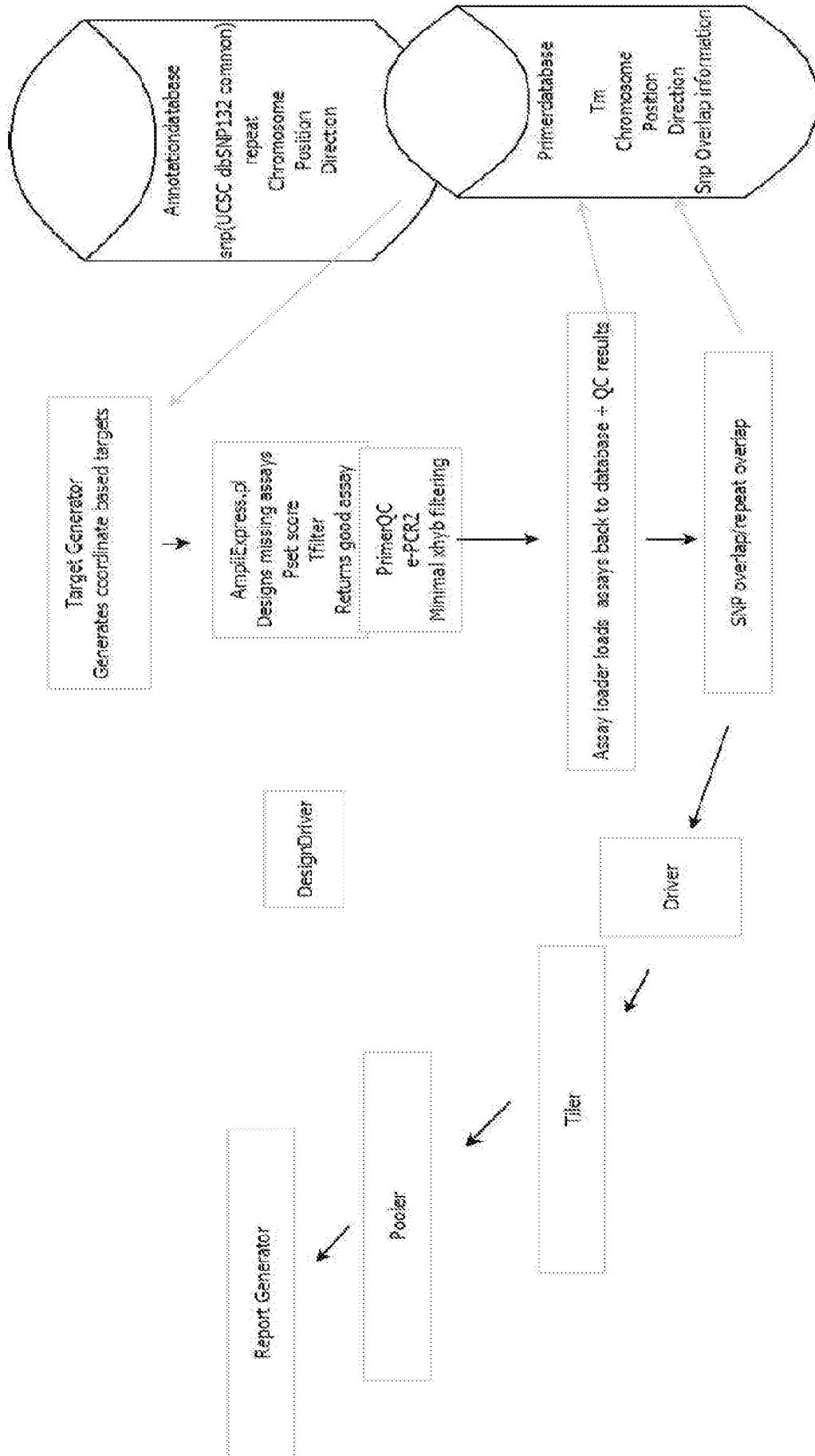


FIG. 4

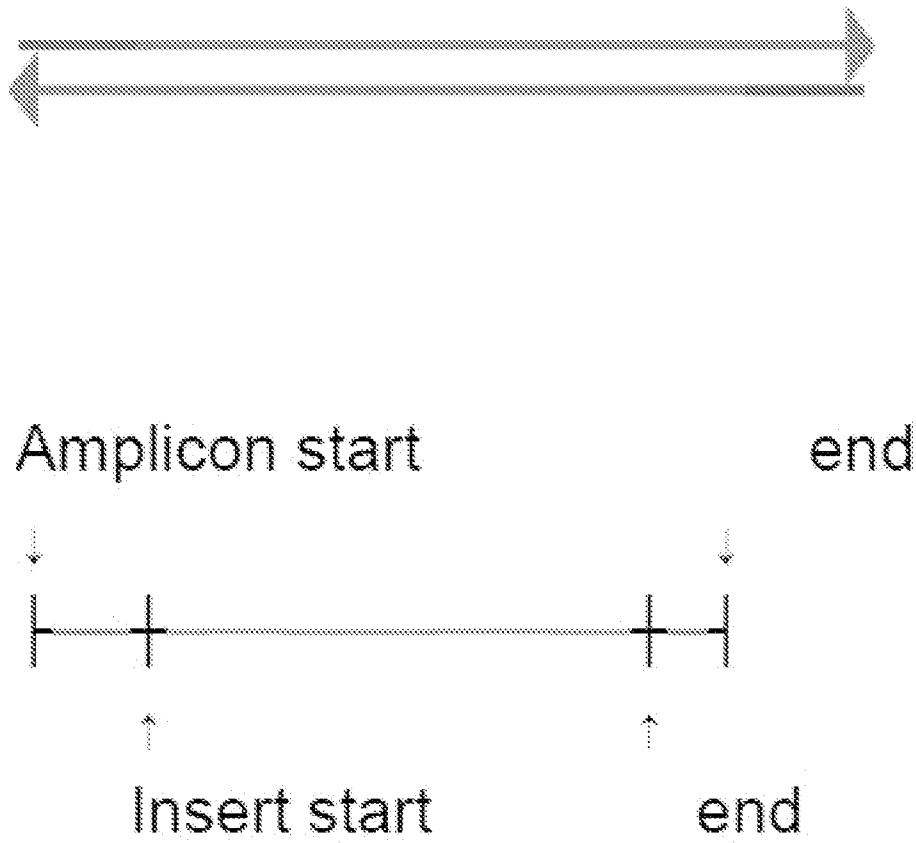


FIG. 5

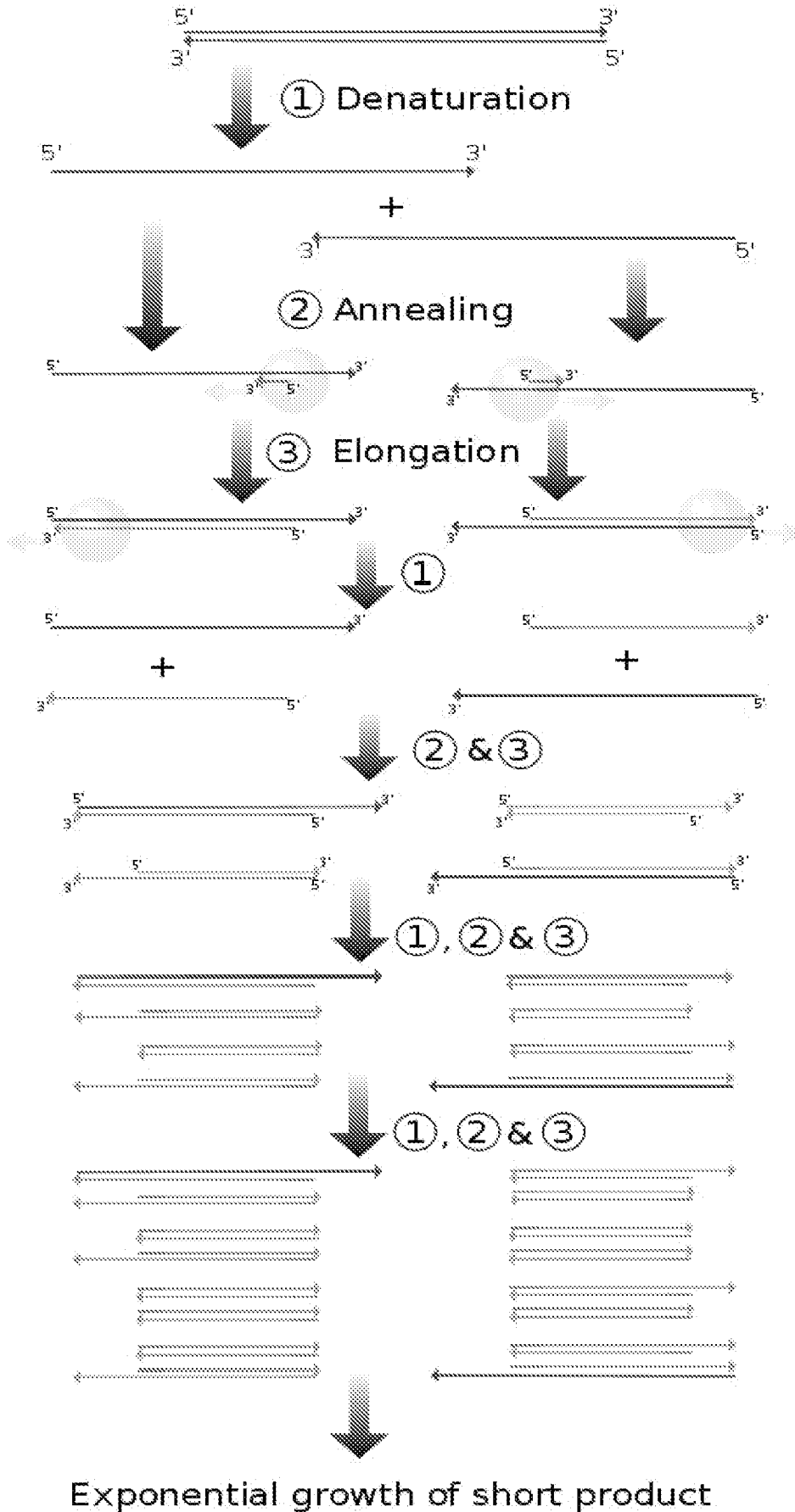
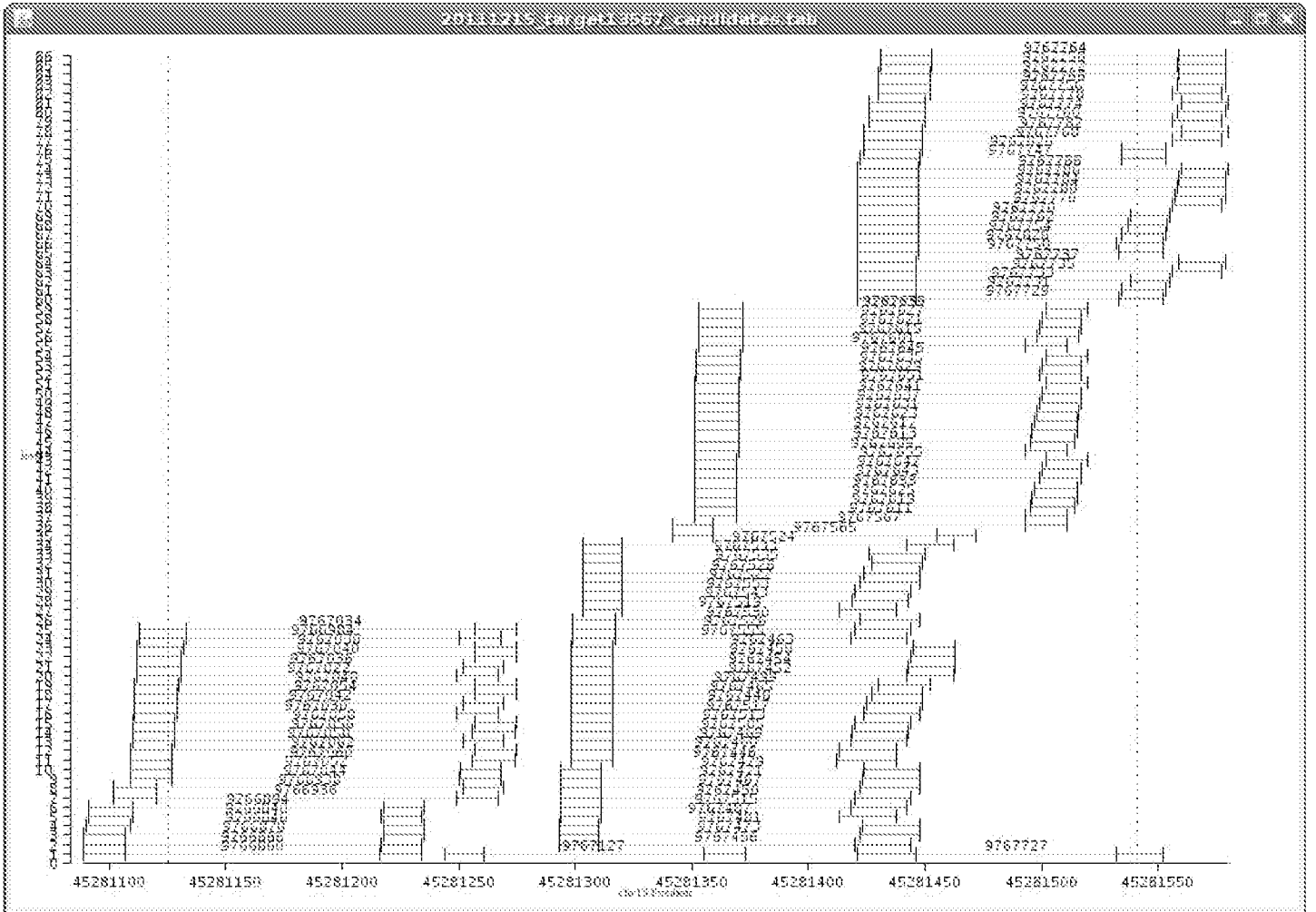


FIG. 6



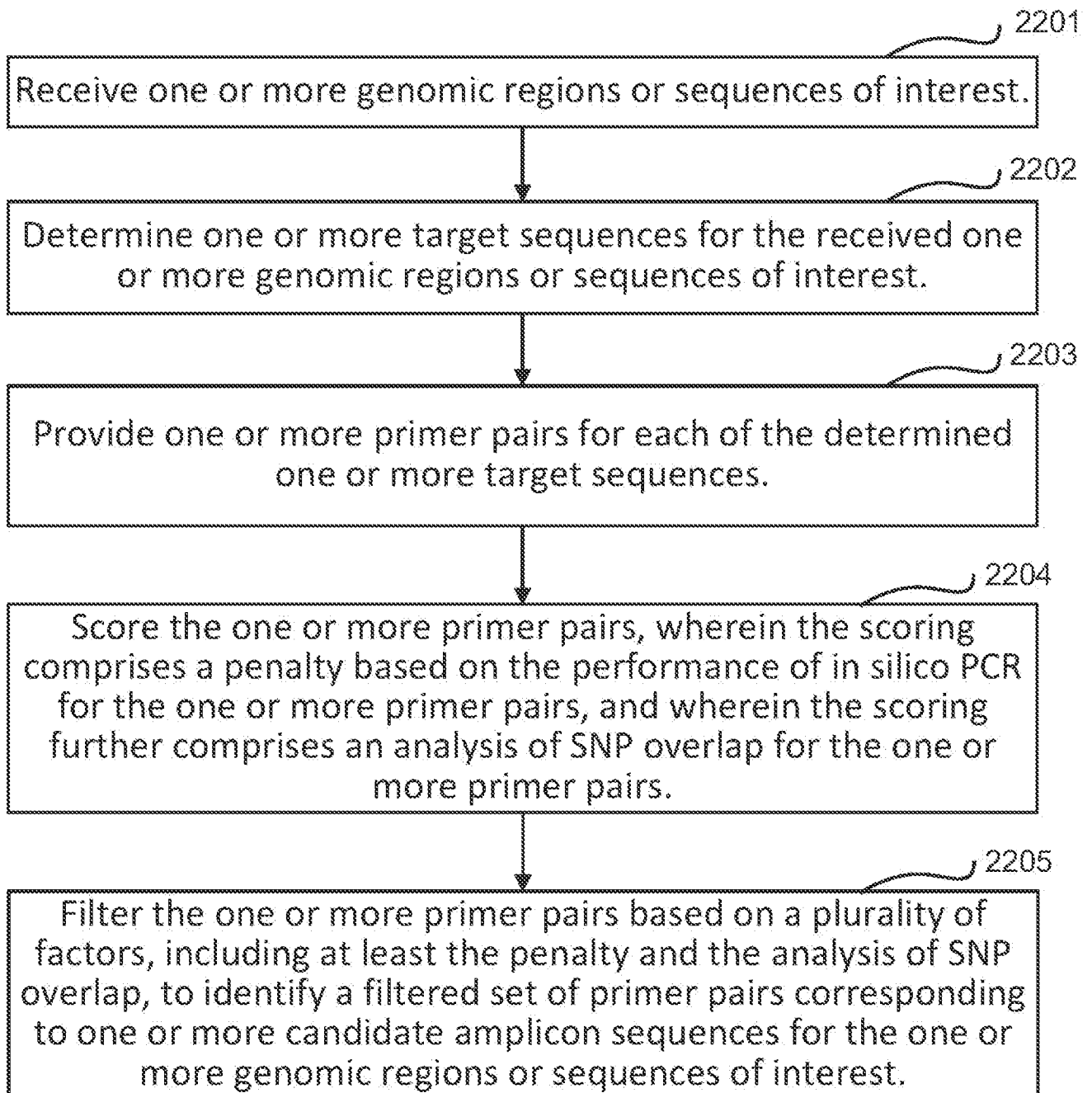


FIG. 8