

COMPOSITIONS AND METHODS FOR IMPROVING LIBRARY ENRICHMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/764,753 filed August 15, 2018, the contents of which is incorporated herein by reference in its entirety.

SUMMARY OF THE INVENTION

[0002] Despite rapid advances in next-generation sequencing (NGS) throughput, classic library preparation and enrichment methods are time-intensive and may result in a throughput-limiting bottleneck. This disclosure describes compositions including a blocker and/or a hybridization buffer, methods for using those compositions, and methods for improving the efficiency of nucleic acid selection prior to sequencing.

[0003] In one aspect, this disclosure describes a hybridization buffer including a crowding agent and at least one of human Cot-1 DNA, a destabilizing agent, salt, and a blocker.

[0004] In another aspect, this disclosure describes a method that includes using the hybridization buffer. This disclosure also describes a kit including the hybridization buffer.

[0005] In a further aspect, this disclosure describes a blocker including an oligonucleotide, wherein the blocker is capable of binding to an adaptor. The adaptor includes a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI). The region of the blocker capable of binding to the index region and/or UMI of the adaptor includes: at least three thymine bases or universal bases and at least one non-universal base.

[0006] In yet another aspect, this disclosure describes a blocker including two non-connected oligonucleotides. The blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI). The non-connected oligonucleotides include bases that do not correspond to the index region and/or the UMI of the adaptor.

[0007] This disclosure also describes a method that includes using the blockers described herein and a kit including the blockers described herein.

[0008] In a further aspect, this disclosure describes a method that includes contacting a library with a blocker in the presence of a hybridization buffer; and contacting the library with a probe, wherein the probe hybridizes to a region of interest within a library member. The method does not include amplifying the library members using PCR prior to sequencing the library members.

[0009] As used herein, the term “nucleic acid” is intended to be consistent with its use in the art and includes naturally occurring nucleic acids or functional analogs thereof. Particularly useful functional analogs are capable of hybridizing to a nucleic acid in a sequence specific fashion or capable of being used as a template for replication of a particular nucleotide sequence. Naturally occurring nucleic acids generally have a backbone containing phosphodiester bonds. An analog structure can have an alternate backbone linkage including any of a variety of those known in the art. Naturally occurring nucleic acids generally have a deoxyribose sugar (e.g., found in deoxyribonucleic acid (DNA)) or a ribose sugar (e.g., found in ribonucleic acid (RNA)). A nucleic acid can contain any of a variety of analogs of these sugar moieties that are known in the art. A nucleic acid can include native or non-native bases. In this regard, a native deoxyribonucleic acid can have one or more bases selected from the group consisting of adenine, thymine, cytosine or guanine and a ribonucleic acid can have one or more bases selected from the group consisting of uracil, adenine, cytosine or guanine. Useful non-native bases that can be included in a nucleic acid are known in the art. The term “target,” when used in reference to a nucleic acid, is intended as a semantic identifier for the nucleic acid in the context of a method or composition set forth herein and does not necessarily limit the structure or function of the nucleic acid beyond what is otherwise explicitly indicated.

[0010] As used herein, the term “ T_m ” refers to the temperature at which half of the DNA strands of a sample are in a double helical state and half of the DNA strands of a sample are in a random coil state.

[0011] As used herein, the term “adaptor” and its derivatives (e.g., universal adaptor, non-target adaptor, etc.) refer generally to any linear, single-stranded oligonucleotide which can be ligated to a nucleic acid molecule of the disclosure. In some embodiments, the adaptor is substantially non-complementary to the 3' end or the 5' end of any target sequence present in the sample. In some embodiments, suitable adaptor lengths are in the range of 10-100

nucleotides, 12-60 nucleotides and 15-50 nucleotides in length. Generally, the adaptor can include any combination of nucleotides and/or nucleic acids. In some aspects, the adaptor can include one or more cleavable groups at one or more locations. In another aspect, the adaptor 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 adaptor can include an index or tag to assist with downstream error correction, identification or sequencing.

[0012] As used herein, the term “universal sequence” refers to a region of sequence that is common to two or more nucleic acid molecules, e.g., adaptor-target adaptor molecules, where the molecules also have regions of sequence that differ from each other. A universal sequence that is present in different members of a collection of molecules can allow capture of multiple different nucleic acids using a population of universal capture nucleic acids that are complementary to a portion of the universal sequence, e.g., a universal extension primer binding site. Non-limiting examples of universal extension primer binding sites include sequences that are identical to or complementary to P5 and P7 primers. Similarly, a universal sequence present in different members of a collection of molecules can allow the replication or amplification of multiple different nucleic acids using a population of universal primers that are complementary to a portion of the universal sequence, e.g., a universal primer binding site. Thus, a universal capture nucleic acid or a universal primer includes a sequence that can hybridize specifically to a universal sequence. Target nucleic acid molecules may be modified to attach universal adaptors, for example, at one or both ends of the different target sequences, as described herein.

[0013] The terms “P5” and “P7” may be used when referring to amplification primers, e.g., universal primer extension primers. The terms “P5” (P5 prime) and “P7” (P7 prime) refer to the complements of P5 and P7, respectively. It will be understood that any suitable amplification primers can be used in the methods presented herein, and that the use of P5 and P7 are exemplary embodiments only. Uses of amplification primers such as P5 and P7 on flow cells are known in the art, as exemplified by the disclosures of WO 2007/010251, WO 2006/064199, WO 2005/065814, WO 2015/106941, WO 1998/044151, and WO 2000/018957. For example, any suitable forward amplification primer, whether immobilized or in solution, can be useful in the methods presented herein for hybridization to a complementary sequence and amplification of a sequence. Similarly, any suitable

reverse amplification primer, whether immobilized or in solution, can be useful in the methods presented herein for hybridization to a complementary sequence and amplification of a sequence. One of skill in the art will understand how to design and use primer sequences that are suitable for capture, and amplification of nucleic acids as presented herein.

[0014] 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 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. Amplification optionally includes linear or exponential replication of a nucleic acid molecule. In some embodiments, such amplification can be 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. 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 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).

[0015] As used herein, “amplification conditions” and its derivatives, generally refer 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 include 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 adaptors, e.g., an adaptor-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^{2+} or Mn^{2+} and can also include various modifiers of ionic strength.

[0016] As used herein, the term “polymerase chain reaction” (“PCR”) refers to the method as described in U.S. Pat. Nos. 4,683,195 and 4,683,202, 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 series 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. The mixture is denatured at a higher temperature first and the primers are then annealed to 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 (referred to as thermocycling) 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.” 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.

[0017] As defined herein “multiplex amplification” refers to the simultaneous amplification of two or more target sequences within a sample. 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. It is also possible to detect the amplified target sequences by several different methodologies (e.g., gel electrophoresis followed by densitometry, quantitation with a bioanalyzer or quantitative PCR, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ³²P-labeled deoxynucleotide triphosphates into the amplified target sequence).

[0018] As used herein, the term “primer” and its derivatives refer generally to any polynucleotide that can hybridize to a target sequence of interest. 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. In some embodiments, the primer is a single-stranded oligonucleotide or polynucleotide. The terms “polynucleotide” and “oligonucleotide” are used interchangeably herein to refer to a polymeric form of nucleotides of any length, and may comprise ribonucleotides, deoxyribonucleotides, analogs thereof, or mixtures thereof. The terms should be understood to include, as equivalents, analogs of either DNA or RNA made from nucleotide analogs and to be applicable to single stranded (such as sense or antisense) and double stranded polynucleotides. The term as used herein also encompasses cDNA, that is complementary or copy DNA produced from an RNA template, for example by the action of reverse transcriptase. This term refers only to the primary structure of the molecule. Thus, the term includes triple-, double- and single-stranded deoxyribonucleic acid (“DNA”), as well as triple-, double- and single-stranded ribonucleic acid (“RNA”).

[0019] As used herein, the terms “ligating,” “ligation,” and their derivatives refer generally to the 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, 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, a target sequence can be ligated to an adaptor to generate an adaptor-target sequence. The skilled person will recognize that a ligation reaction may not result in linking all molecules present in the reaction.

[0020] As used herein, “ligase” and its derivatives refer generally to any agent capable of catalyzing the ligation of two substrate molecules. In some embodiments, a ligase includes an enzyme capable of catalyzing the joining of nicks between adjacent nucleotides of a nucleic acid. In some embodiments, a 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, thermostable T4 DNA ligase, and *E. coli* DNA ligase.

[0021] The term “flow cell” as used herein refers to a chamber comprising a solid surface across which one or more fluid reagents can be flowed. Examples of flow cells and related fluidic systems and detection platforms that can be readily used in the methods of the present disclosure are described, for example, in Bentley *et al.*, *Nature* 456:53-59 (2008); WO 04/018497; WO 91/06678; WO 07/123744; U.S. Pat. No. 7,057,026; U.S. Pat. No. 7,211,414; U.S. Pat. No. 7,315,019; U.S. Pat. No. 7,329,492; U.S. Pat. No. 7,405,281; and U.S. Pat. Publication No. 2008/0108082.

[0022] As used herein, the term “library” refers to a collection of members. In one embodiment, the library includes a collection of nucleic acid members, for example, a collection of whole genomic, subgenomic fragments, cDNA, cDNA fragments, RNA, RNA fragments, or a combination thereof. In some embodiments, a portion or all library members include a non-target adaptor sequence. The adaptor sequence can be located at one or both ends. The adaptor sequence can be used in, for example, a sequencing method

(for example, an NGS method), for amplification, for reverse transcription, or for cloning into a vector.

[0023] “Member” or “library member” or other similar term, as used herein, refers to a nucleic acid molecule, for example, a DNA, RNA, or a combination thereof, that is the member of a library. Typically, a member is a DNA molecule, for example, genomic DNA or cDNA. A member can be fragmented, for example, sheared or enzymatically prepared, genomic DNA. Members include a sequence from a subject and can also include a sequence not derived from a subject, for example, a non-target sequence such as an adaptor sequence, a primer sequence, and/or other sequences that allow for identification, for example, an index.

[0024] As used herein, an “index” (also referred to as an “index region” or “index adaptor”) refers to a nucleic acid tag that can be used to identify a sample or source of the nucleic acid material. When nucleic acid samples are derived from multiple sources, the nucleic acids in each nucleic acid sample can be tagged with different nucleic acid tags such that the source of the sample can be identified. Any suitable index or set of indexes can be used, as known in the art and as exemplified by the disclosures of U.S. Pat. No. 8,053,192, PCT Publication No. WO 05/068656, and U.S. Pat. Publication No. 2013/0274117. In some embodiments, an index can include a six-base Index 1 (i7) sequence, an eight-base Index 1 (i7) sequence, an eight-base Index 2 (i5) sequence, a ten-base Index 1 (i7) sequence, or a ten-base Index 2 (i5) sequence from Illumina, Inc. (San Diego, CA).

[0025] As used herein, the term “unique molecular identifier” or “UMI” or “barcode” refers to a molecular tag that may be attached to a nucleic acid molecule. When incorporated into a nucleic acid molecule, a UMI can be used to correct for subsequent amplification bias by directly counting unique molecular identifiers (UMIs) that are sequenced after amplification.

[0026] As used herein, the term “tagmentation” refers to the modification of DNA by a transposome complex comprising a transposase enzyme complexed with adaptors comprising a double-stranded recognition sequence (hybridized transposon end sequences). Tagmentation results in the simultaneous fragmentation of the DNA and ligation of the adaptors to the 5' ends of both strands of duplex fragments. Following purification to remove the transposase enzyme, gap-filling, and ligation, a fully double-stranded product

results. Additional sequences can be added to the ends of the adapted fragments, for example by PCR, ligation, or any other suitable methodology known to those of skill in the art. See, for example, U.S. Pat. Publication No. 2017/0044525.

[0027] The term “and/or” means one or more of the listed elements or a combination of any two or more of the listed elements.

[0028] The words “preferred” and “preferably” refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

[0029] The term “comprises” and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

[0030] It is understood that wherever embodiments are described herein with the language “include,” “includes,” or “including,” and the like, otherwise analogous embodiments described in terms of “consisting of” and/or “consisting essentially of” are also provided. The term “consisting of” is limited to whatever follows the phrase “consisting of.” That is, “consisting of” indicates that the listed elements are required or mandatory, and that no other elements may be present. The term “consisting essentially of” indicates that any elements listed after the phrase are included, and that other elements than those listed may be included provided that those elements do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements.

[0031] Unless otherwise specified, “a,” “an,” “the,” and “at least one” are used interchangeably and mean one or more than one.

[0032] As used herein, the term “each,” when used in reference to a collection of items, is intended to identify an individual term in the collection but does not necessarily refer to every term in the collection unless the context clearly dictates otherwise.

[0033] The recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

[0034] For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

[0035] The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

[0036] Reference throughout this specification to “one embodiment,” “an embodiment,” “certain embodiments,” or “some embodiments,” etc., means that a particular feature, configuration, composition, or characteristic described in connection with the embodiment is included in at least one embodiment of the disclosure. Thus, the appearances of such phrases in various places throughout this specification are not necessarily referring to the same embodiment of the disclosure. Furthermore, the particular features, configurations, compositions, or characteristics may be combined in any suitable manner in one or more embodiments.

[0037] Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0038] All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

[0039] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

BRIEF DESCRIPTION OF THE FIGURES

[0040] FIG. 1A – FIG. 1B are schematics showing an exemplary embodiment of a hybrid capture during enrichment and comparative off-target capture. FIG. 1A. A probe includes a region designed to hybridize a region of interest within the target genome and a ligand that permits subsequent capture of the probe (e.g., a biotin group). Once hybridization is complete and a DNA template-probe hybrid is formed, a capture means (that is, a component having affinity for the probe including, for example, a streptavidin-coated magnetic bead) is used to bind the probe that is hybridized to the DNA target, removing the target from a pool of oligonucleotides. FIG. 1B. Unwanted or off-target oligonucleotides may be recovered during hybrid capture due to interactions between terminal adaptor sequences in the target sequences and those in off-target library preparation products.

[0041] FIG. 2 shows an exemplary mechanism by which blockers prevent hybridization of adaptor sequences and prevent off-target capture.

[0042] FIG. 3A – FIG. 3I show diagrams of exemplary blockers. FIG. 3A. Diagram of NEXTERA blockers with bases that are complementary to an 8- or 10-base index region of the adaptor. FIG. 3B. Diagram of NEXTERA blockers modified with 8 or 10 deoxyinosine bases in the region of the blocker that correspond with the index region of the adaptor. FIG. 3C. Diagram of TRUSEQ blockers modified with 6, 8, or 10 deoxyinosine bases in the region of the blocker that corresponds with the index region of the adaptor. FIG. 3D. Diagram of NEXTERA and TRUSEQ blockers with 6 or 8 thymines in the region of the blocker that corresponds with the index region of the adaptor. FIG. 3E. Diagram of split NEXTERA blockers that include a component that corresponds to a region 5' to an 8- or 10-base index region and a component that corresponds to a region 3' to the index region. These constructs do not include any bases that correspond with the index region of the adaptor (indicated by an 8- or 10-base gap that corresponds to the index region). FIG. 3F. Diagram of split TRUSEQ blockers that include a component that corresponds to a region 5' to an 8- or 10-base index region and a component that corresponds to a region 3' to the index region. These constructs do not include any bases correspond to the index region of the adaptor (indicated by an 8- or 10-base gap corresponds with the index region). FIG. 3G. Diagram of NEXTERA blockers that correspond to only a portion of the region of the adaptor 3' of the index region. FIG. 3H. Diagram of TRUSEQ blockers that pair to only a portion of the region of the adaptor 3' of the index region. FIG. 3I. Diagram of NEXTERA or

TRUSEQ blockers that correspond to only a portion of the region of the adaptor 5' of the index region (e.g., p5 or p7 sequence).

[0043] FIG. 4 is a graph of the Padded Read Enrichment results (a measure of the amount of on-target DNA captured) obtained using blockers as described in Example 2.

[0044] FIG. 5 shows a diagram of blockers including a modified G (+G) or a random (A, T, G, or C) (N) nucleotide at the fifth position of the portion of the blocker sequence that corresponds to the index sequence, as described in Example 3.

[0045] FIG. 6A – FIG. 6B show blocker constructs and results as described in Example 4. FIG. 6A shows a diagram of various blocker constructs including (1) a pair of split blockers with a first sequence that is complementary to a 25-30-mer region 5' of the index region and a second sequence complementary to a 34-35-mer region 3' of the index region, (2) a blocker that pairs to only a 3' portion of the adaptor (“Inner” blocker), (3) a blocker that pairs to only a 5' portion of the adaptor (“Outer” blocker), and (4) a short (8 nucleotide) blocker that corresponds with the index region (“Short8nt”). FIG. 6B shows Padded Read Enrichment results achieved using XGEN Blocking Oligos (Integrated DNA Technologies, Coralville, IA), split blockers, or blockers that bind to only a portion of the adaptor.

[0046] FIG. 7A – FIG. 7F show results using an enhanced hybridization buffer including dextran sulfate, as described in Example 5. FIG. 7A shows Padded Read Enrichment results achieved using differing concentrations of dextran sulfate in the hybridization buffer. FIG. 7B shows Padded Read Enrichment results achieved using 100 µL hybridization volume and four different probe panels and three different buffer conditions (IDT hybridization buffer, Illumina non-enhanced hybridization buffer, and Illumina enhanced hybridization buffer). FIG. 7C shows Padded Read Enrichment and uniformity of coverage results for all four probe panels at different hybridization incubation times and with and without an additional temperature ramping step. The dotted black line in the left panel indicates the result of hybridization in buffer without dextran sulfate. Three sets of sequencing results are shown including NOVASeq (FIG. 7D), NEXTSeq (FIG. 7E), and ISeq (FIG. 7F) (percent reads identified (PF) for NOVASeq and NEXTSeq, and index percent (%) for ISeq) and associated coefficients of variation (CV) for runs using dextran sulfate hybridization buffer and 30 µL library volume.

[0047] FIG. 8A – FIG. 8C show the results of hybridization assays described in Example 6. FIG. 8A shows Padded Read Enrichment results when using modified blockers, a wash temperature increase, and/or a crowding agent (dextran sulfate) in the hybridization buffer. FIG. 8B shows Padded Read Enrichment results achieved using the methods described in Example 6 in nine probe panels using enhanced hybridization buffer with xGEN blockers, as compared to results of IDT exome panel using the IDT xGEN LOCKDOWN kit protocol. FIG. 8C shows detection of somatic variants by single-hybridization enrichment protocol using a 12 gene, 535 probe panel to enrich libraries generated from the Horizon Discover HD701 quantitative multiplex (QM) DNA standard.

[0048] FIG. 9A – FIG. 9D show schematics of exemplary workflows. eBBN indicates bead-based tagmentation using bead-bound transposome complexes; H indicates hybridization; C indicates capture; W indicates wash; E indicates elution; PCR indicates PCR amplification; S indicates sequencing; Q indicates quantification; SpVac indicates Speed Vacuum concentration. FIG. 9A shows a schematic of an exemplary workflow for hybrid capture showing library preparation using a bead-based tagmentation protocol; any suitable library preparation method may be used prior to enrichment. Notably, only one round of hybridization and capture is included. FIG. 9B shows a schematic of an exemplary workflow for hybrid capture. FIG. 9C shows a schematic of an exemplary workflow for PCR-free hybrid capture. FIG. 9D shows a schematic of an exemplary workflow for PCR-free hybrid capture with a shortened hybridization time.

[0049] FIG. 10 shows Padded Read Enrichment results achieved using BN001 and BN002 (non-modified blockers); BX003 and BX007 (G-Clamp modified blockers); BN007 and BN008 (blockers modified with BNA and deoxyinosine); BN005 and BN006 (BNA and modified sequence-specific blockers); or BN023, BN024, BN025, and BN026 (split blockers modified with LNA) as described in Example 8.

[0050] FIG. 11 shows Padded Read Enrichment results achieved using BN001 and BN002 (non-modified blockers) or BN023, BN024, BN025, and BN026 (LNA modified split blockers) when the corresponding adaptor includes an 8-nucleotide index or a 10-nucleotide index, as described in Example 9.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0051] This disclosure describes compositions including a blocker and/or a hybridization buffer, and methods for improving the efficiency of nucleic acid selection prior to

sequencing including methods of using those compositions and methods including of hybrid capture.

Enrichment by Hybrid Capture

[0052] A variety of methods may be used to enrich for desired sequences from a complex pool of nucleic acids. These methods include the polymerase chain reaction (PCR), molecular inversion probes (MIPs), or sequence capture by hybrid formation (“hybrid capture”). See, for example, Mamanova *et al.*, *Nat. Methods* 7:111-118 (2010)); U.S. Pat. Publication No. 2014/0031240; and U.S. Pat. Publication No. 2017/0114404.

[0053] Next generation sequencing (NGS) applications typically use the hybrid capture method of enrichment. A prepared pool of NGS templates – a library – is heat denatured and mixed with a pool of capture probe oligonucleotides (“probes”). The probes are designed to hybridize to the regions of interest within the target genome and are usually 60 to 200 bases in length and further are modified to contain a ligand that permits subsequent capture of the bound probes. A common capture method incorporates a biotin group (or groups) on the probes. After hybridization to form the DNA template-probe hybrids is complete, capture is performed with a component having affinity for only the probe, as shown in one embodiment in FIG. 1A. For example, streptavidin-coated magnetic beads can be used to bind the biotin moiety of biotinylated-probes that are hybridized to the desired DNA targets from the library. Washing removes unbound nucleic acids, reducing the complexity of the retained material. The retained material is then eluted from the magnetic beads and introduced into automated sequencing processes.

[0054] Though DNA hybridization with the probes can be exquisitely specific, unwanted sequences remain in the enriched pool following completion of the hybrid capture method. The largest fraction of these unwanted sequences is present due to undesired hybridization events between library members having no complementarity to the probes and library members that do (that is, an on-target library member). Two types of sequences lead to undesired hybridizations during hybrid capture methods: (1) highly repetitive DNA elements that are found in endogenous genomic DNA; and (2) the terminal adaptor sequences that are engineered into each of the library members.

[0055] The repetitive endogenous DNA elements, such as an Alu sequence or Long interspersed nuclear element (LINE) sequence, present in one DNA fragment in the

complex pool can hybridize to another similar element present in another unrelated DNA fragment. These fragments, which may originally derive from very different locations within the genome, become linked during the hybridization process of the hybrid capture method. If one of these DNA fragments represents a desired fragment that contains a binding site for a probe, the unwanted fragment will be captured along with the desired fragment. This class of off-target library members can be reduced by adding an excess of the repeat elements to the hybridization buffer of the hybridization reaction. Most commonly, human Cot-1 DNA (which binds Alu, LINE, and other repeat sites in the target and blocks the ability of NGS templates to interact with each other on that basis) is added to the hybridization buffer.

[0056] Off-target (also referred to as non-target) library members may also be captured due to interactions between terminal adaptor sequences in individual library members. Typically, library members include a segment of sequence from a gene of interest, for example, a segment for sequencing. If a member is on-target, the sequence from the gene of interest forms a duplex with the capture probe. On-target sequences may include, for example, an exon or an intron (or fragment thereof), a coding region or a non-coding region, an enhancer, an untranslated region, a specific SNP, etc. Typically, library members also include one or more non-target sequences. These non-target sequences typically do not include a target sequence of interest but do include, for example, an adaptor. Because a pool of library members typically will contain at least some identical terminal adaptor sequences, the adaptor sequences are present at a very high effective concentration(s) in the hybridization solution. Consequently, library members containing off-target sequences can anneal to captured target sequences through portions of their appended adaptor sequences, thereby resulting in capture of off-target sequences along with on-target library members (for example, by a concatenation or “daisy chain” of sequences being linked together), as shown in one embodiment in FIG. 1B. Because the annealed target sequence includes a binding site for a probe, the entire daisy chain may be captured. In this way, capture of a single desired fragment can bring along a large number of undesired fragments, reducing the overall efficiency of enrichment for the desired segment.

[0057] In some aspects, this disclosure describes methods and compositions for minimizing selection of an off-target nucleic acid by hybrid capture.

Blocker Oligonucleotide

[0058] In one aspect, this disclosure describes a “blocker oligonucleotide” (also referred to herein as a “blocker”) and a method of preventing selection of an off-target nucleic acid during hybrid capture by using the blocker to bind to (e.g., form a duplex with) at least a portion of an adaptor sequence. When used, as shown in one embodiment in FIG. 2, the blocker’s binding to the adaptor sequence prevents interactions between adaptor sequences in off-target library preparation products – including, for example, the formation of concatenated chains – that can result in unwanted library members being recovered during hybrid capture.

[0059] In some embodiments, at least two blockers can be used, with a first blocker binding to (e.g., forming a duplex with) a first adaptor sequence, and a second blocker binding to (e.g., forming a duplex with) a second adaptor sequence. In some embodiments, the first adaptor sequence may be at the 5' end of a library member and the second adaptor sequence may be at the 3' end of a library member. In some embodiments, a plurality of different blockers may be used.

[0060] In some embodiments, the blocking oligonucleotide forms a duplex with an adaptor sequence of at least 1, at least 2, at least 5, at least 10, at least 20, at least 50, at least 100, or at least 200 library members, and the resulting duplex has a T_m that is higher than the T_m of a duplex formed by an adaptor sequence to a background nucleic acid including, for example, the complement of the adaptor sequence.

[0061] In some embodiments, a blocker-adaptor complex exhibits a greater T_m than an adaptor-adaptor complex. Such increased T_m means that a blocker-adaptor complex is more likely to form before an adaptor-adaptor complex, which may prevent off target capture (e.g., by preventing the formation of daisy chains). In some embodiments, the T_m of the blocker-adaptor oligonucleotide duplex is at least 1.5°C, at least 2°C, at least 3°C, at least 4°C, at least 5°C, at least 10°C, at least 15°C, at least 20°C, or at least 25°C greater than the T_m of an adaptor duplex with its exact complement. In some embodiments, the T_m of the blocker-adaptor oligonucleotide duplex is up to 2°C, up to 3°C, up to 4°C, up to 5°C, up to 10°C, up to 15°C, up to 20°C, up to 25°C, or up to 30°C greater than the T_m of an adaptor duplex with its exact complement.

[0062] In some embodiments, the blocking oligonucleotide has an association rate with an adaptor sequence of at least 1, at least 2, at least 5, at least 10, at least 20, at least 50, at

least 100, or at least 200 library members, that is higher than the association rate of the adaptor sequence to a background nucleic acid including, for example, the complement of the adaptor sequence. In some embodiments, the association rate is at least 2-fold greater, at least 4-fold greater, at least 6-fold greater, at least 8-fold greater, or at least 10-fold greater. In some embodiments, the association rate is up to 4-fold greater, up to 6-fold greater, up to 8-fold greater, up to 10-fold greater, or up to 12-fold greater.

[0063] In some embodiments, the blocking oligonucleotide has a dissociation rate with an adaptor sequence of at least 1, at least 2, at least 5, at least 10, at least 20, at least 50, at least 100, or at least 200 library members that is lower than the dissociation rate of the adaptor sequence to a background nucleic acid including, for example, the complement of the adaptor sequence. In some embodiments, the dissociation rate is at least 2-fold lower, at least 4-fold lower, at least 6-fold lower, at least 8-fold lower, or at least 10-fold lower. In some embodiments, the association rate is up to 4-fold lower, up to 6-fold lower, up to 8-fold lower, up to 10-fold lower, or up to 12-fold lower.

[0064] In some embodiments, a duplex formed between the blocking oligonucleotide and an adaptor sequence of at least 1, at least 2, at least 5, at least 10, at least 20, at least 50, at least 100, or at least 200 library members is longer than the duplex formed between the adaptor sequence and its complement including, for example, between the Watson and Crick strands of a double-stranded adaptor. In some embodiments, the duplex between a blocking oligonucleotide and an adaptor sequence is at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, or at least 20 nucleotides longer than the duplex formed between the adaptor sequence and its complement.

[0065] In some embodiments, the blocker preferably includes a modification that increases the T_m of the blocker relative to an oligonucleotide having the same sequence as the blocker that does not include the modification. For example, in some embodiments, the blocker may be a “ T_m -enhanced oligonucleotide,” that is an oligonucleotide that includes at least one modified group that provides an increased thermal melting temperature value for a duplex nucleic acid that includes as a hybridization partner the oligonucleotide relative to a duplex nucleic acid that includes as a hybridization partner an oligonucleotide having identical nucleobase composition and unmodified groups. A “ T_m -enhanced oligonucleotide” is further described in U.S. Pat. Publication No. 2014/0031240.

[0066] In some embodiments, the blocker includes one or more non-naturally-occurring nucleotides. In some embodiments, a duplex formed between a blocking oligonucleotide having non-naturally-occurring nucleotides and the adaptor sequence of at least 1, at least 2, at least 5, at least 10, at least 20, at least 50, at least 100, or at least 200 library members, has a value for a parameter related to the binding interaction (for example, affinity, association rate, inverse of dissociation rate, or T_m) that is higher than the value for the non-target nucleic acid sequence to a background nucleic acid, for example, other complementary non-target nucleic acid sequences.

[0067] In some embodiments, the blocker includes a modified base. Any suitable modified base may be included in a blocker. In some embodiments, the modification preferably includes a T_m -enhancing modification, that is, a modification that increases T_m of a blocker-adaptor complex including a modified base relative to the T_m of a blocker-adaptor complex that has the same sequence but does not include a modified base. Such a T_m -enhancing modification may include, for example, a DNA or RNA oligonucleotide modified to capture low GC regions; a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (also referred to as a bicyclic nucleic acid or BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; a 5'-Pyrene cap; etc. In some embodiments, an LNA includes the ribose moiety of a nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. Exemplary BNA are disclosed in, for example, U.S. Pat. No. 7,399,845, U.S. Pat. No. 7,427,672, U.S. Pat. No. 7,547,684, U.S. Pat. No. 7,696,345, U.S. Pat. No. 7,741,457, U.S. Pat. No. 8,022,193, U.S. Pat. No. 8,268,980, U.S. Pat. No. 8,278,425, U.S. Pat. No. 8,278,426, U.S. Pat. No. 8,846,637, U.S. Pat. No. 8,846,639, and U.S. Pat. No. 9,546,368. In some embodiments, a BNA may include a constrained ethyl nucleic acid from Ionis Pharmaceuticals (Carlsbad, CA) or a 2'-O,4'-aminoethylene bridged nucleic acid from Biosynthesis, Inc. (Lewisville, TX). In some embodiments, a PNA includes repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. In some embodiments, a tricyclic nucleic acid includes a tricyclic nucleic acid disclosed in, for example, U.S. Pat. No. 9,221,864. Exemplary phosphoramidites include 1-[5'-O-(4,4'-Dimethoxytrityl)- β -D-2'-deoxyribofuranosyl]-9-(2-trifluoroacetamidoethoxy)-1,3-diaza-2-oxophenoxazine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite (AP-dC-CE

phosphoramidite), 5'-Dimethoxytrityl-Uridine,2'-O-methyl,3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (2'-OMe-U-CE phosphoramidite), 5'-Dimethoxytrityl-N-acetyl-5-methyl-Cytidine,2'-O-methyl,3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (2'-OMe-5-Me-C-CE phosphoramidite), 5'-Dimethoxytrityl-N-acetyl-Cytidine,2'-O-methyl,3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (2'-OMe-Ac-C-CE phosphoramidite), etc.

[0068] In some embodiments, a modification that increases the T_m of the blocker relative to an oligonucleotide having the same sequence as the blocker that does not include the modification may include a non-base modification. Such modification can include, for example, a minor groove binder (MGB), spermine, G-clamp, or a Uaq anthraquinone cap.

[0069] In some embodiments, a blocking oligonucleotide is at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 nucleotides in length. In some embodiments, a blocking oligonucleotide is up to 45, up to 50, up to 55, up to 60, up to 70, up to 80, up to 90, up to 100, up to 150, or up to 200 nucleotides in length.

[0070] In some embodiments, a blocking oligonucleotide will include a plurality of modifications that increase the T_m of the blocker. In some embodiments, the preferred number of modifications is that number which provides an increase in the optimal T_m value under stringent conditions ($0.1 \times SSC$) ("optimal enhanced T_m value") of at least about $1.4^\circ C$ for a duplex DNA containing the modification(s) as one complementary strand. In some embodiments, the preferred number of modifications in a T_m -enhanced blocking oligonucleotide provides for a T_m of the blocker-adaptor oligonucleotide duplex of at least $1.5^\circ C$, at least $2^\circ C$, at least $3^\circ C$, at least $4^\circ C$, at least $5^\circ C$, at least $10^\circ C$, at least $15^\circ C$, at least $20^\circ C$, or at least $25^\circ C$ greater than the T_m of an adaptor duplex with its exact complement. In some embodiments, the preferred number of modifications in a T_m -enhanced blocking oligonucleotide provides for a T_m of the blocker-adaptor oligonucleotide duplex of up to $2^\circ C$, up to $3^\circ C$, up to $4^\circ C$, up to $5^\circ C$, up to $10^\circ C$, up to $15^\circ C$, up to $20^\circ C$, up to $25^\circ C$, or up to $30^\circ C$ greater than the T_m of an adaptor duplex with its exact complement.

[0071] In some embodiments, the blocking oligonucleotide will include modifications of at least 2 percent (%), at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the bases in the blocking oligonucleotide. In some embodiments, the blocking oligonucleotide will include

modifications of up to 5%, up to 10%, up to 20%, up to 30%, up to 40%, up to 50%, up to 60%, up to 70%, up to 80%, up to 90%, or up to 100% of the bases in the blocking oligonucleotide.

[0072] In some embodiments, modification to the blocker will be included in a particular pattern. For example, a modified base may be included every 2 bases (see, e.g., Table 2A, BN021 and BN022), every 3 bases (see, e.g., Table 2A, BN035 and BN036), every 4 bases, or every 5 bases, or some combination thereof (see, e.g., Table 2A, BN036). In some embodiments, each base in the blocker may be modified (see, e.g., Table 2A, BN027 and BN028). In some embodiments, guanine – which has a high affinity than other nucleotides when modified – may be preferentially modified. For example, in some embodiments, an LNA or BNA form of guanine may be included in the blocker.

[0073] In some embodiments, the blocker may include a terminal modification. For example, the blocker may include a 3' terminal group that precludes the availability of the blocker to serve as a primer for DNA synthesis. Such 3' terminal group may include, for example, 3'-dC, 2',3'-ddC (also referred to herein as 3ddc), inverted dT, 3'-spacer C3 (also referred to herein as 3SpC3), etc.

[0074] The blocker may bind to (e.g., form a duplex with) any suitable adaptor sequence (including any portion thereof). As used herein, the portion of the blocker sequence that binds to (e.g., forms a duplex with) a portion of the adaptor sequence is referred to as the portion of the blocker sequence that “corresponds” to the portion of the adaptor sequence. In some embodiments, the corresponding sequence may be the exact complement of the adaptor sequence. In some embodiments, however, up to 1, up to 2, up to 3, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9, or up to 10 bases in the corresponding sequence may not be complementary.

[0075] In some exemplary embodiments, the blocker may bind to an adaptor as shown in Table 1. (Adaptor sequences are shown in Table 1A and additional information about the sequence components is shown in Table 1B.) In some exemplary embodiments, the blocker may bind to an adaptor including a sequence disclosed in Illumina Adapter Sequences (available on the World Wide Web at support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-07.pdf)

[0076] In some embodiments, the blocker binds to an adaptor wherein the adaptor includes a universal sequence including, for example, a universal adaptor and/or a universal primer sequence. In some embodiments, the universal primer sequence may include P5, P7, P5', and/or P7'. In some embodiments, the universal primer sequence may include V2.A14.METS sequence, V2.B15.METS sequence, the complement of V2.A14.METS sequence, and/or the complement of V2.B15.METS sequence. For example BN001, as shown in Table 2A, includes P5 and V2.A14.METS sequence; BN002, as shown in Table 2A, includes P7 and V2.B15.METS sequence; BN003, as shown in Table 2A, includes P5' and the reverse complement of V2.A14.METS sequence; BN004, as shown in Table 2A, includes P7' and the reverse complement of V2.B15.METS sequence.

[0077] In some sequencing applications, it is desirable for library members to include at least one index and/or a UMI sequence. Thus, in some embodiments, a blocker binds to an adaptor where the adaptor includes at least one of an index and a UMI. U.S. Pat. Publication No. 2014/0031240 describes “three approaches can be used to make blocking oligonucleotides” for adaptors that include an index (which U.S. Pat. Publication No. 2014/0031240 refers to as a “barcode domain”): “1) synthesize a series of blockers which are perfect match to each adaptor, 2) synthesize a single blocker with an N-mer domain to pair with the barcode domain of the adaptor, or 3) synthesize a single blocker with a universal base domain to pair with the barcode domain of the adaptor.” As each different index and/or UMI has a unique sequence, a fully complementary blocker as in approach 1 would be a sequence that include a perfect complementary match to each index and/or UMI sequence present (*see* FIG. 3A). If many distinct indexes and/or UMIs are used, however, including a blocker having a perfect complementary match would require tens or hundreds of unique blockers for multiplex amplification – an approach that is not cost effective.

[0078] In some aspects, this disclosure describes blockers for adaptors that include an index and/or a UMI including, and methods of using such blockers, for example, a blocker that includes thymine in the region of the blocker that corresponds to the index and/or UMI (FIG. 3D & Example 2); a blocker than includes universal bases and at least one non-universal base (FIG. 5 & Example 3); or a blocker that does not include an index region (including, for example a “split blocker” or a blocker that pairs to only part of an adaptor) (FIG. 3E-FIG. 3I, FIG. 6A, & Examples 4, 8, and 9).

[0079] In some embodiments, the blocker includes at least one of the sequences of Table 2A, Table 2B, Table 2C, or Table 3. In some embodiments, the sequences of Table 2A may be used following a tagmentation (e.g., NEXTERA) library preparation (Illumina, Inc., San Diego, CA). In some embodiments, the sequences of Table 2B may be used following a ligation-based library preparation and/or a TRUSEQ preparation (Illumina, Inc., San Diego, CA). In some embodiments, the sequences of Table 2C may be used following a tagmentation (e.g., NEXTERA) library preparation (Illumina, Inc., San Diego, CA). In some embodiments, if a sequence of Table 2 includes a BNA-modified nucleic acid, the BNA-modified nucleic acid may include a 2'-O,4'-aminoethylene bridged nucleic acid from Biosynthesis, Inc. (Lewisville, TX). In some embodiments, if a sequence of Table 2 includes a LNA-modified nucleic acid, the LNA-modified nucleic acid may include an LNA-containing oligonucleotide from Qiagen (Hilden, Germany). Additional information about the components of the sequences of Table 2A, Table 2B, and Table 2C may be found in Table 1B and Table 2D.

[0080] In another aspect, the disclosure describes a kit that includes a blocking oligonucleotide. The blocking oligonucleotide may include a blocker as described herein. In some embodiments, the blocking oligonucleotide is a plurality of blocking oligonucleotides. The kit may further include one or more of: a hybridization buffer, a probe, a panel of probes, and a flow cell. The hybridization buffer may include a hybridization buffer as described elsewhere in this disclosure. In some embodiments, the probe(s) are as described elsewhere in this disclosure. In some embodiments the components may be provided in separate containers. For example, the blocking oligonucleotide may be provided in a first container and another component, for example, a hybridization buffer, or a probe or panel of probes may be provided in a different container(s). Alternatively, the blocking oligonucleotide and the hybridization buffer may be provided in a first container, and a probe or panel of probes may be provided in a different container(s).

Table 1A – Exemplary Adaptor Sequences

SEQ ID NO	Ref. No.	Oligo Name	Sequence
1	TN001	NEXTERA 8nt adaptor - i5	5'- AATGATACGGGACCGACCGAGATCTACAC[i5]TCGTGGGACGGTCAGATGTGTATAAGAGACAG
2	TN002	NEXTERA 8nt adaptor - i7	5'- CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
3	TN003	NEXTERA 10nt adaptor - i5	5'- AATGATACGGGACCGACCGAGATCTACAC[i5]TCGTGGGACGGTCAGATGTGTATAAGAGACAG
4	TN004	NEXTERA 10nt adaptor - i7	5'- CAAGCAGAAGACCGGCATACGAGAT[i7]GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
5	TN005	NEXTERA short adaptor - i5	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGT
6	TN006	NEXTERA short adaptor - i7	5'- CTGTCTCTTATACACATCTCCGAGCCACGAGAC
7	TN007	NEXTERA nrUMI adaptor - i5	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTNNNNNT
8	TN008	NEXTERA nrUMI adaptor - i7	/5Phos/NNNNNCTGTCTTATACACATCTCCGAGCCACGAGAC
9	TL001	TruSeq universal adaptor - i5	5'- AATGATACGGGACCGACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT
10	TL002	TruSeq 6nt adaptor - i7	5'- GATCGGAAGAGCACACCGTCTGAACTCCAGTCAC[i7]ATCTCGTATGCCGCTCTTCTGCTTG
11	TL003	TruSeq 8nt adaptor - i5	5'- AATGATACGGGACCGACCGAGATCTACAC[i5]ACACTCTTCCCTACACGACGCTCTTCCGATCT
12	TL004	TruSeq 8nt adaptor - i7	5'- GATCGGAAGAGCACACCGTCTGAACTCCAGTCAC[i7]ATCTCGTATGCCGCTCTTCTGCTTG
13	TL005	TruSeq 10nt adaptor - i5	5'- AATGATACGGGACCGACCGAGATCTACAC[i5]ACACTCTTCCCTACACGACGCTCTTCCGATCT
14	TL006	TruSeq 10nt adaptor - i7	5'- GATCGGAAGAGCACACCGTCTGAACTCCAGTCAC[i7]ATCTCGTATGCCGCTCTTCTGCTTG
15	TL007	TruSeq short adaptor - i5	5'- ACACCTCTTCCCTACACGACGCTCTCCGATCT
16	TL008	TruSeq short adaptor - i7	/5Phos/GATCGGAAGAGCACACCGTCTGAACTCCAGTCAC
17	TL009	TruSeq nrUMI adaptor - i5	5'- ACACCTCTTCCCTACACGACGCTCTTCCGATCTNNNNNT
18	TL010	TruSeq nrUMI adaptor - i5	/5Phos/NNNNNAGATCGGAAGAGCACACGCTCTGAACCTCCAGTCAC

Table 1B

SEQ ID NO	Description	Sequence
19	p5 sequence	AATGATACGGCGACACCGA
20	p7 sequence	TCGTATGCCGCTCTCTGCTTG
21	P7 reverse complement (P7')	CAAGCAGAAGACGGCATACGA
22	TruSeq.i7 adaptor region	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC
23	TruSeq.i5 adaptor region	ACACTCTTTCCCTACACGACGCTCTTCCGATC
24	V2.A14: METS sequence	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
25	V2.B15: METS sequence	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
	Index Sequence (8 or 10 nucleotides)	[i7] or [i5]
26	N-mer domain (a mix of 25% of each A, T, C, G) (e.g., a UMI)	NNNNNN
	5' phosphorylation	/5Phos/

Table 2A – Exemplary Blocker Sequences

SEQ ID NO	Ref No	Oligo Name	Sequence
27	BN001	NEXTERA Blocker1 i5	5' - AATGATACGGCGACCGAGATCTACACTTTTTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG/3ddC/-3'
28	BN002	NEXTERA Blocker2 i7	5' - CAAGCAGAAGACGGCATACGAGATTTTTTGTCTCTGGGCTCGGAGATGTGTATAAGAGACAG/3ddC/-3'
29	BN003	NRCBLK1PrimerRC	5' - GCT GTC TCT TAT ACA CAT CTG ACG CTG CCG ACG ATT TTT TTT GTG TAG ATC TCG GTG GTC GCC GTA TCA TT
30	BN004	NRCBLK2PrimerRC	5' - GCT GTC TCT TAT ACA CAT CTC CGA GCC CAC GAG ACT TTT TTT TAT CTC GTA TGC CGT CTT CTG CTT G
31	BN005	TiS17 with basepairing index sequence example	5' - AATGATACGGCGACCGAC+CGAGA+TCTA+CACG+CGTA+AGATCG+TCGG+CAGCG+TCAGATG+TGTATAA+GAGACAG/3SpC3/
32	BN006	TiS701 with basepairing index sequence example	5' - CAAGCAGAAAGA+CGGCA+TACGAGATT+CGCCTTAG+TCT+CGTGGGC+TCGGAGA+TGTGTATAA+GAGACAG/3SpC3/
33	BN007	TiBNA7	5' - AATGATACGGCGACCGAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +TCG+TCG+GCA+GCG+TCAGATG+TGTATAA+GAGA+CAG/3SpC3/
34	BN008	TiBNA8	5' - CAAGCAGAAAGA+CGGCA+TACGAGAT+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +GTC+TCG+TGGGC+TCGG+AGA+TGTG+TATAA+GAGA+CAG/3SpC3/

35	BN009	TiLNA7	5' - AATGATACGGCGACCAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +TCG+TCG+GCA+GCG+TCAGATG+TGATATAA+GAGA+CAG/3SpC3/
36	BN010	TiLNA8	5' - CAAGCAGAAAGA+CGGCA+TACGAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +GTC+TCG+TGGGC+TCGG+AGA+TGTG+TATAA+GAGA+CAG/3SpC3/
37	BN011	TiBNA11	5' - AATGATACGGCGACCAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// /ideoxyl//ideoxyl//+TCG+TCG+GCA+GCG+TCAGATG+TGATATAA+GAGA+CAG/3SpC3/
38	BN012	TiBNA12	5' - CAAGCAGAAAGA+CGGCA+TACGAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// ideoxyl//ideoxyl//+GTC+TCG+TGGGC+TCGG+AGA+TGTG+TATAA+GAGA+CAG/3SpC3/
39	BN013	TiBNA15G	5' - AATGATACGGCGACCAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//+G/ideoxyl// ideoxyl//ideoxyl//+TCG+TCG+GCA+GCG+TCAGATG+TGTA+TAA+GAGA+CAG/3SpC3/
40	BN014	TiBNA16G	5' - CAAGCAGAAAGA+CGGCA+TACGAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//+G/ideoxyl// /ideoxyl//+GTC+TCG+TGGGC+TCGG+AGA+TGTG+TA+TAA+GAGA+CAG/3SpC3/
41	BN015	TiBNA15N	5' - AATGATACGGCGACCAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl// ideoxyl//ideoxyl//+TCG+TCG+GCA+GCG+TCAGATG+TGTA+TAA+GAGA+CAG/3SpC3/
42	BN016	TiBNA16N	5' - CAAGCAGAAAGA+CGGCA+TACGAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// ideoxyl//+GTC+TCG+TGGGC+TCGG+AGA+TGTG+TA+TAA+GAGA+CAG/3SpC3/
43	BN017	TiLNA11	5' - AATGATACGGCGACCAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// /ideoxyl//ideoxyl//+TCG+TCG+GCA+GCG+TCAGATG+TGATATAA+GAGA+CAG/3SpC3/
44	BN018	TiLNA12	5' - CAAGCAGAAAGA+CGGCA+TACGAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// ideoxyl//ideoxyl//+GTC+TCG+TGGGC+TCGG+AGA+TGTG+TATAA+GAGA+CAG/3SpC3/
45	BN019	TiLNA15G	5' - AATGATACGGCGACCAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//+G/ideoxyl// ideoxyl//ideoxyl//+TCG+TCG+GCA+GCG+TCAGATG+TGTA+TAA+GAGA+CAG/3SpC3/
46	BN020	TiLNA16G	5' - CAAGCAGAAAGA+CGGCA+TACGAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//+G/ideoxyl// /ideoxyl//+GTC+TCG+TGGGC+TCGG+AGA+TGTG+TA+TAA+GAGA+CAG/3SpC3/
47	BN021	TiLNA15N	5' - AATGATACGGCGACCAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl// ideoxyl//ideoxyl//+TCG+TCG+GCA+GCG+TCAGATG+TGTA+TAA+GAGA+CAG/3SpC3/
48	BN022	TiLNA16N	5' - CAAGCAGAAAGA+CGGCA+TACGAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//N/ideoxyl// ideoxyl//+GTC+TCG+TGGGC+TCGG+AGA+TGTG+TA+TAA+GAGA+CAG/3SpC3/
49	BN023	P5LNA	5' - AATGA+TAC+GGC+GAC+CAC+CGA+GAT+CTA+CAC/3SpC3/
50	BN024	P7LNA	5' - CAAGC+AGA+AGA+CGG+CAT+ACG+A+GA+T/3SpC3/
51	BN025	TiLNAS1	5' - TCGTC+GGC+AGC+GTC+AGA+TGT+ATA+TAA+GAG+ACA+G/3SpC3/
52	BN026	TiLNAS2	5' - GTCT+CGT+GGGC+TCG+GAG+ATG+TGT+ATAA+GAG+ACA+G/3SpC3/
53	BN027	TiLNA17	5' - GGCGAC+CAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +TCG+TCG+GCA+GCG+TCA+GATG+TG/3SpC3/

54	BN028	TiLNA18	5' - GGCA+TAC+GAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +GTC+TCG+TGG+GC+TCGG+AGA+TGTG+TATAA+GAG/3SpC3/
55	BN029	P5BNA-All	5' - +A+T+G+A+T+A+C+G+G+C+G+A+C+C+A+C+G+A+G+A+T+C+T+A+C+A+C/3SpC3/
56	BN030	P7BNA-All	5' - +C+A+A+G+C+A+G+A+G+A+G+C+G+C+A+T+A+C+G+A+G+A+T/3SpC3/
57	BN031	TiBNA1-All	5' - +T+C+G+T+C+G+G+C+A+G+C+G+T+C+A+G+A+T+G+T+G+T+A+T+A+G+A+G+A+G/3SpC3/
58	BN032	TiBNA2-All	5' - +G+T+C+T+C+G+T+G+G+G+C+T+C+G+G+G+A+G+A+T+G+T+G+T+A+T+A+G+A+G+A+G/3SpC3/
59	BN033	P5BNA-half	5' - AA+TG+AT+AC+GG+CG+AC+CA+CC+GA+GA+TC+TA+CA+C/3SpC3/
60	BN034	P7BNA-half	5' - C-AA+GC+AG+AA+GA+GC+GC+AT+A+C+G+A+G+A+T/3SpC3/
61	BN035	TiBNA1-half	5' - TC+GT+CG+GC+AG+CG+TC+AG+AT+GT+GT+AT+AA+GA+GA+CA+G/3SpC3/
62	BN036	TiBNA2-half	5' - G+TC+TC+GT+GG+GC+TC+GG+AG+AT+GT+GT+AT+AA+GA+GA+CA+G/3SpC3/
63	BN037	P5BNA-Alt	5' - A+AT+GA+TA+CG+GC+GA+CC+AC+CG+AG+AT+CT+AC+A+C/3SpC3/
64	BN038	P7BNA-Alt	5' - +CA+AG+CA+GA+AG+AC+GG+CA+T+A+C+G+A+G+A+T/3SpC3/
65	BN039	TiBNA1-Alt	5' - T+CG+TC+GG+CA+GC+GT+CA+GA+TG+TG+TA+TA+AG+AG+AC+A+G/3SpC3/
66	BN040	TiBNA2-Alt	5' - +GT+CT+CG+TG+GG+CT+CG+GA+GA+TG+TG+TA+TA+AG+AG+AC+A+G/3SpC3/
67	BN041	TiBNA3	5' - AATGATACGGCGACCGAGATCTACACTTTTTTTCG+TCGG+CAGCG+TCAGATG+TGATATA+GAGACAG/3ddC/
68	BN042	TiBNA4	5' - 5'CAAGCAGAAGACGGCA+TACGAGATTTTTTTCG+TCT+CGTGGG+TCGAGATG+TGATATA+GAGACAG/3ddC/
69	BN043	TiBNA1	5' - AATGATACGGCGACCGAGAT+CTACACTTTTTTTT+CGTCGGCGGTCAGATGTGTATAAGAGA+CAG/3ddC/
70	BN044	TiBNA2	5' - CAAGCAGAAGACGGCATACGAGATTTTTTTTGT+CTCGTGGGCT+CGGAGATGTGTATAAGAGA+CAG/3ddC/

Table 2B – Exemplary Blocker Sequences

SEQ ID NO	Ref No	Oligo Name	Sequence
71	BL001	CT3 Blocker1 i7	5' - CAAGCAGAAGACGGCATACGAGATTTTTTTGTGACTGGAGTTCAGACGTGTGCTCTCCGATC/3ddC/
72	BL002	CT3 Blocker2 i5	5' - AATGATACGGCGACCGAGATCTACACTTTTCCCTACAGACGCTCTCCGATC/3ddC/
73	BL003	TSLTLNA13	5' - AATGATACGGCGACCGAC+CGA+GAT+CTA+CAG+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/
74	BL004	TSLTLNA14	5' - CAAGCAGAAGACGGG+CAT+ACG+AGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +GTG+ACT+GGA+GTT+CAG+ACG+TGT+GCT+CTTCCG+ATC/3ddC/
75	BL005	TSHTLNA13	5' - AATGATACGGCGACCGAC+CGA+GAT+CTA+CAC/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/
76	BL006	TSHTLNA14	5' - CAAGCAGAAGACGGG+CAT+ACG+AGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/
77	BL007	TSHTLNA15	5' - AATGATACGGCGACCGAC+CGA+GAT+CTA+CAC/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// /ideoxyl//ideoxyl//ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/

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78	BL008	TSHTLNA16	5'- CAAGCAGAAACACGG+CAT+ACG+AGA+T/ideoxy///ideoxy///ideoxy///ideoxy//+G/ideoxy//+G/ideoxy///ideoxy///ideoxy// /ideoxy//+GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/
79	BL009	TSHTLNA15G	5'- AATGATACGGCGACAC+CGA+GAT+CTA+CAC/ideoxy///ideoxy///ideoxy///ideoxy//+G/ideoxy///ideoxy///ideoxy// /ideoxy//ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/
80	BL010	TSHTLNA16G	5'- CAAGCAGAAACACGG+CAT+ACG+AGA+T/ideoxy///ideoxy///ideoxy///ideoxy//+G/ideoxy///ideoxy///ideoxy// /ideoxy//+GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/
81	BL011	TSHTLNA15N	5'- AATGATACGGCGACAC+CGA+GAT+CTA+CAC/ideoxy///ideoxy///ideoxy///ideoxy//N/ideoxy///ideoxy///ideoxy// /ideoxy///ideoxy//ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/
82	BL012	TSHTLNA16N	5'- CAAGCAGAAACACGG+CAT+ACG+AGA+T/ideoxy///ideoxy///ideoxy///ideoxy//N/ideoxy///ideoxy///ideoxy// /ideoxy//+GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/
83	BL013	TSLTBNA13	5'- AATGATACGGCGACAC+CGA+GAT+CTA+CAC/ideoxy///ideoxy///ideoxy///ideoxy//+G/ideoxy///ideoxy///ideoxy// /ideoxy//+GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/
84	BL014	TSLTBNA14	5'- CAAGCAGAAACACGG+CAT+ACG+AGA+T/ideoxy///ideoxy///ideoxy///ideoxy//+G/ideoxy///ideoxy///ideoxy// +GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/
85	BL015	TSHTBNA13	5'- AATGATACGGCGACAC+CGA+GAT+CTA+CAC/ideoxy///ideoxy///ideoxy///ideoxy///ideoxy///ideoxy// /ideoxy//ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/
86	BL016	TSHTBNA14	5'- CAAGCAGAAACACGG+CAT+ACG+AGA+T/ideoxy///ideoxy///ideoxy///ideoxy///ideoxy///ideoxy// +GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/
87	BL017	TSHTBNA15	5'- AATGATACGGCGACAC+CGA+GAT+CTA+CAC/ideoxy///ideoxy///ideoxy///ideoxy///ideoxy///ideoxy// /ideoxy///ideoxy//ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/
88	BL018	TSHTBNA16	5'- CAAGCAGAAACACGG+CAT+ACG+AGA+T/ideoxy///ideoxy///ideoxy///ideoxy///ideoxy///ideoxy// /ideoxy///ideoxy//+GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/

Table 2C – Exemplary Blocker Sequences

SEQ ID	Ref No	Oligo Name	Sequence
89	BX001	NEXTERA Blocker1_2X	5' - AATGATAXGGCGACCGACCGAGATCTACACTTTTTTTTCGTCGGCAGCGTCAGATGTGTATAAGAGAXAG/3ddC/
90	BX002	NEXTERA Blocker1_3X	5' - AATGATAXGGCGACCGACCGAGATXTACACTTTTTTTTCGTCGGCAGCGTCAGATGTGTATAAGAGAXAG/3ddC/
91	BX003	NEXTERA Blocker1_2Xalt	5' - AATGATACGGCGACCGACCGAGATXTACACTTTTTTTTCGTCGGCAGCGTCAGATGTGTATAAGAGAXAG/3ddC/
92	BX004	NEXTERA Blocker1_3Xalt	5' - AATGATACGGCGACCGACCGAGATXTACACTTTTTTTXGTCTGGCAGCGTCAGATGTGTATAAGAGAXAG/3ddC/
93	BX005	NEXTERA Blocker2_2X	5' - CAAGXAGAAGACGGCATACGAGATTTTTTTTGTCTCGTGGCTCGGAGATGTGTATAAGAGAXAG/3ddC/
94	BX006	NEXTERA Blocker2_3X	5' - CAAGXAGAAGACGGCATAXGAGATTTTTTTTGTCTCGTGGCTCGGAGATGTGTATAAGAGAXAG/3ddC/
95	BX007	NEXTERA Blocker2_2Xalt	5' - CAAGCAGAAGACGGCATACGAGATTTTTTTTGTCTCGTGGCTCGGAGATGTGTATAAGAGAXAG/3ddC/
96	BX008	NEXTERA Blocker2_3Xalt	5' - CAAGCAGAAGACGGCATACGAGATTTTTTTTGTCTCGTGGCTXGGAGATGTGTATAAGAGAXAG/3ddC/
97	BX009	NEXTERA Blocker1_5X4Y	5' - AATGATAXGGCGACCGACCGAGATXTACACTTTTTTTTCGTCGGXAGCGTCAGAYGYVAYAAAGAGAXAG/3ddC/-3'
98	BX010	NEXTERA Blocker2_5X4Y	5' - CAAGXAGAAGAXGGCATAXGAGATTTTTTTTGTCTCGTGGXTCTGGAGAYGYVAYAAAGAGAXAG/3ddC/-3'
99	BX011	NEXTERA Blocker1_5X4Y-outer	5' - AATGATAXGGCGACCGACCGAGATXTACAC
100	BX012	NEXTERA Blocker1_5X4Y-inner	5' - TCGTCGGXAGCGTCAGAYGYVAYAAAGAGAXAG/3ddC/-3'
101	BX013	NEXTERA Blocker2_5X4Y-outer	5' - CAAGXAGAAGAXGGCATAXGAGAT
102	BX014	NEXTERA Blocker2_5X4Y-inner	5' - GTCTCGTGGXTCTGGAGAYGYVAYAAAGAGAXAG/3ddC/-3'

Table 2D

Thymine bases at index position in oligos (8 nucleotides)	TTTTTTT (SEQ ID NO: 103)
/ideoxyl/	2'-deoxyinosine
/3ddC/	2',3' Dideoxycytosine
/3spC3/	3' Spacer C3
LNA- or BNA-modified base (as indicated in the "Oligo Name" column of Table 2A)	+A +C +T or +G
AP-dC-CE Phosphoramidite	X
2'-Ome-U-CE Phosphoramidite	Y

Blocker with Thymine

[0081] In some embodiments, a blocker may include at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases in the region of the blocker corresponding to the index region of the adaptor and/or the UMI of the adaptor (e.g., the region of the blocker that binds to the index region and/or UMI of the adaptor).

[0082] In some embodiments, the region of the blocker corresponding to the index region of the adaptor and/or the UMI of the adaptor (e.g., that binds to the index region and/or UMI of the adaptor) includes as many thymine bases as the number of bases in the index region of the adaptor and/or the UMI of the adaptor.

[0083] In some embodiments, the blocker includes at least one sequence of Table 2A, Table 2B, Table 2C, or Table 3. Some exemplary embodiments of such a blocker are described in Example 2.

Blocker with Universal Bases and at least one Non-Universal Base

[0084] In some embodiments, a blocker may include universal bases and at least one non-universal base in the region of the blocker that corresponds to the index region. As used herein, a “universal base” is a modified nucleobase that hybridizes to at least two of the common bases (e.g., deoxyadenosine (A), deoxythymidine (T), deoxycytidine (C), deoxyguanosine (G), and uridine (U)). In some embodiments, a universal base hybridizes to any of the common bases. As used herein, a “non-universal base” is a nucleobase (including, in some embodiments, a modified nucleobase) that hybridizes only in a traditional Watson-Crick base pair interaction (e.g., deoxyadenosine(A)-deoxythymidine (T) or deoxyguanosine (G)-deoxycytidine (C)).

[0085] In some embodiments, the at least one non-universal base may be included every third base of the blocker sequence corresponding to the index region. In some embodiments, the at least one non-universal base may be at the third and/or the fifth positions (from 5' end) of the blocker sequence corresponding to the index region. In some embodiments, the at least one non-universal base may be at the second and/or the fourth positions of the blocker sequence corresponding to the index region. In some embodiments, the at least one non-universal base includes a modified guanine. In some embodiments, the

modified guanine may be LNA-modified or BNA-modified. In some embodiments, the at least one non-universal base includes a random (A, T, G, or C) nucleotide.

[0086] Without wishing to be bound by theory, it is believed that including a modified guanine or a random nucleotide in addition to the universal bases in the region of the blocker that corresponds to the index region (as shown in one embodiment in FIG. 5), improves affinity of the blocker for a library member. Some exemplary embodiments of such a blocker are described in Example 3.

[0087] The universal base may include any known universal base. In some embodiments, a universal base used in the index region can include, for example, 2'-deoxyinosine, 2'-deoxynebularine, 3-nitropyrrole 2'-deoxynucleoside, or 5-nitroindole 2'-deoxynucleoside. In some embodiments, a combination of universal bases may be used in the index region.

[0088] The modified guanine may be a guanine modified in any suitable way including, for example, an LNA-modified guanine, a BNA-modified guanine, etc. In some embodiments, the modified guanine is preferably an LNA-modified guanine.

[0089] In some embodiments, the blocker includes at least one sequence of Table 2A, Table 2B, Table 2C, or Table 4.

Split Blockers

[0090] In some embodiments, a blocker may include at least two non-connected oligonucleotides, wherein the non-connected oligonucleotides include bases that do not correspond to the index region and/or the UMI of the adaptor (e.g., include bases that do not bind the index region and/or the UMI of the adaptor) or include bases that only partially correspond to the index region and/or the UMI of the adaptor (e.g., include bases that bind to only a portion of the index region and/or the UMI of the adaptor). In some embodiments, the blocker includes four non-connected oligonucleotides.

[0091] In some embodiments, the at least two non-connected oligonucleotides hybridize to the same strand of the target sequence. In some embodiments, at least a portion of the blocker will correspond to the 5' end of the adaptor and at least a portion of the blocker will correspond to the 3' end of the same adaptor. In some embodiments, including, for example, when the blocker includes four non-connected oligonucleotides, two non-connected oligonucleotides may hybridize to the same strand of a first target sequence, and two non-connected oligonucleotides may hybridize to the same strand of a second target

sequence. Exemplary embodiments of such blockers are shown in FIG. 3E-FIG. 3I and FIG. 6A and are described in Examples 4, 8, and 9.

[0092] In some embodiments, at least a portion of the blocker will correspond to a universal extension primer. In some embodiments, at least a portion of the blocker will correspond to P5, P7, P5', and/or P7'. In some embodiments, at least a portion of the blocker will correspond to V2.A14.METS, V2.B15.METS, the complement of V2.A14.METS, and/or the complement of V2.B15.METS.

[0093] In some embodiments, the blocker will include the same number of bases as the adaptor excluding the portion of the adaptor containing the bases of the index region and/or the UMI of the adaptor. In some embodiments the blocker will include one base fewer, two bases fewer, three bases fewer, four bases fewer, or five bases fewer than the number of bases making up the portion of the adaptor excluding the bases of the index region and/or the UMI of the adaptor.

[0094] In some embodiments, a blocker including more bases may be preferred including, for example, when increased enrichment performance is desired. In some embodiments, a blocker including fewer bases may be preferred including, for example, when the cost of the blocker, synthesis purity and/or yield are a factor.

[0095] In some embodiments, up to five bases of the blocker, up to four bases of the blocker, up to three bases of the blocker, up to two bases of the blocker, only one base of the blocker, or no bases of the blocker will pair with the index region of the adaptor and/or the UMI of the adaptor.

[0096] In some embodiments, the blocker includes at least one sequence (or portion of a sequence) of Table 2A, Table 2B, Table 2C, or Table 5. In some embodiments, the blocker includes at least one of BN023, BN024, BN025, and BN026 of Table 2A. In some embodiments, the blocker includes BN023 and BN025 of Table 2A. In some embodiments, the blocker includes BN024 and BN026 of Table 2A.

Hybridization Buffer

[0097] In another aspect, this disclosure describes a hybridization buffer including a crowding agent and methods of using that hybridization buffer. As used herein, a “crowding agent” includes a compound that allows for, enhances, or facilitates molecular crowding. In some embodiments, a crowding agent includes an inert macromolecule used at a concentration that alters a DNA-protein interaction. A “hybridization buffer” is defined

as any buffer in which a nucleotide-probe hybrid may be formed as part of a hybrid capture assay.

[0098] In some embodiments, the crowding agent may include at least one of dextran, dextran sulfate, polyethylene glycol (PEG), Ficoll, glycerol, betaine, etc. Dextran and dextran sulfate may include high molecular weight dextran (e.g., dextran having a molecular weight of at least 500,000 Da) and/or low molecular weight dextran (e.g., dextran having a molecular weight of up to 10,000 Da, up to 50,000 Da, or up to 100,000 Da). In some embodiments, the crowding agent preferably includes dextran sulfate.

[0099] In some embodiments, the crowding agent may be included in the hybridization buffer in an amount of at least 0.5% weight/volume (w/v), at least 1% (w/v), at least 1.5% (w/v), at least 2% (w/v), at least 3% (w/v), or at least 4% (w/v). In some embodiments, the crowding agent may be included in the hybridization buffer in an amount of up to 1% (w/v), up to 1.5% (w/v), up to 2% (w/v), up to 3% (w/v), up to 4% (w/v), up to 5% (w/v), up to 6% (w/v), up to 8% (w/v), or up to 10% (w/v).

[00100] As described in some embodiments in Example 5 (see, e.g., FIGs. 7D-7F), inclusion of a crowding agent (e.g., dextran sulfate) in a hybridization buffer may, in some embodiments, allow for decreased hybridization time and/or hybridization in larger volumes (e.g., higher dilution of target oligonucleotide) of hybridization buffer than hybridization buffer without a crowding agent. For example, inclusion of dextran sulfate in hybridization buffer may be capable of reducing the hybridization time from overnight to approximately 80 minutes at a single temperature or 60 minutes with an additional temperature ramping step.

[00101] In some embodiments, inclusion of a crowding agent may improve the enrichment specificity. For example, one embodiment is shown in Example 5 (see FIG. 8A), wherein the enrichment specificity was increased from 45% to 80-95%. In some embodiments, improvement of the enrichment specificity due to the inclusion of a crowding agent may be more pronounced with a shorter incubation time.

[00102] In some embodiments, inclusion of a crowding agent in the hybridization buffer allows an enrichment reaction to happen more quickly with a lower concentration of DNA (including, for example, in a larger relative volume of hybridization buffer). Such an increase may be used to facilitate “pooling-by-volume” and/or elimination of a sample concentration step. Generally, to achieve a fast hybridization reaction requires the use of a

high concentration of DNA resulting from the library preparation and a high concentration of probes and, therefore, in some embodiments, a correspondingly small volume of hybridization buffer (e.g., less than 20 μ L). Achieving this small volume requires a sample concentration step after library preparation (with resulting increases in the time and cost of the assay as a whole). With the inclusion of a crowding agent in the hybridization buffer, an enrichment reaction may be achieved more quickly, at a lower DNA concentration and/or probe concentration (and, in some embodiments, in a larger volume). In some embodiments, inclusion of a crowding agent in the hybridization buffer allows the combination of multiple library preparation samples without a concentration step prior to hybridization. In some embodiments, the samples resulting from different library preparations may be pooled by volume, as further described in this disclosure, allowing for multiplex enrichment. The “plexy” or “plex” of a given multiplex enrichment refers to the number of different library preparations that are combined before enrichment (e.g., hybridization and capture). In some embodiments, the plexy of multiplex enrichment can be up to 2-plex, up to 4-plex, up to 6-plex, up to 12-plex, up to 24-plex, up to 48-plex, up to 96-plex, or higher.

[00103] In some embodiments, a hybridization buffer including a crowding agent further includes at least one of human Cot-1 DNA, a destabilizing agent, salt, and a blocker. In some embodiments, the components of hybridization buffer such as Cot-1 DNA and a blocker are kept separated from the components of hybridization buffer including a destabilizing agent and a crowding agent until the hybridization reaction is performed.

[00104] In some embodiments, a hybridization buffer may include human Cot-1 DNA in an amount of at least 0.05 mg/mL, at least 0.1 mg/mL, at least 0.2 mg/mL, or at least 0.3 mg/mL. In some embodiments, a hybridization buffer may include human Cot-1 DNA in an amount of up to 0.1 mg/mL, up to 0.2 mg/mL, up to 0.3 mg/mL, or up to 0.5 mg/mL. In some embodiments, a hybridization buffer may preferably include human Cot-1 DNA in an amount of 0.2 mg/mL.

[00105] In some embodiments, a hybridization buffer may include a destabilizing agent. In some embodiments, the destabilizing agent may include formamide and/or urea. In some embodiments, the hybridization buffer may include at least 1% (v:v), at least 5% (v:v), or at least 10% (v:v) formamide. In some embodiments, the hybridization buffer may include up to 5% (v:v), up to 10% (v:v), or up to 15% (v:v) formamide. In some embodiments, the

hybridization buffer may include at least 1 M urea, at least 2 M urea, or at least 4 M urea. In some embodiments, the hybridization buffer may include up to 2 M urea, up to 4 M urea, or up to 6 M urea. In some embodiments, a hybridization buffer may preferably include 10% formamide.

[00106] In some embodiments, a hybridization buffer may include a buffer. Any suitable buffer may be used. In some embodiments, the buffer may include a phosphate including, for example, potassium phosphate or sodium phosphate; Tris-HCl; piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES); Piperazine-N,N'-bis(3-propanesulfonic acid) (PIPES); 2-(N-morpholino)ethanesulfonic acid (MES); (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), etc. In some embodiments, a hybridization buffer may include at least 40 mM, at least 50 mM, at least 55 mM, at least 60 mM, at least 65 mM, or at least 70 mM phosphate. In some embodiments, a hybridization buffer may include up to 50 mM, up to 55 mM, up to 60 mM, up to 65 mM, up to 70 mM, up to 75 mM, or up to 80 mM phosphate. In some embodiments, a phosphate containing buffer may include monobasic dihydrogen phosphate and/or dibasic monohydrogen phosphate. In some embodiments, a phosphate containing buffer may include KH_2PO_4 - K_2HPO_4 . In some embodiments, a hybridization buffer may preferably include 66.6 mM KH_2PO_4 - K_2HPO_4 .

[00107] In some embodiments, a hybridization buffer may include a salt. Any suitable salt may be included. In some embodiments, the salt may be, for example, NaCl, or a sodium citrate (e.g., trisodium citrate), etc. In some embodiments, a hybridization buffer may include at least 0.1 M, at least 0.2 M, at least 0.3 M, at least 0.4 M, at least 0.5 M, at least 0.6 M, at least 0.7 M, at least 0.8 M, at least 0.9 M, or at least 1 M salt. In some embodiments, a hybridization buffer may include up to 0.2 M, up to 0.3 M, up to 0.4 M, up to 0.5 M, up to 0.6 M, up to 0.7 M, up to 0.8 M, up to 0.9 M, up to 1 M, up to 2 M, up to 3 M, or up to 4 M salt. In some embodiments, a hybridization buffer may preferably include 0.8 M NaCl.

[00108] In some embodiments, a hybridization buffer may include a detergent including, for example, an anionic detergent (e.g., sodium dodecyl sulfate, a sulfonic acid salt, an alcohol sulfate, an alkylbenzene sulfonate, a phosphoric acid ester, or a carboxylic acid salt), a nonionic detergent (e.g., a polyoxyethylene or glycoside detergent, such as a Tween, Triton, or Brij detergent), a cationic detergent (e.g., a quarternary ammonium cationic surfactant), or a zwitterionic detergent (e.g., CHAPS). In some embodiments, the detergent

is an anionic detergent. In some embodiments, the detergent is a nonionic detergent. In some embodiments, the detergent is TWEEN 20, TWEEN 80, sodium dodecyl sulfate (SDS), etc. In some embodiments, a detergent may make up at least 0.001% volume/volume (v/v), at least 0.01% (v/v), at least 0.05% (v/v), at least 0.1% (v/v), at least 1% (v/v), or at least 5% (v/v) of the hybridization buffer. In some embodiments, a detergent may make up to 0.01% (v/v), up to 0.05% (v/v), up to 0.1% (v/v), up to 1% (v/v), up to 5% (v/v), or up to 10% (v/v) of the hybridization buffer. In some embodiments, a hybridization buffer may preferably include 0.04% (v/v) TWEEN 20.

[00109] For example, in some embodiments, a hybridization buffer may include 0.5% to 10% dextran sulfate, 0.05 mg/mL to 0.5 mg/mL human Cot-1 DNA, 1% to 15% (v/v) formamide, 40 mM to 80 mM $\text{KH}_2\text{PO}_4 - \text{K}_2\text{HPO}_4$, 0.1 M to 4 M NaCl, and 0.001% to 10% (v/v) Tween 20. In some embodiments, a hybridization buffer may include (for example, in a final reaction) 1.5% dextran sulfate, 0.2 mg/mL Cot-1, 10% (v/v) formamide, 66.6 mM $\text{KH}_2\text{PO}_4 - \text{K}_2\text{HPO}_4$, 0.8 M NaCl, and 0.04% (v/v) TWEEN 20.

[00110] In some embodiments, a hybridization buffer may include a blocker. In some embodiments, the blocker is as described elsewhere in this disclosure. In some embodiments, the blocker is present at a concentration of at least 0.001 mM, at least 0.005 mM, at least 0.01 mM, at least 0.05 mM, or at least 0.1 mM. In some embodiments, the blocker is present at a concentration of up to 0.01 mM, up to 0.05 mM, up to 0.1 mM, up to 0.2 mM, or up to 0.5 mM.

[00111] In another aspect, the disclosure describes a kit that includes a hybridization buffer, for example, as described herein. In an embodiment the kit further includes at least one of a blocker and a probe. In embodiments the components are provided in separate containers. For example, the hybridization buffer may be provided in a first container and another component, for example, a blocker or a probe may be provided in a different container(s). In some embodiments, the blocker and/or the probe are as described elsewhere in this disclosure.

Hybrid Capture

[00112] In a further aspect, this disclosure describes a method of hybrid capture. In some embodiments, the method includes steps including hybridizing probes to library members and capturing probes. In some embodiments, the method further includes pooling libraries prior to hybridizing probes. In some embodiments, the method further includes amplifying

the captured sequences after capture. In some embodiments, the method may be used in combination with a blocker oligonucleotide as described in this disclosure. In some embodiments, the method may include the use of a hybridization buffer including a crowding agent as described in this disclosure.

Pooling Libraries

[00113] In some embodiments, samples resulting from different library preparations are combined prior to hybridization. In some embodiments, each library to be combined preferably includes sequences with index regions that are distinguishable from the index regions of the sequences in any other library to be combined.

[00114] In some embodiments, the samples resulting from different library preparations may be pooled by volume, allowing for multiplex enrichment. The “plexy” or “plex” of a given multiplex enrichment refers to the number of different library preparations that are combined before enrichment (e.g., hybridization and capture). In some embodiments, the plexy of multiplex enrichment can be up to 2-plex, up to 4-plex, up to 6-plex, up to 12-plex, up to 24-plex, up to 48-plex, up to 96-plex, or higher.

[00115] In some embodiments, a sample resulting from a library preparation results from a tagmentation reaction. *See, e.g.*, U.S. Pat. No. 9,574,226. In some embodiments, a sample resulting from a library preparation results from a NEXTERA library preparation (Illumina, Inc., San Diego, CA). In some embodiments, the library preparation can be directly added into a hybridization reaction.

[00116] In some embodiments, the DNA concentration of each sample resulting from a library preparation being combined will be at least 0.1 ng/μL, at least 0.3 ng/μL, at least 0.4 ng/μL, at least 0.5 ng/μL, at least 1 ng/μL, at least 2 ng/μL, at least 4 ng/μL, at least 6 ng/μL, at least 8 ng/μL, at least 10 ng/μL, at least 12 ng/μL, at least 20 ng/μL, at least 50 ng/μL, at least 60 ng/μL, at least 100 ng/μL, or at least 200 ng/μL. In some embodiments, the DNA concentration of each sample resulting from a library preparation being combined will be up to 0.4 ng/μL, up to 0.5 ng/μL, up to 1 ng/μL, up to 2 ng/μL, up to 4 ng/μL, up to 6 ng/μL, up to 8 ng/μL, up to 10 ng/μL, up to 12 ng/μL, up to 15 ng/μL, up to 20 ng/μL, up to 50 ng/μL, up to 100 ng/μL, up to 120 ng/μL, up to 200 ng/μL, up to 300 ng/μL, or up to 400 ng/μL. For example, in some embodiments, the DNA concentration of each sample resulting from a library preparation will be in a range of 0.3 ng/μL to 400 ng/μL. For example, in some embodiments, the DNA concentration of each

sample resulting from a library preparation will be in a range of 0.1 ng/ μ L to 120 ng/ μ L. For example, in some embodiments, the DNA concentration of the combination of the samples resulting from the library preparations will be in a range of 0.1 ng/ μ L to 120 ng/ μ L (e.g., in 100 μ L). For example, in some embodiments, the DNA concentration of each sample resulting from a library preparation will be in a range of 0.5 ng/ μ L to 12 ng/ μ L. In some embodiments, the DNA concentration of the combination of the samples resulting from the library preparations will be in a range of 0.5 ng/ μ L to 12 ng/ μ L (e.g., in 100 μ L). For example, in some embodiments, the DNA concentration of each sample resulting from a library preparation will be in a range of 0.5 ng/ μ L to 120 ng/ μ L. In some embodiments, the DNA concentration of the combination of the samples resulting from the library preparations will be in a range of 0.5 ng/ μ L to 120 ng/ μ L (e.g., in 100 μ L).

[00117] In some embodiments, at least 1 μ L, at least 2 μ L, at least 3 μ L, at least 5 μ L, at least 10 μ L, at least 15 μ L, at least 20 μ L, or at least 25 μ L of each sample resulting from a library preparation will be combined. In some embodiments, up to 2 μ L, up to 3 μ L, up to 5 μ L, up to 10 μ L, up to 15 μ L, up to 20 μ L, up to 25 μ L, up to 30 μ L, up to 40 μ L, or up to 50 μ L of each sample resulting from a library preparation will be combined.

[00118] In some embodiments, at least 10 ng, at least 15 ng, at least 25 ng, at least 50 ng, at least 100 ng, at least 200 ng, at least 500 ng, at least 1,000 ng, or at least 5,000 ng of DNA from each library preparation will be combined. In some embodiments, up to 15 ng, up to 25 ng, up to 50 ng, up to 100 ng, up to 200 ng, up to 500 ng, up to 1,000 ng, up to 5,000 ng, up to 10,000 ng, or up to 12,000 ng of DNA from each library preparation will be combined. For example, in some embodiments, 10 ng to 12,000 ng of DNA from each library preparation may be combined.

Hybridizing Probes

[00119] In some aspects, a method of hybrid capture includes contacting the library with a probe wherein the probe hybridizes to a region of interest within a library member. The region of interest is separate from the adaptor region and includes genomic material of interest. The probe includes a ligand that allows for subsequent capture of the probe. In some embodiments, the ligand preferably includes a biotin group.

[00120] The library is contacted with the probe in the presence of a hybridization buffer. In some embodiments, the hybridization buffer may include a crowding agent, as described in

this disclosure. In some embodiments, the hybridization buffer may include a blocker, as described in this disclosure.

[00121] In some embodiments, a method of hybrid capture includes a heat-denaturation step. For example, a hybridization mixture including library members, blockers, and a probe in hybridization buffer may be heated to at least 90°C, at least 92°C, or at least 95°C. In some embodiments, the hybridization mixture may be heated up to 92°C, up to 95°C, up to 97°C, or up to 100°C. In some embodiments, the hybridization mixture preferably further includes Cot-1 DNA. In some embodiments, the hybridization buffer may include a blocker prior to being added to the hybridization mixture; in some embodiments, the blocker may be added to the hybridization mixture independently of the hybridization buffer.

[00122] In some embodiments, the DNA concentration in the hybridization mixture (e.g., the final hybridization reaction) will be at least 0.1 ng/μL, at least 0.3 ng/μL, at least 0.4 ng/μL, at least 0.5 ng/μL, at least 1 ng/μL, at least 2 ng/μL, at least 4 ng/μL, at least 6 ng/μL, at least 8 ng/μL, at least 10 ng/μL, at least 12 ng/μL, at least 15 ng/μL, at least 20 ng/μL, at least 50 ng/μL, at least 75 ng/μL, at least 100 ng/μL, at least 120 ng/μL, at least 150 ng/μL, at least 200 ng/μL. In some embodiments, the DNA concentration in the hybridization mixture (e.g., the final hybridization reaction) will be up to 0.3 ng/μL, up to 0.4 ng/μL, up to 0.5 ng/μL, up to 1 ng/μL, up to 2 ng/μL, up to 4 ng/μL, up to 6 ng/μL, up to 8 ng/μL, up to 10 ng/μL, up to 12 ng/μL, up to 15 ng/μL, up to 20 ng/μL, up to 50 ng/μL, up to 75 ng/μL, up to 100 ng/μL, up to 120 ng/μL, up to 150 ng/μL, up to 200 ng/μL, or up to 500 ng/μL. For example, in some embodiments, the DNA concentration in the hybridization mixture (e.g., the final hybridization reaction) will be in a range of 0.1 ng/μL to 120 ng/μL.

[00123] In some embodiments, the method may include the use of a volume of hybridization mixture of up to 150 μL, up to 140 μL, up to 130 μL, up to 120 μL, up to 110 μL, up to 100 μL, up to 90 μL, up to 80 μL, up to 70 μL, up to 60 μL or up to 50 μL. In some embodiments, the method may include the use of a volume of hybridization mixture of at least 10 μL, at least 20 μL, at least 30 μL, at least 40 μL, at least 50 μL, at least 60 μL, or at least 70 μL.

[00124] In some embodiments, the hybridization mixture may be heated at a rate of at least 1°C/minute, at least 1.5°C/minute, at least 2°C/minute, at least 2.5°C/minute, at least

3°C/minute, or at least 5°C/minute. In some embodiments, the hybridization mixture may be heated at a rate of up to 1.5°C/minute, up to 2°C/minute, up to 2.5°C/minute, up to 3°C/minute, up to 5°C/minute, or up to 10°C/minute. In some embodiments, the hybridization mixture may be maintained at a temperature for a heat-denaturation for at least 1 minute, at least 2 minutes, at least 5 minutes, at least 10 minutes, or at least 20 minutes. In some embodiments, the hybridization mixture may be maintained at a temperature for a heat-denaturation for up to 2 minutes, up to 5 minutes, up to 10 minutes, up to 20 minutes, or longer.

[00125] Following the heat-denaturation step, a method of hybrid capture includes cooling the hybridization mixture. As the hybridization mixture cools from the heat-denaturation step temperature to a hybridization temperature, probe:target hybrids (that is, probe:library member hybrids) will form.

[00126] In some embodiments, the hybridization temperature may be at least 50°C, at least 55°C, at least 56°C, at least 57°C, at least 58°C, at least 59°C, at least 60°C, at least 61°C, at least 62°C, at least 63°C, at least 64°C, at least 65°C, or at least 70°C. In some embodiments, the hybridization temperature may be up to 56°C, up to 58°C, up to 60°C, up to 61°C, up to 62°C, up to 63°C, up to 64°C, up to 65°C, or up to 70°C. In some embodiments, the hybridization may include a ramp-down of temperature from a denaturing temperature to the hybridization temperature. In some embodiments, the temperature ramp may include a rate of at least -5°C/minute, at least -2°C/minute, at least -1°C/minute, at least -0.5°C/minute, or at least -0.2°C/minute.

[00127] In some embodiments, the method may include maintaining the library in contact with the probe and/or maintaining the hybridization mixture at a hybridization temperature for up to 3 days, up to 2 days, up to 24 hours, up to 20 hours, up to 16 hours, up to 12 hours, up to 6 hours, up to 3 hours, up to 2 hours, up to 90 minutes, up to 1 hour, or up to 30 minutes. In some embodiments, the method may include maintaining the library in contact with the probe and/or maintaining the hybridization mixture at a hybridization temperature for at least 30 minutes, at least 45 minutes, at least 1 hour, at least 90 minutes, at least 2 hours, at least 3 hours, at least 6 hours, at least 12 hours, at least 16 hours, at least 20 hours, or at least 24 hours. In some embodiments, including when the T_m of the blocker is higher than the T_m of the adaptor, blocker:adaptor hybrids will form before

adaptor:adaptor hybrids form, thereby preventing the formation of “daisy chains” or concatenation of adaptor:adaptor hybrids.

Capture

[00128] In some aspects, a method of hybrid capture includes capturing the probe once it has hybridized to a member of the library using a capture means. The probe may be captured by any suitable means. For example, when the probe includes a biotin group, the probe may be captured using a streptavidin bead including, for example, a streptavidin-coated magnetic bead. In some embodiments, the probe may include FITC, and the capture means may include anti-FITC. In some embodiments, the probe may include a hapten and the capture means may include a molecule (e.g., an antibody) that binds that hapten. Other means for capture may be used, such as isotachopheresis, size exclusion chromatography, liquid chromatography, magnetic means, and the like.

[00129] In some embodiments, including for example when the capture means includes a streptavidin-coated bead, the method may include forming a capture mixture including the probe (including, e.g., a probe:target hybrid) and the capture means.

[00130] In some embodiments, the method may include maintaining the capture mixture at a capture temperature. In some embodiments, the capture temperature may be at least 50°C, at least 55°C, at least 56°C, at least 57°C, at least 58°C, at least 59°C, at least 60°C, at least 61°C, at least 62°C, at least 63°C, at least 64°C, at least 65°C, or at least 70°C. In some embodiments, the capture temperature may be up to 56°C, up to 58°C, up to 60°C, up to 61°C, up to 62°C, up to 63°C, up to 64°C, up to 65°C, or up to 70°C. In some embodiments, the capture mixture may be maintained at the capture temperature for at least 2 minutes, at least 5 minutes, at least 10 minutes, or at least 15 minutes. In some embodiments, the capture mixture may be maintained at the capture temperature for up to 5 minutes, up to 10 minutes, up to 15 minutes, up to 20 minutes, up to 30 minutes, up to 45 minutes, or longer. In some embodiments, and/or shake-mixing may be added every 5 or 10 minutes.

[00131] In some embodiments, the method may further include washing the captured probe (the captured probe may include, for example, a probe:target hybrid and/or captured library members). In some embodiments, the captured probe may be washed at least one time, at least two times, at least three times, or more. In some embodiments, the captured probe may be washed up to one time, up to two times, up to three times, or up to five times.

[00132] In some embodiments, washing the captured library members may include heating the captured probe in a wash buffer to a wash temperature. In some embodiments, the wash buffer may include a detergent. Any suitable buffer may be used including, for example, Tris-HCl; piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES); Piperazine-N,N'-bis(3-propanesulfonic acid) (PIPES); 2-(N-morpholino)ethanesulfonic acid (MES); (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), etc. Any suitable detergent may be used including, for example, TWEEN 20, TWEEN 80, sodium dodecyl sulfate (SDS), etc. In some embodiments, the wash buffer may include a compound intended to reduce the formation of secondary structure in GC-rich regions including, for example, betaine. In some embodiments, the wash buffer may include an iron chelating agent including, for example, deferoxamine mesylate, EGTA, EDTA, etc.

[00133] A wash temperature may be at least 20°C, at least 23°C, at least 25°C, at least 30°C, at least 35°C, at least 40°C, at least 45°C, at least 50°C, at least 55°C, at least 56°C, at least 57°C, at least 58°C, at least 59°C, at least 60°C, at least 61°C, at least 62°C, at least 63°C, at least 64°C, at least 65°C, or at least 70°C. In some embodiments, a wash temperature may be up to 30°C, up to 35°C, up to 40°C, up to 45°C, up to 50°C, up to 55°C, up to 56°C, up to 60°C, up to 61°C, up to 62°C, up to 63°C, up to 64°C, up to 65°C, or up to 70°C.

[00134] In some embodiments, the method may further include eluting the captured library members from the capture means and/or from the probe. For example, the captured library members may be eluted from a streptavidin bead and/or from a biotinylated probe. In embodiments wherein the streptavidin bead includes a magnetic streptavidin bead, elution may include the use of a magnet. In some embodiments, elution may include the use of a strong base including, for example, NaOH, KOH, Ca(OH)₂, etc.

[00135] In some embodiments, a solid support media may be used as a capture means including, for example a solid support media containing streptavidin. In some embodiments, the capture means may be washed under successively more stringent conditions at a temperature below that of the estimated probe:target T_m value and/or above the T_m value of the adaptor.

[00136] In some embodiments, captured library members can be eluted from a ligand (e.g., eluted from a biotinylated probe) or a capture means (e.g., eluted from a streptavidin bead) and directly loaded onto the flow cell.

[00137] In some embodiments, at least 60 femtomoles, at least 80 femtomoles, at least 90 femtomoles, at least 100 femtomoles, or at least 150 femtomoles of DNA can be eluted. In some embodiments, up to 150 femtomoles, up to 500 femtomoles, up to 1 picomole, up to 2 picomoles, or up to 3 picomoles of DNA may be eluted. For example, in some embodiments, 100 femtomoles to 2 picomoles of DNA may be eluted.

[00138] In some embodiments, the eluted library members may be loaded onto the flow cell in a volume of less than 100 μL , a volume of less than 90 μL , a volume of less than 80 μL , a volume of less than 70 μL , or a volume of less than 60 μL . In some embodiments, the captured library members may be loaded onto a flow cell in a volume of approximately 55 μL .

[00139] In some embodiments, following elution, the eluant may have a DNA concentration of at least 1.1 picomolar (pM), at least 1.2 pM, at least 1.3 pM, at least 10 pM, or at least 100 pM. In some embodiments, the eluant may have a DNA concentration of up to 100 pM, up to 200 pM, up to 250 pM, or up to 300 pM. For example, in some embodiments, the the eluant may have a DNA concentration in the range of 1.3 pM to 250 pM. In some embodiments, the eluant may be loaded onto the flow cell without further dilution and/or without altering the DNA concentration.

Amplification

[00140] In another aspect, this disclosure describes a method that includes amplifying the library members using PCR following hybrid capture but prior to sequencing the captured library members. Alternatively, this disclosure describes a method that does not include exposing the captured library member to amplification conditions following hybrid capture prior to sequencing the library members (e.g., does not include exposing the captured library members to a PCR step to amplify captured library members prior to sequencing the library members). Methods of sequencing the captured library members typically include amplifying the library members (e.g., using a PCR step) prior to sequencing the library members.

[00141] The amount of DNA captured during hybridization and hybrid capture can be too low to quantitate directly either by fluorometric or analytic methods (e.g., Bioanalyzer). Amplifying the library enables quantification and quality control of the process.

[00142] Further, it is typical to dilute a sample that has been amplified using PCR after hybrid capture and prior to sequencing. For example, the typical quantification of an

Illumina, Inc.'s NEXTERA Rapid Capture sample after PCR is in the mid-nanomolar range, whereas sequencing concentrations are in the low picomolar range. Thus, several orders of magnitude more molecules are generated by amplifying the captured library members than are required for sequencing.

[00143] In some embodiments, this disclosure describes a method of sequencing captured library members following hybrid capture. Such a method may include directly loading the captured library members and/or the eluted library members onto a flow cell (e.g., using a direct flowcell loading jig, as described, for example, in U.S. Provisional Pat. Application No. 62/564,466, filed on September 28, 2017).

[00144] In some embodiments, a method may further include sequencing the captured library members after hybrid capture.

EMBODIMENTS

Exemplary Blocker Embodiments

[00145] 1. A blocker comprising an oligonucleotide;

wherein the blocker is capable of binding to an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI);

and wherein the region of the blocker capable of binding to the index region and/or UMI of the adaptor comprises:

at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases; or
universal bases and at least one non-universal base.

[00146] 2. The blocker of Blocker Embodiment 1, wherein the region of the blocker capable of binding to the index region or UMI of the adaptor comprises as many thymine bases as the number of bases in the index region and/or the UMI of the adaptor.

[00147] 3. The blocker of Blocker Embodiment 1, wherein the at least one non-universal base is located in the region of the blocker sequence corresponding to the index region and/or UMI and wherein the non-universal base is at the third position or the fifth position or both of the blocker sequence, relative to the 5' end of the index region.

- [00148] 4. The blocker of Blocker Embodiment 1 or Blocker Embodiment 3, wherein the at least one non-universal base comprises a modified guanine.
- [00149] 5. The blocker of any one of Blocker Embodiments 1, 3, or 4, wherein the at least one non-universal base comprises a random nucleotide.
- [00150] 6. The blocker of any one of Blocker Embodiments 1, 3, 4, or 5, wherein the universal base comprises 2'-deoxyinosine, 2'-deoxynebularine, 3-nitropyrrole 2'-deoxynucleoside, or 5-nitroindole 2'-deoxynucleoside.
- [00151] 7. The blocker of any one of the previous Blocker Embodiments, wherein the universal primer sequence comprises at least one of P5, P7, P5', P7', V2.A14.METS, V2.B15.METS, the complement of V2.A14.METS, and the complement of V2.B15.METS.
- [00152] 8. The blocker of any one of the previous Blocker Embodiments, wherein the blocker comprises a modification that increases the T_m of the blocker relative to the same blocker that does not include the modification.
- [00153] 9. The blocker of any one of the previous Blocker Embodiments, wherein the blocker comprises at least one of a DNA or RNA oligonucleotide modified to capture low GC regions; a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap.
- [00154] 10. The blocker of any one of the previous Blocker Embodiments, wherein the blocker comprises a modified base at every base, at least every 2 bases, at least every 3 bases, at least every 4 bases, or at least every 5 bases.
- [00155] 11. The blocker of any one of the previous Blocker Embodiments, wherein the blocker comprises a 3' terminal group that precludes the availability of the blocker to serve as a primer for DNA synthesis.
- [00156] 12. The blocker of Blocker Embodiment 11, wherein the 3' terminal group comprises 3'-dC, 2',3'-ddC, inverted dT, or 3'-spacer C3.
- [00157] 13. The blocker of any one of the previous Blocker Embodiments, wherein the blocker comprises a sequence of Table 2A, Table 2B, Table 2C, or Table 3.
- [00158] 14. A kit comprising the blocker of any one of the preceding Blocker Embodiments.

Exemplary Split Blocker Embodiments

- [00159]** 1. A blocker comprising two non-connected oligonucleotides; wherein the blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI); and wherein the non-connected oligonucleotides comprise bases that do not correspond to the index region and/or the UMI of the adaptor.
- [00160]** 2. The blocker of Split Blocker Embodiment 1, wherein the non-connected oligonucleotides do not comprise bases corresponding to the index region and/or the UMI of the adaptor.
- [00161]** 3. The blocker of any one of the previous Split Blocker Embodiments, wherein the universal primer sequence includes at least one of P5, P7, P5', P7', V2.A14.METS, V2.B15.METS, the complement of V2.A14.METS, and the complement of V2.B15.METS.
- [00162]** 4. The blocker of any one of the previous Split Blocker Embodiments, wherein the blocker comprises a modification that increases the T_m of the blocker relative to the same blocker that does not include the modification.
- [00163]** 5. The blocker of any one of the previous Split Blocker Embodiments, wherein the blocker comprises at least one of a DNA or RNA oligonucleotide modified to capture low GC regions; a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap.
- [00164]** 6. The blocker of any one of the previous Split Blocker Embodiments, wherein the blocker comprises a modified base at every base, at least every 2 bases, at least every 3 bases, at least every 4 bases, or at least every 5 bases.
- [00165]** 7. The blocker of any one of the previous Split Blocker Embodiments, wherein the blocker comprises a 3' terminal group that precludes the availability of the blocker to serve as a primer for DNA synthesis.
- [00166]** 8. The blocker of Split Blocker Embodiment 11, wherein the 3' terminal group comprises 3'-dC, 2',3'-ddC, inverted dT, or 3'-spacer C3.
- [00167]** 9. The blocker of any one of the previous Split Blocker Embodiments, wherein the blocker comprises a sequence of Table 2A, Table 2B, Table 2C, or Table 3.

[00168] 10. The blocker of any of the previous Split Blocker Embodiments, wherein the blocker comprises at least one of BN023, BN024, BN027, and BN028 of Table 2A.

[00169] 11. A kit comprising the blocker of any one of the preceding Split Blocker Embodiments.

Exemplary Hybridization Buffer Embodiments

[00170] 1. A hybridization buffer comprising a crowding agent.

[00171] 2. The hybridization buffer of Hybridization Buffer Embodiment 1, wherein the crowding agent comprises at least one of dextran, dextran sulfate, polyethylene glycol (PEG), Ficoll, glycerol, and betaine.

[00172] 3. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the crowding agent is included in the hybridization buffer in an amount of at least 0.5%, at least 1%, at least 1.5%, at least 2%, at least 3%, or at least 4%.

[00173] 4. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the crowding agent is included in the hybridization buffer in an amount of up to 1%, up to 1.5%, up to 2%, up to 3%, up to 4%, up to 5%, up to 6%, up to 8%, or up to 10%.

[00174] 5. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises at least one of human Cot-1 DNA, a destabilizing agent, salt, and a blocker.

[00175] 6. The hybridization buffer of Hybridization Buffer Embodiment 5, wherein the hybridization buffer comprises Cot-1 DNA in an amount of at least 0.05 mg/mL, at least 0.1 mg/mL, at least 0.2 mg/mL, or at least 0.3 mg/mL.

[00176] 7. The hybridization buffer of Hybridization Buffer Embodiment 5 or 6, wherein the hybridization buffer comprises Cot-1 DNA in an amount of up to 0.1 mg/mL, up to 0.2 mg/mL, up to 0.3 mg/mL, or up to 0.5 mg/mL.

[00177] 8. The hybridization buffer of any one of Hybridization Buffer Embodiments 5 to 7, wherein the destabilizing agent comprises at least 1% (v/v), at least 5% (v/v), or at least 10% (v/v) of the hybridization buffer.

[00178] 9. The hybridization buffer of any one of Hybridization Buffer Embodiments 5 to 8, wherein the destabilizing agent comprises up to 5% (v/v), up to 10% (v/v), or up to 15% (v/v) of the hybridization buffer.

[00179] 10. The hybridization buffer of any one of Hybridization Buffer Embodiments 5 to 9, wherein the destabilizing agent comprises formamide.

[00180] 11. The hybridization buffer of any one of Hybridization Buffer Embodiments 5 to 10, wherein the hybridization buffer comprises at least 0.1 M, at least 0.2 M, at least 0.3 M, at least 0.4 M, at least 0.5 M, at least 0.6 M, at least 0.7 M, at least 0.8 M, at least 0.9 M, or at least 1 M salt.

[00181] 12. The hybridization buffer of any one of Hybridization Buffer Embodiments 5 to 11, wherein the hybridization buffer comprises up to 0.2 M, up to 0.3 M, up to 0.4 M, up to 0.5 M, up to 0.6 M, up to 0.7 M, up to 0.8 M, up to 0.9 M, up to 1 M, up to 2 M, up to 3 M, or up to 4 M salt.

[00182] 13. The hybridization buffer of any one of Hybridization Buffer Embodiments 5 to 11, wherein the salt comprises NaCl.

[00183] 14. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises a phosphate.

[00184] 15. The hybridization buffer of Hybridization Buffer Embodiment 14, wherein the hybridization buffer comprises at least 40 mM, at least 50 mM, at least 55 mM, at least 60 mM, at least 65 mM, or at least 70 mM phosphate.

[00185] 16. The hybridization buffer of Hybridization Buffer Embodiments 14 of 15, wherein the hybridization buffer comprises up to 50 mM, up to 55 mM, up to 60 mM, up to 65 mM, up to 70mM, up to 75 mM, or up to 80 mM phosphate.

[00186] 17. The hybridization buffer of any one of Hybridization Buffer Embodiments 14 to 16, wherein the phosphate comprises KH_2PO_4 - K_2HPO_4 .

[00187] 18. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises a detergent.

[00188] 19. The hybridization buffer of Hybridization Buffer Embodiment 18, wherein the hybridization buffer comprises at least 0.001% (v/v), at least 0.01% (v/v), at least 0.05% (v/v), at least 0.1% (v/v), at least 1% (v/v), or at least 5% (v/v) detergent.

[00189] 20. The hybridization buffer of Hybridization Buffer Embodiment 18 or 19, wherein the hybridization buffer comprises up to 0.01% (v/v), up to 0.05% (v/v), up to 0.1% (v/v), up to 1% (v/v), up to 5% (v/v), or up to 10% (v/v) detergent.

[00190] 21. The hybridization buffer of any one of Hybridization Buffer Embodiments 18 to 20, wherein the detergent comprises TWEEN 20.

[00191] 22. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises
0.5% to 10% dextran sulfate,
0.05 mg/mL to 0.5 mg/mL human Cot-1 DNA,
1% to 15% (v/v) formamide,
40 mM to 80 mM KH_2PO_4 - K_2HPO_4 ,
0.1 M to 4 M NaCl, and
0.001% to 10% (v/v) Tween 20.

[00192] 23. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises
1.5% Dextran Sulfate,
0.2 mg/mL human Cot-1 DNA,
10% (v/v) formamide,
66.6 mM KH_2PO_4 - K_2HPO_4 ,
0.2 mM Enhanced adaptor blockers, (0.1 mM for each adaptor)
0.8 M NaCl, and
0.04% (v/v) Tween 20.

[00193] 24. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises a blocker.

[00194] 25. The hybridization buffer of Hybridization Buffer Embodiment 24, wherein the blocker comprises a T_m -enhanced oligonucleotide.

[00195] 26. The hybridization buffer of Hybridization Buffer Embodiment 24 or 25, wherein the blocker comprises a plurality of T_m -enhancing modifications.

[00196] 27. The hybridization buffer of Hybridization Buffer Embodiment 26, wherein the plurality of modifications that increase the T_m of the blocker comprise at least one of a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap.

[00197] 28. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises a blocker,
wherein the blocker comprises an oligonucleotide;

wherein the blocker is capable of binding to an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI);

and wherein the region of the blocker capable of binding to the index region and/or UMI of the adaptor comprises:

at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases; or

universal bases and at least one non-universal base.

[00198] 29. The hybridization buffer of any one of the preceding Hybridization Buffer Embodiments, wherein the hybridization buffer comprises a blocker,

wherein the blocker comprises two non-connected oligonucleotides;

wherein the blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI);

and wherein the non-connected oligonucleotides comprise bases that do not correspond to the index region of the adaptor and/or the UMI of the adaptor.

[00199] 30. The hybridization buffer of Hybridization Buffer Embodiments 28 or 29, wherein the blocker comprises a plurality of T_m -enhancing modifications.

[00200] 31. A kit comprising the hybridization buffer of any one of the preceding Hybridization Buffer Embodiments.

Exemplary Methods of Using a Hybridization Buffer (Buffer Method Embodiments)

[00201] 1. A method comprising using a hybridization buffer comprising a crowding agent for hybrid capture.

[00202] 2. The method of Buffer Method Embodiment 1, wherein the crowding agent comprises at least one of dextran, dextran sulfate, polyethylene glycol (PEG), Ficoll, glycerol, and betaine.

[00203] 3. The method of any one of the preceding Buffer Method Embodiments, wherein the crowding agent is included in the hybridization buffer in an amount of at least 0.5% (w/v), at least 1% (w/v), at least 1.5% (w/v), at least 2% (w/v), at least 3% (w/v), or at least 4% (w/v).

[00204] 4. The method of any one of the preceding Buffer Method Embodiments, wherein the crowding agent is included in the hybridization buffer in an amount of up to 1% (w/v), up to 1.5% (w/v), up to 2% (w/v), up to 3% (w/v), up to 4% (w/v), up to 5% (w/v), up to 6% (w/v), up to 8% (w/v), or up to 10% (w/v).

[00205] 5. The method of any one of the preceding Buffer Method Embodiments, wherein the hybridization buffer comprises at least one of human Cot-1 DNA, a destabilizing agent, and salt.

[00206] 6. The method of Buffer Method Embodiment 5, wherein the hybridization buffer comprises human Cot-1 DNA in an amount of at least 0.05 mg/mL, at least 0.1 mg/mL, at least 0.2 mg/mL, or at least 0.3 mg/mL.

[00207] 7. The method of Buffer Method Embodiment 5 or 6, wherein the hybridization buffer comprises human Cot-1 DNA in an amount of up to 0.1 mg/mL, up to 0.2 mg/mL, up to 0.3 mg/mL, or up to 0.5 mg/mL.

[00208] 8. The method of any one of Buffer Method Embodiments 5 to 7, wherein the destabilizing agent comprises at least 1% (v/v), at least 5% (v/v), or at least 10% (v/v) of the hybridization buffer.

[00209] 9. The method of any one of Buffer Method Embodiments 5 to 8, wherein the destabilizing agent comprises up to 5% (v/v), up to 10% (v/v), or up to 15% (v/v) of the hybridization buffer.

[00210] 10. The method of any one of Buffer Method Embodiments 5 to 9, wherein the destabilizing agent comprises formamide.

[00211] 11. The method of any one of Buffer Method Embodiments 5 to 10, wherein the hybridization buffer comprises at least 0.1 M, at least 0.2 M, at least 0.3 M, at least 0.4 M, at least 0.5 M, at least 0.6 M, at least 0.7 M, at least 0.8 M, at least 0.9 M, or at least 1 M salt.

[00212] 12. The method of any one of Buffer Method Embodiments 5 to 11, wherein the hybridization buffer comprises up to 0.2 M, up to 0.3 M, up to 0.4 M, up to 0.5 M, up to 0.6 M, up to 0.7 M, up to 0.8 M, up to 0.9 M, up to 1 M, up to 2 M, up to 3 M, or up to 4 M salt.

[00213] 13. The method of any one of Buffer Method Embodiments 5 to 12, wherein the salt comprises NaCl.

- [00214]** 14. The method of any one of the preceding Buffer Method Embodiments, wherein the hybridization buffer comprises a phosphate.
- [00215]** 15. The method of Buffer Method Embodiment 14, wherein the hybridization buffer comprises at least 40 mM, at least 50 mM, at least 55 mM, at least 60 mM, at least 65 mM, or at least 70 mM phosphate.
- [00216]** 16. The method of Buffer Method Embodiments 14 or 15, wherein the hybridization buffer comprises up to 50 mM, up to 55 mM, up to 60 mM, up to 65 mM, up to 70 mM, up to 75 mM, or up to 80 mM phosphate.
- [00217]** 17. The method of any one of Buffer Method Embodiments 14 to 16, wherein the phosphate comprises KH_2PO_4 - K_2HPO_4 .
- [00218]** 18. The method of any one of the preceding Buffer Method Embodiments, wherein the hybridization buffer comprises a detergent.
- [00219]** 19. The method of Buffer Method Embodiment 17, wherein the hybridization buffer comprises at least 0.001% (v/v), at least 0.01% (v/v), at least 0.05% (v/v), at least 0.1% (v/v), at least 1% (v/v), or at least 5% (v/v) detergent.
- [00220]** 20. The method of Buffer Method Embodiment 18 or 19, wherein the hybridization buffer comprises up to 0.01% (v/v), up to 0.05% (v/v), up to 0.1% (v/v), up to 1% (v/v), up to 5% (v/v), or up to 10% (v/v) detergent.
- [00221]** 21. The method of any one of Buffer Method Embodiments 18 to 20, wherein the hybridization buffer comprises TWEEN 20.
- [00222]** 22. The method of any one of the preceding Buffer Method Embodiments, wherein the hybridization buffer comprises:
0.5% to 10% dextran sulfate,
0.05 mg/mL to 0.5 mg/mL Cot-1,
1% to 15% (v/v) formamide,
40 mM to 80 mM KH_2PO_4 - K_2HPO_4 ,
0.1 M to 4 M NaCl, and
0.001% to 10% (v/v) TWEEN 20.
- [00223]** 23. The method of any one of the preceding Buffer Method Embodiments, wherein the hybridization buffer comprises:
1.5% dextran sulfate,
0.2 mg/mL Cot-1,

10% (v/v) formamide,
66.6 mM KH_2PO_4 - K_2HPO_4 ,
0.8 M NaCl, and
0.04% (v/v) TWEEN 20.

[00224] 24. The method of any one of the preceding Buffer Method Embodiments, wherein the hybridization buffer comprises a blocker.

[00225] 25. The method of Buffer Method Embodiment 24, wherein the blocker is present at a concentration of at least 0.001 mM, at least 0.005 mM, at least 0.01 mM, at least 0.05 mM, or at least 0.1 mM.

[00226] 26. The method of Buffer Method Embodiment 24 or 25, wherein the blocker is present at a concentration of up to 0.01 mM, up to 0.05 mM, up to 0.1 mM, up to 0.2 mM, or up to 0.5 mM.

[00227] 27. The method of any one of the preceding Buffer Method Embodiments, wherein the method comprises forming the hybridization buffer from a first composition comprising human Cot-1 DNA and a blocker and a second composition comprising formamide and dextran sulfate.

[00228] 28. The method of any one of the preceding Buffer Method Embodiments, wherein the method comprises contacting a library with the hybridization buffer, wherein the hybridization buffer comprises a blocker.

[00229] 29. The method of Buffer Method Embodiment 28, wherein the method comprises combining samples from at least two library preparations prior to contacting the library with the blocker in the presence of the hybridization buffer.

[00230] 30. The method of Buffer Method Embodiment 29, wherein at least 1 μL , at least 2 μL , at least 3 μL , at least 5 μL , at least 10 μL , at least 15 μL , or at least 20 μL of a sample resulting from each library preparation are combined.

[00231] 31. The method of Buffer Method Embodiment 29 or 30, wherein up to 2 μL , up to 3 μL , up to 5 μL , up to 10 μL , up to 15 μL , up to 20 μL , up to 25 μL , up to 30 μL , up to 40 μL , or up to 50 μL of a sample resulting from each library preparation are combined.

[00232] 32. The method of any one of Buffer Method Embodiments 29 to 31, wherein at least 10 ng, at least 15 ng, at least 25 ng, at least 50 ng, at least 100 ng, at least 200 ng, at least 500 ng, at least 1,000 ng, or at least 5,000 ng of DNA from each library preparation are combined.

[00233] 33. The method of any one of Buffer Method Embodiments 29 to 32, wherein up to 15 ng, up to 25 ng, up to 50 ng, up to 100 ng, up to 200 ng, up to 500 ng, up to 1,000 ng, up to 5,000 ng, up to 10,000 ng, or up to 12,000 ng of DNA from each library preparation are combined.

[00234] 34. The method of any one of Buffer Method Embodiments 29 to 33, wherein the DNA concentration of each sample resulting from a library preparation being combined is at least 0.1 ng/ μ L, at least 0.3 ng/ μ L, at least 0.4 ng/ μ L, at least 0.5 ng/ μ L, at least 1 ng/ μ L, at least 2 ng/ μ L, at least 4 ng/ μ L, at least 6 ng/ μ L, at least 8 ng/ μ L, at least 10 ng/ μ L, at least 12 ng/ μ L, at least 20 ng/ μ L, at least 50 ng/ μ L, at least 60 ng/ μ L, at least 100 ng/ μ L, or at least 200 ng/ μ L.

[00235] 35. The method of any one of Buffer Method Embodiments 29 to 34, wherein the DNA concentration of each sample resulting from a library preparation being combined is up to 0.4 ng/ μ L, up to 0.5 ng/ μ L, up to 1 ng/ μ L, up to 2 ng/ μ L, up to 4 ng/ μ L, up to 6 ng/ μ L, up to 8 ng/ μ L, up to 10 ng/ μ L, up to 12 ng/ μ L, up to 15 ng/ μ L, up to 20 ng/ μ L, up to 50 ng/ μ L, up to 100 ng/ μ L, up to 120 ng/ μ L, up to 200 ng/ μ L, up to 300 ng/ μ L, or up to 400 ng/ μ L.

[00236] 36. The method of any one of Buffer Method Embodiments 29 to 35, wherein the DNA concentration after the library preparations are combined is in a range of 0.3 ng/ μ L to 400 ng/ μ L.

[00237] 37. The method of any one of Buffer Method Embodiments 29 to 36, wherein the method further comprises contacting library members with a probe, wherein the probe hybridizes to a region of interest within a library member, and wherein the probe comprises a ligand.

[00238] 38. The method of Buffer Method Embodiment 37, wherein the ligand comprises a biotin group.

[00239] 39. The method of any one Buffer Method Embodiments 29 to 38, wherein the method comprises forming a hybridization mixture comprising library members, the hybridization buffer (wherein the hybridization buffer comprises a blocker), and a probe.

[00240] 40. The method of Buffer Method Embodiment 39, wherein the hybridization mixture further comprises human Cot-1 DNA.

[00241] 42. The method of Buffer Method Embodiment 39 or 40, wherein the method comprises using a volume of hybridization mixture of up to 150 μ L, up to 140 μ L, up to

130 μL , up to 120 μL , up to 110 μL , up to 100 μL , up to 90 μL , up to 80 μL , up to 70 μL , up to 60 μL or up to 50 μL .

[00242] 42. The method of any one of Buffer Method Embodiments 39 to 41, wherein the method comprises using a volume of hybridization mixture of at least 10 μL , at least 20 μL , at least 30 μL , at least 40 μL , at least 50 μL , at least 60 μL , or at least 70 μL .

[00243] 43. The method of any one of Buffer Method Embodiments 39 to 42, wherein the DNA concentration in the hybridization mixture is at least at least 0.1 ng/ μL , at least 0.3 ng/ μL , at least 0.4 ng/ μL , at least 0.5 ng/ μL , at least 1 ng/ μL , at least 2 ng/ μL , at least 4 ng/ μL , at least 6 ng/ μL , at least 8 ng/ μL , at least 10 ng/ μL , at least 12 ng/ μL , at least 15 ng/ μL , at least 20 ng/ μL , at least 50 ng/ μL , at least 75 ng/ μL , at least 100 ng/ μL , at least 120 ng/ μL , at least 150 ng/ μL , at least 200 ng/ μL .

[00244] 44. The method of any one of Buffer Method Embodiments 39 to 43, wherein the DNA concentration in the hybridization mixture is up to 0.3 ng/ μL , up to 0.4 ng/ μL , up to 0.5 ng/ μL , up to 1 ng/ μL , up to 2 ng/ μL , up to 4 ng/ μL , up to 6 ng/ μL , up to 8 ng/ μL , up to 10 ng/ μL , up to 12 ng/ μL , up to 15 ng/ μL , up to 20 ng/ μL , up to 50 ng/ μL , up to 75 ng/ μL , up to 100 ng/ μL , up to 120 ng/ μL , up to 150 ng/ μL , up to 200 ng/ μL , or up to 500 ng/ μL .

[00245] 45. The method of any one of Buffer Method Embodiments 37 to 44, wherein the library is in contact with the probe for up to 3 days, up to 2 days, up to 24 hours, up to 20 hours, up to 16 hours, up to 12 hours, up to 6 hours, up to 3 hours, up to 2 hours, up to 90 minutes, up to 1 hour, or up to 30 minutes.

[00246] 46. The method of any one of Buffer Method Embodiments 37 to 45, wherein the library is in contact with the probe for at least 30 minutes, at least 45 minutes, at least 1 hour, at least 90 minutes, at least 2 hours, at least 3 hours, at least 6 hours, at least 12 hours, at least 16 hours, at least 20 hours, or at least 24 hours.

[00247] 47. The method of any one of Buffer Method Embodiments 39 to 46, wherein method comprises maintaining the hybridization mixture at a hybridization temperature and wherein the hybridization temperature is at least 50°C, at least 55°C, at least 56°C, at least 57°C, at least 58°C, at least 59°C, at least 60°C, at least 61°C, at least 62°C, at least 63°C, at least 64°C, at least 65°C, or at least 70°C.

[00248] 48. The method of any one of Buffer Method Embodiments 39 to 47, wherein method comprises maintaining the hybridization mixture at a hybridization temperature and

wherein the hybridization temperature is up to 56°C, up to 58°C, up to 60°C, up to 61°C, up to 62°C, up to 63°C, up to 64°C, up to 65°C, or up to 70°C.

[00249] 49. The method of any one of Buffer Method Embodiments 39 to 48, wherein the method comprises maintaining the hybridization mixture at a hybridization temperature for up to 3 days, up to 2 days, up to 24 hours, up to 20 hours, up to 16 hours, up to 12 hours, up to 6 hours, up to 3 hours, up to 2 hours, up to 90 minutes, up to 1 hour, or up to 30 minutes.

[00250] 50. The method of any one of Buffer Method Embodiments 39 to 49, wherein the method comprises maintaining the hybridization mixture at a hybridization temperature for at least 30 minutes, at least 45 minutes, at least 1 hour, at least 90 minutes, at least 2 hours, at least 3 hours, at least 6 hours, at least 12 hours, at least 16 hours, at least 20 hours, or at least 24 hours.

[00251] 51. The method of any one of Buffer Method Embodiments 37 to 50, wherein the method further comprises capturing the probe after it has hybridized using a capture means.

[00252] 52. The method of Buffer Method Embodiment 51, wherein capturing the probe comprises using a streptavidin bead.

[00253] 53. The method of any one of Buffer Method Embodiments 37 to 52, wherein the method comprises forming a capture mixture including the probe and a capture means.

[00254] 54. The method of Buffer Method Embodiment 53, wherein the method comprising maintaining the capture mixture at a capture temperature of at least 50°C, at least 55°C, at least 56°C, at least 57°C, at least 58°C, at least 59°C, at least 60°C, at least 61°C, at least 62°C, at least 63°C, at least 64°C, at least 65°C, or at least 70°C.

[00255] 55. The method of Buffer Method Embodiment 53 or 54, wherein the method comprising maintaining the capture mixture at a capture temperature of up to 56°C, up to 58°C, up to 60°C, up to 61°C, up to 62°C, up to 63°C, up to 64°C, up to 65°C, or up to 70°C.

[00256] 56. The method of any one of Buffer Method Embodiments 53 to 55, wherein the method comprises maintaining the capture mixture at a capture temperature for at least 2 minutes, at least 5 minutes, at least 10 minutes, or at least 15 minutes.

[00257] 57. The method of any one of Buffer Method Embodiments 53 to 56, wherein the method comprises maintaining the capture mixture at a capture temperature for up to 5 minutes, up to 10 minutes, up to 15 minutes, up to 20 minutes, up to 30 minutes, or up to 45 minutes.

[00258] 58. The method of any one of Buffer Method Embodiments 53 to 57, wherein the method comprises washing the captured probe.

[00259] 59. The method of any one of Buffer Method Embodiments 53 to 58, wherein the method further comprises eluting the captured library members from the capture means into an eluant.

[00260] 60. The method of any one of Buffer Method Embodiments 53 to 59, wherein the method further comprises eluting the captured library members from the probe into an eluant.

[00261] 61. The method of Buffer Method Embodiment 60, wherein at least 60 femtomoles, at least 80 femtomoles, at least 90 femtomoles, at least 100 femtomoles, or at least 150 femtomoles of DNA is eluted.

[00262] 62. The method of Buffer Method Embodiment 60 or 61, wherein up to 150 femtomoles, up to 500 femtomoles, up to 1 picomole, up to 2 picomoles, or up to 3 picomoles of DNA is eluted.

[00263] 63. The method of any one of Buffer Method Embodiments 60 to 62, wherein the DNA concentration of the eluant is at least 1.1 pM, at least 1.2 pM, at least 1.3 pM, at least 10 pM, or at least 100 pM.

[00264] 64. The method of any one of Buffer Method Embodiments 60 to 63, wherein the DNA concentration of the eluant is up to 100 pM, up to 200 pM, up to 250 pM, or up to 300 pM.

[00265] 65. The method of any one of Buffer Method Embodiments 60 to 64, wherein the DNA concentration of the eluant is in the range of 1.3 pM to 250 pM.

[00266] 66. The method of any one of Buffer Method Embodiments 37 to 65, wherein the method further comprises sequencing the library members.

[00267] 67. The method of Buffer Method Embodiment 66, wherein the method comprises amplifying the library members prior to sequencing the library members.

[00268] 68. The method of Buffer Method Embodiment 66, wherein the method does not comprise amplifying the library members prior to sequencing the library members.

[00269] 69. The method of any of Buffer Method Embodiments 24 to 68, wherein the blocker comprises a T_m -enhanced oligonucleotide.

[00270] 70. The method of any of Buffer Method Embodiments 24 to 69, wherein the blocker comprises a plurality of T_m -enhancing modifications.

[00271] 71. The method of Buffer Method Embodiment 70, wherein the plurality of modifications that increase the T_m of the blocker comprise at least one of a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap.

[00272] 72. The method of any of Buffer Method Embodiments 24 to 71, wherein the blocker comprises an oligonucleotide;
wherein the blocker is capable of binding to an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI);
and wherein the region of the blocker capable of binding to the index region and/or UMI of the adaptor comprises:
at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases; or
universal bases and at least one non-universal base.

[00273] 73. The method of any of Buffer Method Embodiments 24 to 72, wherein the blocker comprises two non-connected oligonucleotides;
wherein the blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI);
and wherein the non-connected oligonucleotides comprise bases that do not correspond to the index region of the adaptor and/or the UMI of the adaptor.

Exemplary Methods of PCR-Free Hybrid Capture (Capture Method Embodiments)

[00274] 1. A method comprising
contacting a library with a blocker in the presence of a hybridization buffer;
and contacting the library with a probe, wherein the probe hybridizes to a region of interest within a library member;
wherein the method does not comprise amplifying the library members using PCR prior to sequencing the library members.

- [00275]** 2. The method of Capture Method Embodiment 1, wherein the probe further comprises a ligand, and wherein the library members are eluted from the ligand prior to sequencing the library members.
- [00276]** 3. The method Capture Method Embodiment 2 wherein the ligand comprises biotin.
- [00277]** 4. The method of either of Capture Method Embodiments 2 or 3, the method comprising loading the library members onto a flow cell after elution from the ligand.
- [00278]** 5. The method of any one of Capture Method Embodiments 1 to 4, wherein the method further comprises capturing the probe after it has hybridized using a capture means.
- [00279]** 6. The method of Capture Method Embodiment 5, wherein the capture means comprises streptavidin.
- [00280]** 7. The method of either of Capture Method Embodiments 5 or 6, the method comprising loading the library members onto a flow cell after elution from the capture means.
- [00281]** 8. The method of Capture Method Embodiment 4 or 7, the method comprising loading the library members onto a flow cell in a volume of less than 100 μL , a volume of less than 90 μL , a volume of less than 80 μL , a volume of less than 70 μL , or a volume of less than 60 μL .
- [00282]** 9. The method of Capture Method Embodiment 4 or 7, the method comprising loading the library members onto a flow cell at a concentration of at least 1.1 pM, at least 1.2 pM, at least 1.3 pM, at least 10 pM, or at least 100 pM.
- [00283]** 10. The method of Capture Method Embodiment 4, 7, or 9, the method comprising loading the library members onto a flow cell at a concentration of up to 100 pM, up to 200 pM, up to 250 pM, or up to 300 pM.
- [00284]** 11. The method of any one of Capture Method Embodiments 4, or 7-10, the method comprising loading the library members onto a flow cell using a direct flow cell loading jig.
- [00285]** 12. The method of any one of the preceding Capture Method Embodiments, wherein the hybridization buffer comprises a crowding agent.
- [00286]** 13. The method of Capture Method Embodiment 12, wherein the hybridization buffer comprises 0.5% to 10% dextran sulfate,

0.05 mg/mL to 0.5 mg/mL human Cot-1 DNA,
1% to 15% (v/v) formamide,
40 mM to 80 mM KH_2PO_4 - K_2HPO_4 ,
0.1 M to 4 M NaCl, and
0.001% to 10% (v/v) Tween 20.

[00287] 14. The method of any one of the preceding Capture Method Embodiments, wherein the blocker comprises a T_m -enhanced oligonucleotide.

[00288] 15. The method of any one of the preceding Capture Method Embodiments, wherein the blocker comprises a plurality of T_m -enhancing modifications.

[00289] 16. The method of Capture Method Embodiment 15, wherein the plurality of modifications that increase the T_m of the blocker comprise at least one of a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap.

[00290] 17. The method of any one of the preceding Capture Method Embodiments, wherein the blocker comprises an oligonucleotide;
wherein the blocker is capable of binding to an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI);
and wherein the region of the blocker capable of binding to the index region and/or UMI of the adaptor comprises:
at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases; or
universal bases and at least one non-universal base.

[00291] 18. The method of any one of the preceding Capture Method Embodiments, wherein the blocker comprises two non-connected oligonucleotides;
wherein the blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI);

and wherein the non-connected oligonucleotides comprise bases that do not correspond to the index region of the adaptor and/or the UMI of the adaptor.

[00292] The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Embodiments

[00293] The following numbered items provide embodiments as described herein, though the embodiments recited here are not limiting.

[00294] Item 1. A hybridization buffer comprising a crowding agent and at least one of human Cot-1 DNA, a destabilizing agent, salt, and a blocker.

[00295] Item 2. The hybridization buffer of Item 1, wherein the crowding agent comprises at least one of dextran, dextran sulfate, polyethylene glycol (PEG), Ficoll, glycerol, and betaine.

[00296] Item 3. The hybridization buffer of any one of the preceding Items, wherein the hybridization buffer comprises: 0.5% to 10% dextran sulfate, 0.05 mg/mL to 0.5 mg/mL Cot-1, 1% to 15% (v/v) formamide, 40 mM to 80 mM KH_2PO_4 - K_2HPO_4 , 0.1 M to 4 M NaCl, and 0.001% to 10% (v/v) TWEEN 20.

[00297] Item 4. The hybridization buffer of any one of the preceding Items, wherein the blocker comprises a plurality of T_m -enhancing modifications.

[00298] Item 5. A method comprising using the hybridization buffer of any one of the preceding Items.

[00299] Item 6. The method of Item 5, the method comprising forming a hybridization mixture comprising library members, the hybridization buffer, human Cot-1 DNA, a blocker, and a probe.

[00300] Item 7. The method of Item 6, wherein the hybridization mixture comprises samples from at least two library preparations, wherein at least 10 ng, at least 25 ng, at least 50 ng, at least 100 ng, at least 200 ng, or at least 500 ng of DNA from each library preparation are combined.

[00301] Item 8. The method of Item 6 or Item 7, wherein the DNA concentration of the hybridization mixture is in a range of 0.1 ng/ μ L to 120 ng/ μ L.

[00302] Item 9. The method of any one of Items 6 to 8, wherein the method comprises: contacting the library members with the probe, wherein the probe hybridizes to a region of interest within a library member, and wherein the probe comprises a ligand, capturing the probe after it has hybridized using a capture means, and eluting the captured library members from the capture means into an eluant, wherein the DNA concentration of the eluant is in the range of 1.3 pM to 250 pM.

[00303] Item 10. The method of any one of Items 6 to 9, wherein the method further comprises sequencing the library members and wherein the method does not comprise amplifying the library members prior to sequencing the library members.

[00304] Item 11. The method of Item 10, wherein the method comprises loading the library members onto a flow cell using a direct flow cell loading jig.

[00305] Item 12. A blocker comprising an oligonucleotide; wherein the blocker is capable of binding to an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI); and wherein the region of the blocker capable of binding to the index region and/or UMI of the adaptor comprises: at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases; or universal bases and at least one non-universal base.

[00306] Item 13. A blocker comprising two non-connected oligonucleotides; wherein the blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI); and wherein the non-connected oligonucleotides comprise bases that do not correspond to the index region and/or the UMI of the adaptor.

[00307] Item 14. The blocker of Item 12 or 13, wherein the universal primer sequence comprises at least one of P5, P7, P5', P7', V2.A14.METS, V2.B15.METS, the complement of V2.A14.METS, and the complement of V2.B15.METS.

[00308] Item 15. The blocker of any one of Items 12 to 14, wherein the blocker comprises a modification that increases the T_m of the blocker relative to the same blocker that does not include the modification.

[00309] Item 16. The blocker of any one of Items 12 to 15, wherein the blocker comprises at least one of a DNA or RNA oligonucleotide modified to capture low GC regions; a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap.

[00310] Item 17. A method comprising contacting a library with a blocker in the presence of a hybridization buffer; and contacting the library with a probe, wherein the probe hybridizes to a region of interest within a library member; wherein the method does not comprise amplifying the library members using PCR prior to sequencing the library members.

[00311] Item 18. The method of Item 17, wherein the method comprises loading the library members onto a flow cell at a concentration of at least 1.1 pM, at least 1.2 pM, at least 1.3 pM, at least 10 pM, or at least 100 pM and at a concentration of up to 100 pM, up to 200 pM, up to 250 pM, or up to 300 pM.

[00312] Item 19. The method of Item 17 or 18, wherein the blocker comprises a plurality of T_m -enhancing modifications.

[00313] Item 20. The method of any one of Items 17 to 19, wherein the hybridization buffer comprises a crowding agent.

[00314] Item 21. The method of any one of Items 17 to 20, wherein the hybridization buffer comprises 0.5% to 10% dextran sulfate, 0.05 mg/mL to 0.5 mg/mL human Cot-1 DNA, 1% to 15% (v/v) formamide, 40 mM to 80 mM KH_2PO_4 - K_2HPO_4 , 0.1 M to 4 M NaCl, and 0.001% to 10% (v/v) Tween 20.

[00315] Item 22. The method of any one of Items 17 to 21, the method comprising loading the library members onto a flow cell using a direct flow cell loading jig.

[00316] Item 23. A kit comprising the hybridization buffer of any one of Items 1 to 4.

[00317] Item 24. A kit comprising the blocker of any one of Items 12 to 16.

EXAMPLES

Example 1 – Hybridization and Capture*Hybridization*

[00318] A non-enhanced hybridization buffer includes the following components/final concentrations in a 100 μ L hybridization reaction: human Cot-1 0.2 mg/ml, formamide 10% (v/v), KH_2PO_4 - K_2HPO_4 66.6 mM, NaCl 0.8 M, TWEEN 20 0.04% (v/v).

[00319] An enhanced hybridization buffer includes the following components/final concentrations in a 100 μ L hybridization reaction: dextran sulfate 1.5% (w/v), human Cot-1 0.2 mg/ml, formamide 10% (v/v), KH_2PO_4 - K_2HPO_4 66.6 mM, NaCl 0.8 M, TWEEN 20 0.04% (v/v).

[00320] In each case, the 100 μ L final reaction also contains: probes (with at least 500 probes and up to 500,000 probes) at concentration 125 pM per probe (with total probe concentration of at least 30 nM), and DNA samples of up to 30 μ L (50 ng – 6 μ g). If included, blockers are present at 0.2 mM.

[00321] The hybridization reaction is performed in a thermocycler or HYBEX system with accurate temperature control. After denaturing at 95°C for 5 minutes, the hybridization reaction is incubated at 58°C or 62°C for 90 minutes (total hybridization time approximately (~) 100 minutes) or up to 24 hours. Ramping at 2°C per minute starting from 95°C to 58°C or 62°C (additional 20 minutes), can be followed by an incubation at 58°C or 62°C, depending on probe design. (For the experiments described herein the samples are incubated at 62°C.).

Capture

[00322] Targeted DNA is captured using 250 μ L of streptavidin beads SMB (Streptavidin Magnetic Beads, Illumina, Inc., San Diego, CA) for each 100 μ L hybridization reaction at hybridization incubation temperature (e.g., 62°C if hybridization reaction is incubated at 62°C for 90 minutes) for 15 minutes. Targeted DNA is then washed at the same temperature (e.g., 62°C) for 5 minutes for total 4 times using wash buffer EEW (Enhanced Enrichment Wash buffer, Illumina, Inc., San Diego, CA (Tris-HCL, Deferoxamine mesylate (DFO), betaine, and TWEEN 20)). Targeted DNA is eluted using fresh denaturation solution (0.1% (v/v) TWEEN 20 (EE1) and 2 N NaOH (HP3)), neutralized with

ET2 (Elution Target Buffer 2, Illumina, Inc., San Diego, CA (Tris base, Tris Acetate)), and further amplified with a primer cocktail (PPC; sequencing adaptor primer set including 5 μ M P5 and P7 primers, Illumina, Inc.) and Enhanced PCR Mix (EPM) (Illumina, Inc., San Diego, CA) and is cleaned up using 0.9x Solid Phase Reversible Immobilization (SPRI) beads.

Example 2 – Blockers including Thymine

[00323] This Example describes blockers that include thymine in the region of the blocker that corresponds to the index and/or UMI (*see* FIG. 3D).

[00324] Using the blockers of Table 3, Hybridization and Capture are performed as described in Example 1 using enhanced hybridization buffer and 0.2 mM blocker. The resulting captured DNA is sequenced and Padded Read Enrichment is calculated using the Enrichment v3.0 BASESPACE App (Illumina, Inc., San Diego, CA).

[00325] Blockers that include a stretch of T at the index sequence positions require at least 8 or 10 modified nucleotides in the region of the blocker that does not correspond to the index to achieve high capture of on-target DNA (as measured by Padded Read Enrichment).

[00326] Oligos with a sequence complementary to the index region require fewer modified nucleotides (8 nucleotides and 10 nucleotides were tested) and showed higher Padded Read Enrichment. Results are shown in FIG. 4. With the same number of modification (8 or 10 nucleotides) in the blockers, blockers with sequence complementary to index region (the last bar) showed higher Padded Read Enrichment than the blockers with a stretch of T in the index region (labeled as “10mod with (T)8” in FIG. 4).

Table 3

SEQ ID NO	Name	Description	Sequences	Modification
104	NEXTERA Blocker1 (15016424) i5	Illumina NEXTERA i5 blocker	5'- AATGATACGGCGACCACCGAGATCTACACTTTTTTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG/3ddC/	no mod
105	NEXTERA Blocker2 (15016425) i7	Illumina NEXTERA i7 blocker	5'- CAAGCAGAAGACGGCATACGAGATTTTTTTTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG/3ddC/	no mod
106	TiBNA1	NEXTERA Blocker1_3XCG	5' - AATGATACGGCGACCACCGAGAT+CTACACTTTTTTTTT+CGTCGGCAGCGTCAGATGTGTATAAGAGACAG/3ddC/	3 mod
107	TiBNA2	NEXTERA Blocker2_3XCG	5' - CAAGCAGAAGACGGCATACGAGATTTTTTTTTGT+CTCGTGGGCT+CGGAGATGTGTATAAGAGACAG/3ddC/	3 mod
108	TiBNA3	NEXTERA Blocker1_6XCG	5' - AATGATACGGCGACCACCGAGA+TCTACACTTTTTTTTTTCG+TCGG+CAGCG+TCAGATG+TGTATAAGAGACAG/3ddC/	6mod
109	TiBNA4	NEXTERA Blocker2_6XCG	5' - CAAGCAGAAGACGGCA+TACGAGATTTTTTTTG+TCT+CGTGGGC+TCGGAGATG+TGTATAAGAGACAG/3ddC/	6mod
110	TiBNA5	NEXTERA Blocker1_10XCG	5' - AATGATA+CGGCGA+CCAC+CGAGA+TCTA+CACTTTTTTTTTTCG+TCGG+CAGCG+TCAGATG+TGTATAAGAGACAG/3ddC/	10mod
111	TiBNA6	NEXTERA Blocker2_10XCG	5' - CAAG+CAGAAGA+CGGCA+TACGA+GATTTTTTTTG+TCT+CGTGGGC+TCGG+AGATG+TGTATAAGAGACAG/3ddC/	10mod
112	TiS517	SSpecificBlocker 517	5' - AATGATACGGCGACCACCGAGA+TCTA+CACG+CGTA+AGATCG+TCGG+CAGCG+TCAGATG+TGTATAAGAGACAG/3ddC/	10mod
113	TiS701	SSpecificBlocker 701	5' - CAAGCAGAAGA+CGGCA+TACGAGAT+CGCCCTTAG+TCT+CGTGGGC+TCGGAGAGATGTGTATAAGAGACAG/3ddC/	8mod

Table 4

SEQ ID NO	Name	Description	Sequences	Modification
114	BL007	TSHTLNA15G	5'- AATGATACGGCGACCACCGAGA+GAT+CTA+CAC/ideoxyl//ideoxyl//ideoxyl//ideoxyl//+G/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/	G mod in Index
115	BL008	TSHTLNA16G	5'- CAAGCAGAAGACGG+CAC+ACG+AGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//+G/ideoxyl//	G mod in

116	BL009	TSHTLNA15N	ideoxyl//ideoxyl//ideoxyl//+GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/	Index N in Index
117	BL010	TSHTLNA16N	5'- AATGATACGGCGACAC+CGA+GAT+CTA+CAC/ideoxyl//ideoxyl//ideoxyl//N/ideoxyl// ideoxyl//ideoxyl//ideoxyl//ideoxyl//ACAC+TCT+TTC+CCT+ACA+CGA+CGC+TCT+TCC+GAT+C/3ddC/ 5'- CAAAGCAGAAAGACGGG+CAT+ACG+AGA+T/ideoxyl//ideoxyl//ideoxyl//N/ideoxyl// ideoxyl//ideoxyl//ideoxyl//+GTG+ACT+GGA+GTT+CAG+ACG+TGTGCT+CTT+CCG+ATC/3ddC/	Index N in Index

Table 5

SEQ ID NO	Name	Description	Sequences	Modification
118	BN023	P5LNA	5'- AATGA+TAC+GGC+GAC+CAC+CGA+GAT+CTA+CAC/3SpC3/	8mod
119	BN024	P7LNA	5'- CAAGC+AGA+AGA+CGG+CAT+ACG+A+GA+T/3SpC3/	8mod
120	BN025	TiLNAS1	5'- TCGTC+GGC+AGC+GTC+AGA+TGT+GTA+TAA+GAG+ACA+G/3SpC3/	10mod
121	BN026	TiLNAS2	5'- GTCT+CGT+GGGC+TCG+GAG+ATG+TGT+ATA+GAG+ACA+G/3SpC3/	10mod
122	BN027	TiLNA17	5'- GCGCAC+CAC+CGAGA+TCT+ACA+C/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +TCG+TCG+GCA+GCG+TCA+GATG+TG/3SpC3/	
123	BN028	TiLNA18	5'- GGCA+TAC+GAGA+T/ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl//ideoxyl// +GTC+TCG+TGG+GC+TCGG+AGA+TGTG+TATAA+GAG/3SpC3/	

Example 3 – Blockers Including Universal Bases and Modified Bases

[00327] Using 0.2 mM of the blockers of Table 4 in enhanced hybridization buffer, Hybridization and Capture is performed as described in Example 1. As shown in Table 4, the region of the blocker corresponding to the index region includes universal bases (deoxyinosine) and an LNA-modified guanine or a random nucleotide.

[00328] Without wishing to be bound by theory, it is believed that including a modified G or random nucleotide at every third base of the blocker sequence corresponding to the index region (for example at the third and/or the fifth positions (from 5' end) of the blocker sequence corresponding to the index region, as shown in one embodiment in FIG. 5, or at the second and/or the fourth positions of the blocker sequence corresponding to the index region) improves affinity of the blocker for a library member.

Example 4 – Split Blockers

[00329] This Example shows split LNA blockers work well with a library that included an 8-nucleotide index.

[00330] Using the blockers of Table 5, Hybridization and Capture are performed as described in Example 1 using enhanced hybridization buffer. As shown in Table 5, the blockers TiLNAS1 (BN025), TiLNAS2 (BN026), P5LNA (BN023), and P7LNA (BN024) do not include a region corresponding to the index region. P5LNA (BN023), and P7LNA (BN024) each include 8 LNA-modified bases. TiLNAS1 (BN025) and TiLNAS2 (BN026) each include 10 LNA-modified bases. TiLNA17 (BN027) and TiLNA18 (BN028) include universal bases (deoxyinosine) in the region of the blocker corresponding to the index region but include shortened regions of the blocker corresponding to the other regions of the adaptor.

[00331] The resulting captured DNA is sequenced and Padded Read Enrichment is calculated using the Enrichment v3.0 BASESPACE App (Illumina, Inc., San Diego, CA). A diagram of the blockers is shown in FIG. 6A & FIG. 3E – FIG. 3I; results are shown in FIG. 6B. Sample “Split 4 pc” contains TiLNAS1 (BN025), TiLNAS2 (BN026), P5LNA (BN023), and P7LNA (BN024) of Table 5; sample “Inner” contains TiLNAS1 (BN025) and TiLNAS2 (BN026) in Table 5; sample “Outer” contains P5LNA (BN023) and P7LNA (BN024) in Table 5; sample Short8nt includes TiLNA17 (BN027) and TiLNA18 (BN028) in Table 5. Two two-piece blockers (“split blockers”) including BN023, BN024, BN025,

and BN026 work as well as XGEN Blocking Oligos (Integrated DNA Technologies, Coralville, IA), as measured by Padded Read Enrichment; experiments using a blocker that blocked only part of the adaptor do not work as well.

Example 5 – Hybridization Buffer

[00332] This Example shows comparisons of on-target capture using non-enhanced hybridization buffer and enhanced hybridization buffer.

[00333] Hybridization and Capture are performed as described in Example 1 using XGEN Blocking Oligos (Integrated DNA Technologies, Coralville, IA) and enhanced hybridization buffer including the dextran sulfate concentrations indicated in FIG. 7A. The resulting captured DNA is sequenced and Padded Read Enrichment is calculated using the Enrichment v3.0 BASESPACE App (Illumina, Inc., San Diego, CA). Results are shown in FIG. 7A. Inclusion of dextran sulfate allows for fast hybridization (60 minutes to 90 minutes) in a large volume (100 μ L) of hybridization buffer.

[00334] Enrichment using 100 μ L hybridization buffer volume is evaluated using four different probe panels: Coding Exome Oligos (CEX) (Illumina, Inc., San Diego, CA), IDT Exome Research Panel (IDT Exome) (Integrated DNA Technologies, Coralville, IA), TRUSIGHT Cancer Panel (Cancer) (Illumina, Inc., San Diego, CA), and TRUSIGHT One Sequencing Panel (TSO) (Illumina, Inc., San Diego, CA). Hybridization in three hybridization buffers is tested: the IDT buffer from the XGEN LOCKDOWN kit (Integrated DNA Technologies, Coralville, IA), and hybridization buffer with and without dextran sulfate, as described in Example 1. Results are shown in FIG. 7B. Enhanced hybridization buffer provides similar or higher on-target results as compared to the IDT buffer for all four probe panels (ranging from 4,000 to 450,000 probes).

[00335] Two key enrichment metrics (Padded Read Enrichment and Uniformity of Coverage) for the four panels (CEX, IDT Exome, Cancer, and TSO) are obtained at different hybridization incubation times and with and without an additional temperature ramping step using Illumina enhanced hybridization buffer with IDT XGEN blockers. Results are shown in FIG. 7C. The dotted black line in the left panel indicates the result of hybridization in buffer without dextran sulfate.

[00336] Three runs of 12plex library input are pooled by volume to a total of 30 μ L and are enriched using Illumina enhanced hybridization buffer with IDT XGEN blockers.

Samples are run on various Illumina, Inc., sequencers (NOVASEQ, NEXTSEQ, and ISEQ). No sample concentration step is required. Results are shown in FIGs. 7D-7F. The results show that when using an enhanced hybridization buffer including dextran sulfate, up to 30 μ L of samples can be accommodated in a hybridization reaction (e.g., single 15 μ L sample or a 12-plex run at 2.5 μ L each). The use of an enhanced hybridization buffer allows up to 12 samples to be enriched in one reaction without using a speed vacuum, centrifugation column, or SPRI beads to further reduce pool volume.

Example 6 – Improved Adaptors + Enhanced Hybridization Buffer

[00337] Enrichment performance of an IDT exome panel (Cat# 1056114, Integrated DNA Technologies, Coralville, IA) is tested using: (1) IDT buffer (17 μ L) from the XGEN LOCKDOWN kit (Integrated DNA Technologies, Coralville, IA); (2) non-enhanced hybridization buffer with non-enhanced adaptor blockers (NEXTERA Blocker1 i5 (BN001) and NEXTERA Blocker2 i7 (BN002) in Table 2) in a small (11 μ L) reaction volume (TiE0); (3) non-enhanced hybridization buffer with enhanced adaptor blockers (XGEN blockers) in a small (11 μ L) reaction volume (TiE3); (4) non-enhanced hybridization buffer with enhanced adaptor blockers (XGEN blockers) in a large (100 μ L) reaction volume (TiE4); and (5) enhanced hybridization buffer with enhanced adaptor blockers (XGEN blockers) in a large (100 μ L) reaction volume (TiE9). Results are shown in FIG. 8A and demonstrate that enrichment performance is improved when using modified blockers, a wash temperature increase, and/or a crowding agent (dextran sulfate) in the hybridization buffer.

[00338] Enrichment performance (as measured using Padded Read Enrichment) is evaluated in nine probe panels using an IDT enrichment assay (Catalog No. 1080584, Integrated DNA Technologies, Coralville, IA), and in an Illumina enrichment assay with enhanced hybridization buffer. High Padded Read Enrichment is achieved in 100 μ L reaction volume with various probe panels (from 500 – 450,000 probes). Probe panels include: (1) XGEN Exome Research Panel (IDT Exome) (Catalog No. 1056114, Integrated DNA Technologies, Coralville, IA); (2) Twist Human Core Exome (Twist Bioscience, Inc., Catalog No. 100254); (3) Coding Exome Oligos (CEX) (Catalog No. 15034575, Illumina, Inc., San Diego, CA); (4) TruSight One (Illumina Inc, San Diego, CA, Catalog No. FC-141-1007; Probe Catalog No. 15046658); (5) TruSight One Expanded (Illumina, Inc San Diego, Catalog No. FC-141-2007; Probe Catalog No. 20015656); (6) TruSight Cancer

(Illumina, Inc., San Diego, CA, Catalog No. FC-121-0202; Probe Catalog No. 15035642); (7) TruSight Cardio (Illumina, Inc., San Diego, CA, Catalog No. FC-141-1010; Probe Catalog No. 15069654); (8) TruSight RNA Fusion Panel (Illumina Inc, San Diego, CA, Catalog No. 20000906); (9) Oncology DNA Probes Master Pool (OPD1) (Illumina, Inc., Catalog No. OP-101-1004; Probe Catalog No. 20001565). Results are shown in FIG. 8B. The white bar (column 1) shows the performance of IDT exome panel in IDT lockdown enrichment workflow with Nextera Rapid Capture library preparation (Illumina, Inc., San Diego, CA). Gray bars show the performance of the panels with the Illumina Enrichment Assay with enhanced hybridization buffer and IDT xGEN adaptor blockers. The black, horizontal lines show comparative results that are obtained with corresponding panels using a Twist enrichment assay (Twist Bioscience, Inc.) (column 3), Nextera Rapid Capture library preparation (columns 4-8), or the TruSight Tumor 170 workflow with the OPD1 panel (column 10). Performance of each panel in the Illumina Enrichment Assay with enhanced hybridization buffer is evaluated in at least three independent experiments; the error bars indicate standard deviation.

[00339] Somatic variants are detected by a single-hybridization enrichment protocol using enhanced hybridization buffer and XGEN blockers for a 12-gene, 535-probe panel to enrich libraries generated from the Horizon Discover HD701 quantitative multiplex (QM) DNA standard. Data presented in FIG. 8C shows the expected variant frequency (x-axis) versus called variant frequency (y-axis). Each panel in FIG. 8C represents different concentrations of high affinity adaptor blockers (IDT XGEN adaptor blockers, 0.1x (0.02 mM) and 0.5x (0.1 mM) and a negative control (no high affinity adaptor blockers). Each point represents a somatic variant call, colored by read depth. The dashed red line represents a predicted perfect correlation between known variant frequencies and called variant frequencies. The blue and gray shading represent the best fit line and 95% confidence interval, respectively, for called variant frequencies compared to known variant frequencies. The expected variant frequency (x-axis) correlates with the known (called) variant frequency (y-axis) for the IDT XGEN adaptor blockers (0.1X (0.02 mM), 0.5X (0.1 mM)) but does not correlate for a negative control (no high affinity adaptor blockers)).

Example 7A – PCR-Free Hybrid Capture using TRUSEQ Adaptor Ligated Libraries

[00340] This Example shows PCR-free Hybrid Capture.

[00341] Hybridization is performed using the XGEN LOCKDOWN reagents (Integrated DNA Technologies, Coralville, IA), according to package instructions, with the following modifications. Targeted DNA is made using a TRUSEQ adaptor ligation approach and is captured using 100 μ L DYNABEADS M-270 Streptavidin (Integrated DNA Technologies, Coralville, IA). The hybridization is only incubated for 30 minutes instead of the recommended 4 hours. The following protocol is then followed:

1. Incubate the beads with 10 μ L of 0.1 N NaOH, denaturing the captured library from the probes and releasing it into solution.
2. Remove the captured library solution from the beads (using a magnet) and neutralize with 10 μ L of 200 mM Tris.HCl pH 7.0.
3. (optionally) Add denatured PhiX Control v3 (Illumina, Inc., San Diego, CA) to sample, up to 7.5 μ L of resuspension buffer (RSB) (Illumina, Inc., San Diego, CA).
4. Add 27.5 μ L 2x HT1 (Hybridization buffer, Illumina, Inc., San Diego, CA)

[00342] The resulting sample (in 55 μ L 1x HT1 buffer) is loaded directly onto a flow cell using a pipette or using a flow cell loading jig as described, for example, in U.S.

Provisional Pat. Application No. 62/564,466, filed on September 28, 2017. Results are shown in Table 6A. The control (column 1) is run using the same conditions as the PCR free methods except that PCR is performed after the capture. Initial attempts at running a PCR-free enrichment are shown in column 2 and show that too few reads resulted in failure to reach an acceptable level of coverage for an exome. Only ~18x mean target coverage was achieved whereas the goal is to exceed ~50x. For the second attempt (column 3), a direct flow cell loading jig was used, and shows increased DNA input into the hybridization improved the sequenced yield of the PCR-free enrichment method to ~140x, far greater than the ~40x goal. Down sampling the PCR-free data to 70 million reads (column 4) results in metrics that are similar (or even superior) to the control.

Example 7B – PCR free Hybrid Capture using Bead-Based NEXTERA libraries Improves Exome Quality from Short Hybridization Times

[00343] A controlled experiment is performed using similar conditions as in Example 7A, but using libraries that are generated using a bead-based tagmentation library preparation method, and using corresponding blocking oligonucleotides. In this experiment, both hybridization duration and PCR amplification are variables. The recommended 4 hours

hybridization reaction protocol with PCR post capture (in total ~7.5 hours, see IDT XGEN protocol) with sequencing using a NEXTSEQ instrument without direct flow cell loading is used as a control (a schematic of this workflow is shown in FIG. 9B), and is compared to two conditions using only 30-minute hybridizations (with and without PCR post capture) and with direct flow cell loading (FIG. 9C). Shortening the hybridization duration and removing PCR enable an enrichment protocol to be carried out in only ~2 hrs. A schematic of this workflow with the shortened hybridization time and without PCR is shown in FIG. 9D.

[00344] The results are shown in Table 6B. All data is down-sampled to ~23 million clusters. In the first column (control), data indicates a good quality Exome but with long ~7.5hr workflow. When the hybridization is shortened to 30 minutes, the protocol duration is reduced in total to ~4hrs (column 2); however, the number of PCR duplicates increases in comparison to the control. Increased PCR duplicates also reduces the diversity of the library and corresponding coverage at ~20x. When PCR is removed from the 30 minute workflow (column 3), the PCR duplicates are reduced considerably and the diversity increases to a level above that of the control, with a far shorter workflow (2 hours vs. 7.5 hours). This example highlights that the removal of the PCR can improve the quality of hybridization capture reactions by minimizing the duplication of templates during the PCR.

Table 6A

	Control	PCR free	PCR free + direct load	PCR free + direct load
Turnaround time	4hrs	2hrs 17m	2hrs 17m	2hrs 17m
Reads	70,000,000	17,090,392	132,402,247	70,026,594
Downsampled	yes	no	no	yes
Padded Read Enrichment	88%	85%	86%	86%
Uniformity of coverage (Pct > 0.2*mean)	99%	98%	99%	99%
Mean Target Coverage	76.6	17.9	140.3	81.7
Mean Target Coverage (deduped)	60.9	15.8	98.3	64.1
SNVs in target	26,585	21,354	26,758	26,629
Indels in target	2,082	1,078	2,638	2,348

Table 6B

	4hr hyb	30 min hyb	30 min hyb (PCR free)
Hybridization duration	4hrs	30m	30m
PCR after enrichment	YES	YES	No
Total Enrichment TAT	7.5hrs	4hrs	2hrs
Padded Read Enrichment	92.42 ± 1%	87.03 ± 0.35%	84.72 ± 0.76%
Uniformity	98.76 ± 0.14%	98.67 ± 0.04%	98.44 ± 0.13%
% Duplicates	9.015 ± 0.69%	16.04 ± 0.52%	6.02 ± 0.42%
HS library Size	102,789,918 ± 11,527,768	50,909,151 ± 1,527,235	141,515,722 ± 7,601,326
Mean Region Coverage Depth	45.4 ± 1.13	39 ± 0.14	42.2 ± 0
Target Coverage at 20x	89.98 ± 0.91%	85.04 ± 0.22%	87.40 ± 0.77%
SNV recall	95.91 ± 2.74%	97.26 ± 0.0%	97.59 ± 0.24%
SNV precision	99.63 ± 0.05%	99.4 ± 0.05%	99.38 ± 0.01%
Indel Recall	80.87 ± 5.82%	82.91 ± 0.68%	85.68 ± 0.08%
Indel Precision	91.92% ± 0.94%	91.01 ± 0.83%	94.49 ± 0.01%

Example 8 - Blocker Comparisons

[00345] Using 0.2 mM of the blockers BN001 and BN002; BX003 and BX007; BN007 and BN008; BN005 and BN006; or BN023, BN024, BN025, and BN026 (as described in Table 2A) in enhanced hybridization buffer, Hybridization and Capture are performed as described in Example 1. “Non-modified” blockers BN001 and BN002 include thymines in the region of the blocker that corresponds with the index region of the adaptor and bases complementary to the adaptor in the regions of the blocker that correspond with the non-index region of the adaptor; blockers BN001 and BN002 include no modified bases and include a 3'-ddC terminal group. Blockers BX003 and BX007 are modified versions of BN001 and BN002 each having two AP-dC-CE phosphoramidite (G-Clamp) substitutions in the regions of the blocker that correspond with the non-index region of the adaptor. Blockers BN007 and BN008 are modified versions of BN001 and BN002 with universal bases (deoxyinosines) in the region of the blocker that corresponds with the index region of the adaptor and BNA-modified bases in the regions of the blocker that correspond with the non-index region of the adaptor. Blockers BN023 and BN025 are modified versions of the regions of BN001 that correspond with the non-index region of the adaptor and include LNA-modified bases; the blockers also include 3'-spacer C3 instead of 3'-ddC. Blockers BN024 and BN026 are modified versions of the regions of BN002 that correspond with the non-index region of the adaptor and include LNA-modified bases; the blockers also include 3'-spacer C3 instead of 3'-ddC.

[00346] The resulting captured DNA is sequenced and Padded Read Enrichment, a measure of the amount of on-target DNA captured, is calculated. Results are shown in FIG. 10 and indicate that various modifications of the “non-modified” blockers improve the Padded Read Enrichment in hybridization assays.

Example 9 - Split Blocker Comparisons

[00347] Using 0.2 mM BN001 and BN002; or BN023, BN024, BN025, and BN026 (as described in Table 2A) in enhanced hybridization buffer, Hybridization and Capture are performed as described in Example 1. The corresponding adaptor includes an 8-nucleotide index or a 10-nucleotide index. The resulting captured DNA is sequenced, and Padded Read Enrichment is calculated. Results are shown in FIG. 11 and indicate that using “split blockers” (that is, blockers that do not include bases that correspond with the index region

of the adaptor) allow the same blockers to be used with adaptors having various index sequences and index lengths. These results indicate that split blockers may be used in situations where it would be disadvantageous to accommodate a change in index designs.

[00348] The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

What is claimed is:

1. A hybridization buffer comprising a crowding agent and at least one of human Cot-1 DNA, a destabilizing agent, salt, and a blocker.
2. The hybridization buffer of claim 1, wherein the crowding agent comprises at least one of dextran, dextran sulfate, polyethylene glycol (PEG), Ficoll, glycerol, and betaine.
3. The hybridization buffer of claim 1 or claim 2, comprising a crowding agent and a blocker.
4. The hybridization buffer of claim 1 or claim 2, comprising a crowding agent, human Cot-1 DNA, a blocker, a destabilizing agent, a buffer, salt, and a detergent.
5. The hybridization buffer of any one of claims 1 to 4, wherein the hybridization buffer comprises a buffer and the buffer is a phosphate buffer.
6. The hybridization buffer of any one of claims 1 to 5, wherein the hybridization buffer comprises salt and the salt is NaCl or sodium citrate.
7. The hybridization buffer of any one of claims 1 to 6, wherein the hybridization buffer comprises a destabilizing agent and the destabilizing agent is formamide or urea or a mixture thereof.
8. The hybridization buffer of any one of claims 1 to 7, wherein the hybridization buffer comprises a detergent and the detergent is TWEEN 20, TWEEN 80, or sodium dodecyl sulfate (SDS).
9. The hybridization buffer of claim 1, wherein the hybridization buffer comprises:
 - 0.5% to 10% dextran sulfate,
 - 0.05 mg/mL to 0.5 mg/mL human Cot-1 DNA,
 - 1% to 15% (v/v) formamide,

40 mM to 80 mM KH_2PO_4 - K_2HPO_4 ,
0.1 M to 4 M NaCl, and
0.001% to 10% (v/v) TWEEN 20.

10. The hybridization buffer of any one of claims 1 to 9, wherein the hybridization buffer comprises a blocker and the blocker comprises at least one of:

- (a) a plurality of T_m -enhancing modifications;
- (b) a plurality of modifications that increase the T_m of the blocker comprising at least one of a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap;
- (c) an oligonucleotide; wherein the blocker is capable of binding to an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI); and wherein the region of the blocker capable of binding to the index region and/or UMI of the adaptor comprises: at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases; or universal bases and at least one non-universal base; or
- (d) two non-connected oligonucleotides; wherein the blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI); and wherein the non-connected oligonucleotides comprise bases that do not correspond to the index region and/or the UMI of the adaptor.

11. A method of preparing an enriched library comprising forming a hybridization mixture comprising library members, a hybridization buffer of any one of claims 1 to 10, and a probe to form a hybridization mixture, under conditions sufficient for the probe to hybridize to a region of interest within a library member, wherein the probe comprises a ligand.

12. The method of claim 11, comprising:
capturing the probe after it has hybridized using a capture means, and
eluting the captured library members from the capture means into an eluant comprising enriched library members, wherein the DNA concentration of the eluant is in the range of 1.3 pM to 250 pM.
13. The method of claim 11 or claim 12, wherein the hybridization mixture comprises samples from at least two library preparations, wherein at least 10 ng, at least 25 ng, at least 50 ng, at least 100 ng, at least 200 ng, or at least 500 ng of DNA from each library preparation are combined.
14. The method of any one of claims 11 to 13, wherein the DNA concentration of the hybridization mixture is in a range of 0.1 ng/ μ L to 120 ng/ μ L.
15. The method of any one of claims 11 to 14, comprising sequencing the enriched library members and wherein the method does not comprise amplifying the library members prior to capturing the library members.
16. The method of claim 15, wherein the sequencing occurs on a flow cell and the method comprises loading the enriched library members onto the flow cell at a concentration of at least 1.1 pM, at least 1.2 pM, at least 1.3 pM, at least 10 pM, or at least 100 pM and at a concentration of up to 100 pM, up to 200 pM, up to 250 pM, or up to 300 pM.
17. The method of claim 16, comprising loading the enriched library members onto a flow cell using a direct flow cell loading jig.
18. A kit comprising the hybridization buffer of any one of claims 1 to 10 and instructions for use.
19. A blocker comprising an oligonucleotide; wherein the blocker is capable of binding to an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI); and wherein the region of the

blocker capable of binding to the index region and/or UMI of the adaptor comprises: at least three thymine bases, at least four thymine bases, at least five thymine bases, at least six thymine bases, at least seven thymine bases, at least eight thymine bases, at least nine thymine bases, or at least ten thymine bases; or universal bases and at least one non-universal base.

20. A blocker comprising two non-connected oligonucleotides; wherein the blocker is capable of binding to at least a portion of an adaptor, wherein the adaptor comprises a universal primer sequence and at least one of an index region or a unique molecular identifier (UMI); and wherein the non-connected oligonucleotides comprise bases that do not correspond to the index region and/or the UMI of the adaptor.

21. The blocker of claim 19 or claim 20, wherein the universal primer sequence comprises at least one of P5, P7, P5', P7', V2.A14.METS, V2.B15.METS, the complement of V2.A14.METS, and the complement of V2.B15.METS.

22. The blocker of any one of claims 19 to 21, wherein the blocker comprises a modification that increases the T_m of the blocker relative to the same blocker that does not include the modification.

23. The blocker of any one claims 19 to 22, wherein the blocker comprises at least one of a DNA or RNA oligonucleotide modified to capture low GC regions; a crosslinked oligonucleotide; a modified 5-methyl deoxycytidine (5-methyl-dc); 2,6-diaminopurine; a locked nucleic acid (LNA); a bridged nucleic acid (BNA); a tricyclic nucleic acid; a peptide nucleic acid (PNA); a C5-modified pyrimidine base; a propynyl pyrimidine; a morpholino; a phosphoramidite; and a 5'-Pyrene cap.