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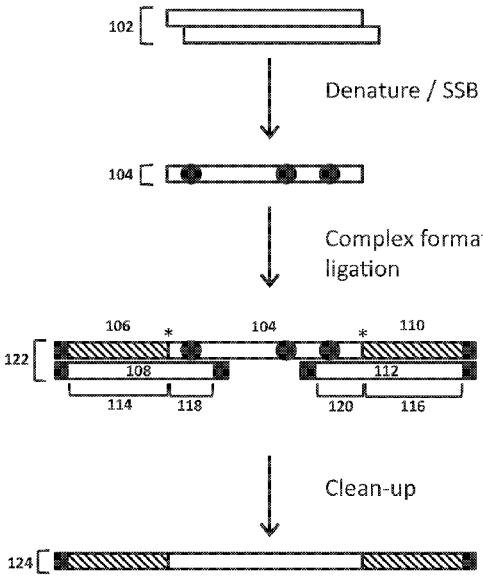
(54) Title: METHODS OF PRODUCING NUCLEIC ACID LIBRARIES AND COMPOSITIONS AND KITS FOR PRACTICING SAME

FIG. 1



(57) Abstract: Provided are methods of producing nucleic acid libraries. The methods include combining single-stranded nucleic acid binding protein-bound single-stranded nucleic acid (SSB-bound ssNA), an adapter oligonucleotide, and a splint oligonucleotide, to form complexes including the splint oligonucleotide hybridized to a terminal region of the SSB-bound ssNA and to the adapter oligonucleotide. An end of the first adapter oligonucleotide is adjacent to an end of the first terminal region of the SSB-bound ssNA, and the methods may further include covalently linking the adjacent ends. Also provided are compositions and kits that find use, e.g., in practicing the methods of the present disclosure.

METHODS OF PRODUCING NUCLEIC ACID LIBRARIES AND COMPOSITIONS AND KITS FOR PRACTICING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 62/681,524, filed June 6, 2018, which application is incorporated herein by reference in its entirety.

INTRODUCTION

Nucleic acid sequencing has become an increasingly important area of genetic research, with uses in diagnostic and other applications. In general, nucleic acid sequencing consists of determining the order of nucleotides for a nucleic acid such as a fragment of RNA or DNA. Relatively short sequences are typically analyzed, and the resulting sequence information may be used in various bioinformatics methods to align fragments against a reference sequence or to logically fit fragments together so as to reliably determine the sequence of much more extensive lengths of genetic material from which the fragments were derived. Automated, computer-based examination of characteristic fragments have been developed, and have been used more recently in genome mapping, analysis of genetic variation between individuals, identification of genes and their function, and the like.

Several methods employed for high throughput DNA sequencing rely on a universal amplification reaction, whereby a DNA sample is randomly fragmented, then treated such that the ends of the different fragments all contain the same DNA sequence. Fragments with universal ends can be amplified in a single reaction with a single pair of amplification primers. The addition of universal priming sequences onto the ends of targets to be amplified by PCR can be achieved by a variety of methods. For example, a universal primer with a universal sequence at its 5′ end and a degenerate sequence at its 3′ end can be used to amplify fragments randomly from a complex target sequence or a complex mixture of target sequences. The degenerate 3′ portion of the primer anneals at random positions on DNA and can be extended to generate a copy of the target that has the universal sequence at its 5′ end.

Alternatively, adapters that contain universal priming sequences can be ligated onto the ends of the target sequences. One or more adapters may be used in a ligation reaction with target sequences. Drawbacks associated with current methods for preparing nucleic acid sequencing libraries via ligation of one or more adapter sequences for universal amplification are the time and expense required by such methods.

SUMMARY

Provided are methods of producing nucleic acid libraries. The methods include combining single-stranded nucleic acid binding protein-bound single-stranded nucleic acid (SSB-bound ssNA), an adapter oligonucleotide, and a splint oligonucleotide, to form complexes including the splint oligonucleotide hybridized to a terminal region of the SSB-bound ssNA and to the adapter oligonucleotide. An end of the first adapter oligonucleotide is adjacent to an end of the first terminal region of the SSB-bound ssNA, and the methods may further include covalently linking the adjacent ends. Also provided are compositions and kits that find use, e.g., in practicing the methods of the present disclosure.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Schematic illustration of a method of producing a nucleic acid library according to one embodiment of the present disclosure.

FIG. 2. Comparison of an example method of the present disclosure to other methods for degraded DNA (top panel) and modern DNA (bottom panel).

FIG. 3. Hair DNA length distribution. Left panel shows the observed length of template molecules produced by the Santa Cruz method (SRL3) from modern hair DNA. As expected for DNA in hair shafts, the lengths of intact molecules is generally short. A similar length distribution is shown in the right panels (SRL4).

FIG. 4. Estimated library complexity (number of unique molecules) in SRL3 and SRL4.

FIG. 5. Library complexity comparison. Sequencing libraries were made using an example method of the present disclosure, SS2.0, BEST, and forked adapter ligation. Library complexity (the number of unique molecules in the library) was estimated from several million reads using Preseq (left) or via qPCR (right) in triplicate experiments. The example method of the present disclosure converts 2 to 3 times more of the extract DNA into sequencing libraries than the next best protocol, SS2.0.

DETAILED DESCRIPTION

Provided are methods of producing nucleic acid libraries. The methods include combining single-stranded nucleic acid binding protein-bound single-stranded nucleic acid (SSB-bound ssNA), an adapter oligonucleotide, and a splint oligonucleotide, to form complexes including the splint oligonucleotide hybridized to a terminal region of the SSB-bound ssNA and to the adapter oligonucleotide. An end of the first adapter oligonucleotide is adjacent to an end of the first terminal region of the SSB-bound ssNA, and the methods

may further include covalently linking the adjacent ends. Also provided are compositions and kits that find use, e.g., in practicing the methods of the present disclosure.

Before the methods, compositions and kits of the present disclosure are described in greater detail, it is to be understood that the methods, compositions and kits are not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the methods, compositions and kits will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the methods, compositions and kits. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the methods, compositions and kits, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the methods, compositions and kits.

Certain ranges are presented herein with numerical values being preceded by the term “about.” The term “about” is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods, compositions and kits belong. Although any methods, compositions and kits similar or equivalent to those described herein can also be used in the practice or testing of the methods, compositions and kits, representative illustrative methods, compositions and kits are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the materials and/or methods in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing

date and should not be construed as an admission that the present methods, compositions and kits are not entitled to antedate such publication, as the date of publication provided may be different from the actual publication date which may need to be independently confirmed.

It is noted that, as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

It is appreciated that certain features of the methods, compositions and kits, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the methods, compositions and kits, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments are specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and explicitly disclosed, to the extent that such combinations embrace operable processes and/or compositions. In addition, all sub-combinations listed in the embodiments describing such variables are also specifically embraced by the present methods, compositions and kits and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present methods. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

METHODS

As summarized above, the present disclosure provides methods of producing nucleic acid libraries. The methods include contacting single-stranded nucleic acid (ssNA) with single-stranded nucleic acid binding protein (SSB) to produce SSB-bound ssNA. The methods further include combining the SSB-bound ssNA, a first adapter oligonucleotide, and a first splint oligonucleotide including an SSB-bound ssNA hybridization region and a first adapter oligonucleotide hybridization region. The combining results in the formation of

complexes including the first splint oligonucleotide hybridized to a terminal region of the SSB-bound ssNA via the SSB-bound ssNA hybridization region, and the first splint oligonucleotide hybridized to the first adapter oligonucleotide via the first adapter oligonucleotide hybridization region, such that an end of the first adapter oligonucleotide is adjacent to an end of the first terminal region of the SSB-bound ssNA. In some embodiments the combining further includes combining the SSB-bound ssNA, a second adapter oligonucleotide, and a second splint oligonucleotide including an SSB-bound ssNA hybridization region and a second adapter oligonucleotide hybridization region, where the formed complexes further include the second splint oligonucleotide hybridized via the SSB-bound ssNA hybridization region to the terminal region of the SSB-bound ssNA opposite the terminal region hybridized to the first splint oligonucleotide, and the second splint oligonucleotide hybridized to the second adapter oligonucleotide via the second adapter oligonucleotide hybridization region, such that an end of the second adapter oligonucleotide is adjacent to the end of the SSB-bound ssNA opposite the end adjacent to the first adapter oligonucleotide.

An example embodiment in which a second adapter oligonucleotide and a second splint oligonucleotide are employed is schematically illustrated in FIG. 1. In this example, ssNA is produced from dsNA (e.g., ssDNA produced from dsDNA) by denaturing the dsNA. Shown at the top of FIG. 1 is dsNA 102. Upon denaturation of the dsNA 102, the resulting ssNA is contacted with single-stranded nucleic acid binding protein (SSB) to produce SSB-bound ssNA. Shown in FIG. 1 is SSB-bound ssNA 104 derived from a strand of dsNA 102. In this example, ssNA 104 is combined with a first adapter oligonucleotide 106 hybridized to a first splint oligonucleotide 108, and a second adapter oligonucleotide 110 hybridized to a second splint oligonucleotide 112. Hybridization of the first splint oligonucleotide 108 to the first adapter oligonucleotide 106 is via a first adapter oligonucleotide hybridization region 114 of the first splint oligonucleotide 108. Hybridization of the second splint oligonucleotide 112 to the second adapter oligonucleotide 110 is via a second adapter oligonucleotide hybridization region 116 of the second splint oligonucleotide 112. The hybridization of the first splint oligonucleotide 108 and the second splint oligonucleotide 112 with the SSB-bound NA 104, the first adapter region 106, and the second adapter region 110 forms a complex 122. Hybridization of the first splint oligonucleotide 108 to a 5' terminal region of SSB-bound ssNA 104 is via a first SSB-bound ssNA hybridization region 118 of the first splint oligonucleotide 108. Hybridization of the second splint oligonucleotide 112 to a 3' terminal region of the SSB-bound ssNA 104 is via a second SSB-bound ssNA hybridization region 120 of the second splint oligonucleotide 112. The splint oligonucleotides are designed such that when the SSB-bound ssNA hybridization regions of the splint

oligonucleotides are hybridized to their respective terminal regions of the SSB-bound ssNA, an end of the adapter oligonucleotide is adjacent to an end of the SSB-bound ssNA. The locations of adjacent ends are indicated by asterisks. These adjacent ends may be covalently linked (e.g., by enzymatic ligation) to produce adapted ssNA (e.g., adapted ssNA 124 shown in FIG. 1) which may then be used in a downstream application of interest (e.g., PCR amplification, next-generation sequencing, and/or the like) facilitated by one or more sequences in the adapter portion of the adapted ssNA. As shown in FIG. 1, upon covalent linkage of the adjacent ends, an optional clean-up step may be performed to separate the adapted ssNA from one or more reagents or components of the formed complexes, e.g., enzyme used for the covalent linkage, splint oligonucleotides, SSB, and/or the like. Suitable approaches for such a clean-up step include, but are not limited to, solid phase reversible immobilization (SPRI – e.g., using magnetic beads) and nucleic acid column purification. In the example shown in FIG. 1, a blocking modification is present at each end of the splint oligonucleotides (black rectangles), and a blocking modification is further present at the end of each adapter oligonucleotide which is not adjacent to the SSB-bound ssNA (black rectangles). The blocking modifications prevent ligation of oligonucleotides and ssNA to those ends.

As summarized above, drawbacks associated with current methods for preparing nucleic acid sequencing libraries via ligation of one or more adapter sequences include the time and expense required by such methods. The methods of the present disclosure constitute an improvement of current state-of-the-art approaches to single-stranded library preparation, such as the approach (designated “ssDNA2.0”) described by Gansauge et al. (2017) *Nucleic Acids Research* 45(10):e79, where the present methods were surprisingly found to be more efficient, require less time, and reduce costs. Aspects of the methods of the present disclosure will now be described in further detail.

The subject methods include contacting single-stranded nucleic acid (ssNA) with single-stranded nucleic acid binding protein (SSB) to produce SSB-bound ssNA. By “single-stranded nucleic acid” or “ssNA” is meant a collection of polynucleotides which are single-stranded (that is, not hybridized intermolecularly or intramolecularly) over 70% or more of their length. In some embodiments, the ssNA is single-stranded over 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, or 99% or more, of the length of the polynucleotides. In certain aspects, the ssNA is single-stranded over the entire length of the polynucleotides.

The ssNA may be (or be prepared from) any nucleic acid sample of interest, including but not limited to, a nucleic acid sample isolated from a single cell, a plurality of cells (e.g., cultured cells), a tissue, an organ, or an organism (e.g., bacteria, yeast, or the

like). Exemplary sample types include but are not limited to blood, serum, saliva, sputum, urine, feces, vomitus, mucus, hair, nail (e.g., fingernail, toenail), swabs (e.g., cheek swabs, throat swabs, vaginal swabs), biopsied tissue (e.g., punch biopsies, fine-needle biopsies, fine-needle aspiration biopsies), cell culture, environmental samples (e.g., water, soil, air, surfaces, touch DNA), and metagenomic samples. In certain aspects, the nucleic acid sample is isolated from a single cell, collection of cells, tissue, organ, and/or the like of an animal. In some cases, the nucleic acid sample comprises cell-free nucleic acids (e.g., cell-free DNA (cfDNA)), such as but not limited to fetal cell free nucleic acids (e.g., cell-free fetal DNA (cffDNA)) or circulating tumor nucleic acids (e.g., circulating tumor DNA (ctDNA)). In some embodiments, the animal is a mammal (e.g., a mammal from the genus *Homo*, a rodent (e.g., a mouse or rat), a dog, a cat, a horse, a cow, or any other mammal of interest). In other aspects, the nucleic acid sample is isolated/obtained from a source other than a mammal, such as bacteria, yeast, insects (e.g., drosophila), amphibians (e.g., frogs (e.g., *Xenopus*)), viruses, plants, or any other non-mammalian nucleic acid sample source.

In some embodiments, the ssNA is from a degraded nucleic acid sample. As used herein, a "degraded nucleic acid sample" is a sample of DNA that has been fragmented by enzymatic, physical, chemical or other processes. Examples of degraded nucleic acid samples are the DNA fragments recovered from bone remains, hair, cell-free DNA from blood plasma, or environmental DNA recovered from soil or water. In certain aspects, when the ssNA is from a degraded nucleic acid sample, the ssNA is from an ancient nucleic acid sample. By "ancient nucleic acid sample" is meant nucleic acid fragments recovered from biological remains. A non-limiting example of an ancient nucleic acid sample of interest is a nucleic acid sample obtained (e.g., isolated) from an extinct organism or animal, e.g., an extinct mammal. In certain aspects, the extinct mammal is from the genus *Homo*. In some embodiments, the ssNA is from a forensic nucleic acid sample. As used herein, a "forensic nucleic acid sample" is a nucleic acid sample relating to (e.g., obtained during the course of) the investigation of a crime.

In certain aspects, the ssNA is from a tumor nucleic acid sample (that is, a nucleic acid sample isolated from a tumor). As used herein, "tumor" refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum,

hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, various types of head and neck cancer, and the like.

In some embodiments, the ssNA is from a cell-free nucleic acid sample, e.g., cell-free DNA, cell-free RNA, or both. In certain aspects, the cell-free nucleic acids are obtained from a body fluid sample selected from the group consisting of: whole blood, blood plasma, blood serum, amniotic fluid, saliva, urine, pleural effusion, bronchial lavage, bronchial aspirates, breast milk, colostrum, tears, seminal fluid, peritoneal fluid, pleural effusion, and stool. In some embodiments, the cell-free nucleic acids are cell-free fetal DNAs. In certain aspects, the cell-free nucleic acids are circulating tumor DNAs. In some embodiments, the cell-free nucleic acids comprise infectious agent DNAs. In certain aspects, the cell-free nucleic acids comprise DNAs from a transplant.

In certain aspects, the ssNA is single-stranded deoxyribonucleic acid (ssDNA). ssDNA of interest includes, but is not limited to, ssDNA derived from double-stranded DNA (dsDNA). For example, the ssDNA may be derived from double-stranded DNA which is denatured (e.g., heat-denatured and/or chemically-denatured) to produce the ssDNA. In some embodiments, the methods include, prior to contacting the ssDNA with SSB, producing the ssDNA by denaturing the dsDNA.

When the ssNA is ssDNA derived from a dsDNA sample, the methods may further include, after formation of the complexes, rehybridizing the ssDNA (which now includes one or more adapters (e.g., sequencing adapters) at one or both ends) to produce dsDNA. If desired, the produced dsDNA may be sequenced. In some embodiments, the rehybridizing is carried out under sufficiently stringent hybridization conditions to produce dsDNAs that resemble the original dsDNAs from which the ssDNA was derived. The sufficiently stringent hybridization conditions may include a selected hybridization temperature, a selected salt concentration, and/or any other convenient hybridization parameters selected to produce dsDNAs that resemble the original dsDNAs from which the ssDNA was derived. One or both ends of at least a subset of such produced dsDNAs will resemble/replicate the ends (e.g., overhangs) of the original dsDNAs. Determining the end/overhang content (e.g., by sequencing) using the methods of the present disclosure may provide a variety of useful information regarding the nucleic acid sample from which the ssDNA was derived. For example, knowing the overhang content is of value in analyzing cell-free DNA (cfDNA), e.g., from blood plasma or another suitable source. It has been shown that cfDNA derives from a variety of sources including blood cells, fetal cells in pregnant women, tumor cells in

individuals having cancer, from transplanted organ tissue in organ transplant recipients, etc. The overhang content provided by embodiments of the methods of the present disclosure can be used to classify sequencing reads, e.g., by source of origin for diagnostic purposes.

Moreover, the end/overhang content may be used to analyze mixed DNA from forensic samples. For example, DNA from semen, blood, or another source of interest may have end characteristics that are diagnostic for that source, and DNA sequences could be partitioned based on this information.

In addition, determining the overhang content in an ancient DNA sample (e.g., a sample from an extinct organism, plant, or animal) provides information useful in characterizing such samples and the organisms, plants, animals, etc. from which the sample is derived. For example, ancient DNA samples (e.g., a DNA sample from an extinct mammal) often include contaminating DNAs (e.g., contaminating bacterial DNA, or the like). In such cases, the DNA sequences of interest may be partitioned from the contaminating DNA sequences based on the types of overhangs detected, when such types of overhangs are associated with a particular source of DNA.

In certain embodiments, the methods of the present disclosure find use in determining the rate and position of base damage in DNA extracts (e.g., ancient DNA extracts), as a function of the length and type of overhang.

Accordingly, in some embodiments, provided are methods that include combining SSB-bound dsDNA-derived ssDNA with the adapter and splint oligonucleotides to form complexes including the SSB-bound dsDNA-derived ssDNA hybridized to the adapter and splint oligonucleotides as described herein, and subsequent to complex formation, rehybridizing the ssDNA to produce dsDNAs (that is, “adapted” dsDNAs which now include one or more adapters (e.g., sequencing adapters) at one or both ends) that resemble the original dsDNAs from which the ssDNA was derived. Such methods may further include sequencing the adapted dsDNAs. In certain aspects, the sequencing is to determine the end/overhang content of the dsDNAs from which the ssDNA was derived. In any embodiments of the methods of the present disclosure which involve sequencing, the methods may include sequencing a subsample of the adapted ssNAs in order to reduce the complexity during sequencing.

In some embodiments, the ssNA is single-stranded ribonucleic acid (ssRNA). RNAs of interest include, but are not limited to, messenger RNA (mRNA), microRNA (miRNA), small interfering RNA (siRNA), transacting small interfering RNA (ta-siRNA), natural small interfering RNA (nat-siRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), long non-coding RNA (lncRNA), non-coding RNA (ncRNA), transfer-messenger RNA (tmRNA), precursor messenger RNA (pre-mRNA),

small Cajal body-specific RNA (scaRNA), piwi-interacting RNA (piRNA), endoribonuclease-prepared siRNA (esiRNA), small temporal RNA (stRNA), signal recognition RNA, telomere RNA, ribozyme, or any combination of such RNA types or subtypes. In some embodiments, when the ssNA is ssRNA, the ssRNA is mRNA.

Approaches, reagents and kits for isolating, purifying and/or concentrating DNA and RNA from sources of interest are known in the art and commercially available. For example, kits for isolating DNA from a source of interest include the DNeasy®, RNeasy®, QIAamp®, QIAprep® and QIAquick® nucleic acid isolation/purification kits by Qiagen, Inc. (Germantown, Md); the DNAzol®, ChargeSwitch®, Purelink®, GeneCatcher® nucleic acid isolation/purification kits by Life Technologies, Inc. (Carlsbad, CA); the NucleoMag®, NucleoSpin®, and NucleoBond® nucleic acid isolation/purification kits by Clontech Laboratories, Inc. (Mountain View, CA). In certain aspects, the nucleic acid is isolated from a fixed biological sample, e.g., formalin-fixed, paraffin-embedded (FFPE) tissue. Genomic DNA from FFPE tissue may be isolated using commercially available kits – such as the AllPrep® DNA/RNA FFPE kit by Qiagen, Inc. (Germantown, Md), the RecoverAll® Total Nucleic Acid Isolation kit for FFPE by Life Technologies, Inc. (Carlsbad, CA), and the NucleoSpin® FFPE kits by Clontech Laboratories, Inc. (Mountain View, CA).

When an organism, plant, animal, etc. from which the nucleic acid sample is obtained (e.g., isolated) is extinct, suitable strategies for recovering such nucleic acids are known and include, e.g., those described in Green et al. (2010) *Science* 328(5979):710-722; Poinar et al. (2006) *Science* 311(5759):392-394; Stiller et al. (2006) *Proc. Natl. Acad. Sci.* 103(37):13578–13584; Miller et al. (2008) *Nature* 456(7220):387-90; Rasmussen et al. (2010) *Nature* 463(7282):757-762; and elsewhere.

As summarized above, the subject methods include contacting the ssNA with single-stranded nucleic acid binding protein (SSB) to produce SSB-bound ssNA. SSB binds in a cooperative manner to ssNA and does not bind well to double-stranded nucleic acid (dsNA). Upon binding ssDNA, SSB destabilizes helical duplexes. SSBs that may be employed when practicing the subject methods include prokaryotic SSB (e.g., bacterial or archaeal SSB) and eukaryotic SSB. Non-limiting examples of SSBs that may be employed when practicing the subject methods include *E. coli* SSB, *E. coli* RecA, Extreme Thermostable Single-Stranded DNA Binding Protein (ET SSB), *Thermus thermophilus* (Tth) RecA, T4 Gene 32 Protein, replication protein A (RPA – a eukaryotic SSB), and the like. ET SSB, Tth RecA, *E. coli* RecA, T4 Gene 32 Protein, as well buffers and detailed protocols for preparing SSB-bound ssNA using such SSBs are available from, e.g., New England Biolabs, Inc. (Ipswich, MA). The inventors have determined that, given equal molarity inputs, a greater input of SSB is beneficial for ssNAs with higher average fragment lengths.

Detailed guidance regarding example approaches for contacting ssNA with SSB to produce SSB-bound ssNA is provided in the Experimental section below.

As summarized above, the subject methods include combining the SSB-bound ssNA, the adapter oligonucleotide, and the splint oligonucleotide that includes an SSB-bound ssNA hybridization region and a adapter oligonucleotide hybridization region, to form the complexes. As used herein, an "oligonucleotide" is a single-stranded multimer of nucleotides from 5 to 500 nucleotides, e.g., 5 to 100 nucleotides. Oligonucleotides may be synthetic or may be made enzymatically, and, in some embodiments, are 5 to 50 nucleotides in length. Oligonucleotides may contain ribonucleotide monomers (i.e., may be oligoribonucleotides or "RNA oligonucleotides"), deoxyribonucleotide monomers (i.e., may be oligodeoxyribonucleotides or "DNA oligonucleotides"), or a combination thereof. Oligonucleotides may be 10 to 20, 20 to 30, 30 to 40, 40 to 50, 50 to 60, 60 to 70, 70 to 80, 80 to 100, 100 to 150 or 150 to 200, or up to 500 nucleotides in length, for example.

An "adapter oligonucleotide" of the present disclosure is an oligonucleotide that includes an adapter or portion thereof. By "adapter" is meant a nucleotide sequence useful for one or more downstream applications (e.g., PCR amplification of the adapted ssNA or derivative thereof, sequencing of the adapted ssNA or derivative thereof, and/or the like). In certain aspects, the adapter or portion thereof present in the adapter oligonucleotide is a sequencing adapter. By "sequencing adapter" is meant one or more nucleic acid domains that include at least a portion of a nucleotide sequence (or complement thereof) utilized by a sequencing platform of interest, such as a sequencing platform provided by Illumina® (e.g., the HiSeq™, MiSeq™ and/or Genome Analyzer™ sequencing systems); Oxford Nanopore™ Technologies (e.g., the MinION™ sequencing system), Ion Torrent™ (e.g., the Ion PGM™ and/or Ion Proton™ sequencing systems); Pacific Biosciences (e.g., a Sequel or PacBio RS II sequencing system); Life Technologies™ (e.g., a SOLiD™ sequencing system); Roche (e.g., the 454 GS FLX+ and/or GS Junior sequencing systems); or any other sequencing platform of interest.

In certain aspects, the sequencing adapter is, or includes, a nucleic acid domain selected from: a domain (e.g., a "capture site" or "capture sequence") that specifically binds to a surface-attached sequencing platform oligonucleotide (e.g., the P5 or P7 oligonucleotides attached to the surface of a flow cell in an Illumina® sequencing system); a sequencing primer binding domain (e.g., a domain to which the Read 1 or Read 2 primers of the Illumina® platform may bind); a unique identifier (e.g., a barcode or other domain that uniquely identifies the 3' region of the oligonucleotide probe, the probe complement oligonucleotide, or both, and/or uniquely identifies the sample source of the rRNA being sequenced to enable sample multiplexing by marking every molecule from a given sample

with a specific barcode or "tag"); a barcode sequencing primer binding domain (a domain to which a primer used for sequencing a barcode binds); a molecular identification domain (e.g., a molecular index tag, such as a randomized tag of 4, 6, or other number of nucleotides) for uniquely marking molecules of interest, e.g., to determine expression levels based on the number of instances a unique tag is sequenced; a complement of any such domains; or any combination thereof. In certain aspects, a barcode domain (e.g., sample index tag) and a molecular identification domain (e.g., a molecular index tag) may be included in the same nucleic acid.

When the adapter oligonucleotide includes one or a portion of a sequencing adapter, one or more additional sequencing adapters and/or a remaining portion of the sequencing adapter may be added using a variety of approaches. For example, additional and/or remaining portions of sequencing adapters may be added by ligation, reverse transcription, PCR amplification, and/or the like. In the case of PCR, an amplification primer pair may be employed that includes a first amplification primer that includes a 3' hybridization region (e.g., for hybridizing to an adapter region of the adapter oligonucleotide) and a 5' region including an additional and/or remaining portion of a sequencing adapter, and a second amplification primer that includes a 3' hybridization region (e.g., for hybridizing to an adapter region of a second adapter oligonucleotide added to the opposite end of an ssNA molecule) and optionally a 5' region including an additional and/or remaining portion of a sequencing adapter.

A "splint oligonucleotide" of the present disclosure is an oligonucleotide that includes an SSB-bound ssNA hybridization region and an adapter oligonucleotide hybridization region. The SSB-bound ssNA hybridization region is a region (nucleotide sequence) that hybridizes to a terminal region of the SSB-bound ssNA. The adapter oligonucleotide hybridization region is a region (nucleotide sequence) that hybridizes to all or a portion of the adapter oligonucleotide. The splint oligonucleotide is designed for simultaneous hybridization to the SSB-bound ssNA and the adapter oligonucleotide such that, upon complex formation, an end of the adapter oligonucleotide is adjacent to an end of the terminal region of the SSB-bound ssNA.

The SSB-bound ssNA hybridization region of the splint oligonucleotide may have any suitable length and sequence. In some embodiments, the length of the SSB-bound ssNA hybridization region is 10 nucleotides or less. In certain aspects, the SSB-bound ssNA hybridization region is from 4 to 20 nucleotides in length, e.g., from 5 to 15, 5 to 10, 5 to 9, 5 to 8, or 5 to 7 (e.g., 6 or 7) nucleotides in length. In some embodiments, the SSB-bound ssNA hybridization region includes (e.g., consists of) a random nucleotide sequence, such that when a plurality of heterogeneous splint oligonucleotides having various random

SSB-bound ssNA hybridization regions are employed, the collection is capable of acting as splint oligonucleotides for a heterogeneous population of SSB-bound ssNAs irrespective of the sequences of the terminal regions of the SSB-bound ssNAs.

Accordingly, in certain aspects, the methods include forming the complexes by combining the SSB-bound ssNA, an adapter oligonucleotide, and a plurality of heterogeneous splint oligonucleotides having various random SSB-bound ssNA hybridization regions capable of acting as splint oligonucleotides for a heterogeneous population of SSB-bound ssNA having terminal regions of undetermined sequence.

In some embodiments, the SSB-bound ssNA hybridization region includes a known sequence designed to hybridize to a SSB-bound ssNA terminal region of known sequence. In certain aspects, two or more heterogeneous splint oligonucleotides having different SSB-bound ssNA hybridization regions of known sequence designed to hybridize to respective SSB-bound ssNA terminal regions of known sequence are employed. Embodiments in which the SSB-bound ssNA hybridization regions have a known sequence find use, e.g., when it is desirable to produce a nucleic acid library from only a subset of SSB-bound ssNAs having terminal regions of known sequence. Accordingly, in certain aspects, the methods include forming the complexes by combining the SSB-bound ssNA, an adapter oligonucleotide, and one or more heterogeneous splint oligonucleotides having one or more different SSB-bound ssNA hybridization regions of known sequence capable of acting as splint oligonucleotides for one or more SSB-bound ssNAs having one or more terminal regions of known sequence.

In certain aspects, the SSB-bound ssNA hybridization region includes one or more universal bases. As used herein, a “universal base” is a base capable of indiscriminately base pairing with each of the four standard nucleotide bases: A,C,G and T. Universal bases that may be incorporated into the SSB-bound ssNA hybridization region include, but are not limited to, 2′-deoxyinosine (dI, dInosine) and 5-nitroindole.

The manner in which the SSB-bound ssNA, the adapter oligonucleotide, and the splint oligonucleotide are combined may vary. In some embodiments, the combining includes combining a complex including the splint oligonucleotide hybridized to the adapter oligonucleotide via the adapter oligonucleotide hybridization region, and the SSB-bound ssNA. In other aspects, the combining includes combining a complex including the splint oligonucleotide hybridized to the SSB-bound ssNA via the SSB-bound ssNA hybridization region, and the adapter oligonucleotide. In still other aspects, the combining includes combining the SSB-bound ssNA, the adapter oligonucleotide, and the splint oligonucleotide, where none of the three components are pre-complexed with (that is – hybridized to) another component prior to the combining.

The combining is carried out under hybridization conditions such that complexes including the splint oligonucleotide hybridized to a terminal region of the SSB-bound ssNA via the SSB-bound ssNA hybridization region, and the splint oligonucleotide hybridized to the adapter oligonucleotide via the adapter oligonucleotide hybridization region. Whether specific hybridization occurs is determined by such factors as the degree of complementarity between the relevant (that is, hybridizing) regions of the splint oligonucleotide, the terminal region of the SSB-bound ssNA, and the adapter oligonucleotide, as well as the length thereof, salt concentration, and the temperature at which the hybridization occurs, which may be informed by the melting temperatures (T_M) of the relevant regions. The melting temperature refers to the temperature at which half of the relevant regions remain hybridized and half of the relevant regions dissociate into single strands. The *Tm* of a duplex may be experimentally determined or predicted using the following formula $Tm = 81.5 + 16.6(\log10[Na^+]) + 0.41$ (fraction G+C) – (60/N), where N is the chain length and $[Na^+]$ is less than 1 M. *See* Sambrook and Russell (2001; Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor N.Y., Ch. 10). Other more advanced models that depend on various parameters may also be used to predict *Tm* of relevant regions depending on various hybridization conditions. Approaches for achieving specific nucleic acid hybridization may be found in, e.g., Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, part I, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier (1993).

The terms "complementary" or "complementarity" as used herein refer to a nucleotide sequence that base-pairs by non-covalent bonds to a region of a target nucleic acid, e.g., the nucleotide sequence of the SSB-bound ssNA hybridization region that hybridizes to the terminal region of the SSB-bound ssNA, and the nucleotide sequence of the adapter oligonucleotide hybridization region that hybridizes to the probe complement oligonucleotide. In the canonical Watson-Crick base pairing, adenine (A) forms a base pair with thymine (T), as does guanine (G) with cytosine (C) in DNA. In RNA, thymine is replaced by uracil (U). As such, A is complementary to T and G is complementary to C. In RNA, A is complementary to U and vice versa. Typically, "complementary" or "complementarity" refers to a nucleotide sequence that is at least partially complementary. These terms may also encompass duplexes that are fully complementary such that every nucleotide in one strand is complementary to every nucleotide in the other strand in corresponding positions. In certain cases, a nucleotide sequence may be partially complementary to a target, in which not all nucleotides are complementary to every nucleotide in the target nucleic acid in all the corresponding positions. For example, the SSB-bound ssNA hybridization region may

be perfectly (i.e., 100%) complementary to the terminal region of the SSB-bound ssNA, or the SSB-bound ssNA hybridization region may share some degree of complementarity which is less than perfect (e.g., 70%, 75%, 85%, 90%, 95%, 99%). The percent identity of two nucleotide sequences can be determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first sequence for optimal alignment). The nucleotides at corresponding positions are then compared, and the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity= # of identical positions/total # of positions×100). When a position in one sequence is occupied by the same nucleotide as the corresponding position in the other sequence, then the molecules are identical at that position. A non-limiting example of such a mathematical algorithm is described in Karlin et al., *Proc. Natl. Acad. Sci.* USA 90:5873-5877 (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) as described in Altschul et al., *Nucleic Acids Res.* 25:389-3402 (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., NBLAST) can be used. In one aspect, parameters for sequence comparison can be set at score=100, wordlength=12, or can be varied (e.g., wordlength=5 or wordlength=20).

The complexes are formed such that an end of the adapter oligonucleotide is adjacent to an end of the terminal region of the SSB-bound ssNA. By "adjacent to" is meant the terminal nucleotide at the end of the adapter oligonucleotide and the terminal nucleotide end of the terminal region of the SSB-bound ssNA are sufficiently proximal to each other that the terminal nucleotides may be covalently linked, e.g., by chemical ligation, enzymatic ligation, or the like. In some embodiments, the ends are adjacent to each other by virtue of the terminal nucleotide at the end of the adapter oligonucleotide and the terminal nucleotide end of the terminal region of the SSB-bound ssNA being hybridized to adjacent nucleotides of the splint oligonucleotide. The splint oligonucleotide may be designed to ensure that the an end of the adapter oligonucleotide is adjacent to an end of the terminal region of the SSB-bound ssNA. Non-limiting examples of such splint oligonucleotides are provided in the Experimental section herein.

Any of the methods described herein may further include covalently linking the adjacent ends of an adapter oligonucleotide and SSB-bound ssNA. The covalent linking may include ligating the adjacent ends. Ligating the adjacent ends may be carried out using any suitable approach. In certain aspects, the ligating is by chemical ligation. In other aspects, the ligating is by enzymatic ligation. Suitable reagents (e.g., ligases and corresponding buffers, etc.) and kits for performing enzymatic ligation reactions are known and available, e.g., the Instant Sticky-end Ligase Master Mix available from New England

Biolabs (Ipswich, MA). Ligases that may be employed include, e.g., T4 DNA ligase (e.g., at low or high concentration), T4 DNA ligase, T7 DNA Ligase, E. coli DNA Ligase, Electro Ligase®, or the like. Conditions suitable for performing the ligation reaction will vary depending upon the type of ligase used. Information regarding such conditions is readily available. When necessary, a phosphate group may be added at the 5' end of the adapter oligonucleotide or SSB-bound ssNA using, e.g., a suitable kinase, such as T4 polynucleotide kinase (PNK). Such kinases and guidance for using such kinases to phosphorylate 5' ends are available, e.g., from New England BioLabs, Inc. (Ipswich, MA).

In some embodiments, the splint oligonucleotide, the adapter oligonucleotide, or both, includes a blocking modification. For example, one or both ends of the splint oligonucleotide may include a blocking modification and/or the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA may include a blocking modification. By "blocking modification" is meant the end is not competent for being linked to the end of any other oligonucleotide components using an approach employed to covalently link the adjacent ends of the adapter oligonucleotide and SSB-bound ssNA. In certain aspects, the blocking modification is a ligation-blocking modification. Examples of blocking modifications which may be included at one or both ends of the splint oligonucleotide and/or the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA, include the absence of a 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA, and an inaccessible 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA. Non-limiting examples of blocking modifications in which an end has an inaccessible 3' OH include: an amino modifier, a spacer, a dideoxy base, an inverted dideoxy base, a 3' phosphate, or the like.

In certain aspects, the splint oligonucleotide, the adapter oligonucleotide, or both, includes one or more non-natural nucleotides (which may also be referred to as nucleotide analogs). Non-limiting examples of non-natural nucleotides that may be included in the splint oligonucleotide, the adapter oligonucleotide, or both are LNA (locked nucleic acid), PNA (peptide nucleic acid), FANA (2'-deoxy-2'-fluoroarabinonucleotide), GNA (glycol nucleic acid), TNA (threose nucleic acid), 2'-O-Me RNA, 2'-fluoro RNA, Morpholino nucleotides, and any combination thereof.

Covalently linking the adjacent ends of an adapter oligonucleotide and SSB-bound ssNA produces adapted ssNA, where "adapted" means the ssNA now includes one or more adapter sequences or subregions thereof. The adapted ssNA may be purified before being used as input in a downstream application of interest. For example, the complexes may be denatured (e.g., heat-denatured) to separate the adapted ssNA from the splint oligonucleotides, the adapted ssNA may be purified from the SSB and/or any other

components present during the contacting and/or combining steps (e.g., by solid phase reversible immobilization (SPRI), column purification, and/or the like), or combinations thereof.

In some embodiments, the one or more adapter sequences or subregions thereof is one or more sequencing adapters or subregions thereof, and the methods further include sequencing at least a portion of the adapted ssNA, or any derivative thereof (e.g., amplicons produced by PCR amplification using the adapted ssNA as template). The sequencing may be carried out on any suitable sequencing platform, including a high-throughput sequencing (HTS) (or "next-generation sequencing (NGS)") platform, or the like. HTS/NGS sequencing platforms of interest include, but are not limited to, a sequencing platform provided by Illumina® (e.g., the HiSeq™, MiSeq™ and/or Genome Analyzer™ sequencing systems); Oxford Nanopore™ Technologies (e.g., a MinION™, GridION x5™, PromethION™, or SmidgION™ nanopore-based sequencing system), Ion Torrent™ (e.g., the Ion PGM™ and/or Ion Proton™ sequencing systems); Pacific Biosciences (e.g., a Sequel or PacBio RS II sequencing system); Life Technologies™ (e.g., a SOLiD sequencing system); Roche (e.g., the 454 GS FLX+ and/or GS Junior sequencing systems); or any other sequencing platform of interest. Detailed protocols for direct sequencing (e.g., by nanopore-based sequencing) or preparing compatible nucleic acid molecules for sequencing on a particular platform (e.g., by amplification, e.g., solid-phase amplification, or the like), sequencing the compatible molecules, and analyzing the sequencing data are available from the manufacturer of the sequencing platform of interest.

As summarized above, the methods of the present disclosure constitute an improvement of current state-of-the-art approaches to single-stranded library preparation, such as the approach (designated "ssDNA2.0") described by Gansauge et al. (2017) *Nucleic Acids Research* 45(10):e79, where the present methods were surprisingly found to be more efficient, require less time, and reduce costs. In some embodiments, when the methods include covalently linking the adjacent ends of an adapter oligonucleotide and SSB-bound ssNA, the total duration of the combining and covalently linking steps is 4 hours or less, 3 hours or less, 2 hours or less, or 1 hour or less. In certain aspects, when the methods include covalently linking the adjacent ends of an adapter oligonucleotide and SSB-bound ssNA, the total duration of the contacting, combining and covalently linking steps is 4 hours or less, 3 hours or less, 2 hours or less, or 1 hour or less. In some embodiments, the efficiency of the methods is such that complexes are formed from 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, or 99% or more of the ssNA contacted with the SSB during the contacting step.

COMPOSITIONS

As summarized above, the present disclosure also provides compositions. The compositions find use in a variety of applications, including, e.g., practicing any of the methods of the present disclosure, including carrying out one or more of any of the steps described above in the Methods section of the present disclosure. As such, the compositions may include any of the oligonucleotides (including pluralities/collections of heterogeneous oligonucleotides), ssNA, SSB, other reagents, etc. described above in the Methods section of the present disclosure, in any combination.

In certain aspects, provided are compositions that include SSB-bound ssNA, a first adapter oligonucleotide, and a first splint oligonucleotide including an SSB-bound ssNA hybridization region and a first adapter oligonucleotide hybridization region. Such compositions may further include a second adapter oligonucleotide and a second splint oligonucleotide including an SSB-bound ssNA hybridization region and a second adapter oligonucleotide hybridization region.

In certain aspects, provided are compositions that include complexes including a splint oligonucleotide hybridized to an adapter oligonucleotide via the adapter oligonucleotide hybridization region, present as hybridized complexes (e.g., in the absence of SSB-bound ssNA). In other aspects, provided are compositions that include complexes including a splint oligonucleotide hybridized to SSB-bound ssNA via the SSB-bound ssNA hybridization region, present as hybridized complexes.

The ssNA may be ssDNA. When the ssNA is ssDNA, the ssDNA may be derived from dsDNA. In some embodiments, the ssNA is ssRNA. In some embodiments, the ssNA is from a degraded nucleic acid sample. In certain aspects, when the ssNA is from a degraded nucleic acid sample, the ssNA is from an ancient nucleic acid sample, such as a nucleic acid sample obtained (e.g., isolated) from an extinct organism or animal, e.g., an extinct mammal. In certain aspects, the extinct mammal is from the genus *Homo*. In some embodiments, the ssNA is from a forensic nucleic acid sample.

The compositions of the present disclosure may further include a reagent for covalently linking an adapter oligonucleotide end to an end of the SSB-bound ssNA. In some embodiments, the reagent is a chemical ligation reagent or an enzymatic ligation reagent, e.g., a ligase.

The compositions of the present disclosure may include the one or more components present in a container. Suitable containers include, but are not limited to, tubes, vials, and plates (e.g., a 96- or other-well plate).

In certain aspects, the compositions include the one or more components in a liquid medium. The liquid medium may be an aqueous liquid medium, such as water, a buffered solution, and the like. One or more additives such as a salt (e.g., NaCl, $MgCl_2$, KCl, $MgSO_4$), a buffering agent (a Tris buffer, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 2-(N-Morpholino)ethanesulfonic acid (MES), 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES), 3-(N-Morpholino)propanesulfonic acid (MOPS), N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), etc.), a solubilizing agent, a detergent (e.g., a non-ionic detergent such as Tween-20, etc.), a nuclease inhibitor, glycerol, a chelating agent, and the like may be present in such compositions.

KITS

As summarized above, the present disclosure provides kits. The kits find use in a variety of applications, including, e.g., practicing any of the methods of the present disclosure, including carrying out one or more of any of the steps described above in the Methods section of the present disclosure. As such, the kits may include any of the oligonucleotides (including pluralities/collections of heterogeneous oligonucleotides), ssNA, SSB, other reagents, etc. described above in the Methods section of the present disclosure, in any combination.

In some embodiments, a kit of the present disclosure includes single-stranded nucleic acid binding protein (SSB, e.g., single-stranded DNA binding protein, single-stranded RNA binding protein, or both), a first adapter oligonucleotide, a first splint oligonucleotide comprising an SSB-bound ssNA hybridization region and a first adapter oligonucleotide hybridization region, and instructions for using the SSB, first adapter oligonucleotide, and first splint oligonucleotide to produce a nucleic acid library. In certain aspects, such a kit further includes a second adapter oligonucleotide, and a second splint oligonucleotide including an SSB-bound ssNA hybridization region and a second adapter oligonucleotide hybridization region, where the instructions are for using the SSB, first adapter oligonucleotide, first splint oligonucleotide, second adapter oligonucleotide, and second splint oligonucleotide to produce a nucleic acid library.

The kits of the present disclosure may further include a reagent for covalently linking an adapter oligonucleotide end to an end of SSB-bound ssNA. In some embodiments, the reagent is a chemical ligation reagent or an enzymatic ligation reagent, e.g., a ligase.

In some embodiments, a splint oligonucleotide, an adapter oligonucleotide, or both, includes a blocking modification. For example, one or both ends of the splint oligonucleotide may include a blocking modification and/or the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA may include a blocking modification.

In certain aspects, the blocking modification is a ligation-blocking modification. Examples of blocking modifications which may be included at one or both ends of the splint oligonucleotide and/or the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA, include the absence of a 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA, and an inaccessible 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA. Non-limiting examples of blocking modifications in which an end has an inaccessible 3' OH include: an amino modifier, a spacer, a dideoxy base, an inverted dideoxy base, a 3' phosphate, or the like.

In some embodiments, one or more splint oligonucleotides provided in a kit of the present disclosure includes an SSB-bound ssNA hybridization region that includes (e.g., consists of) a random nucleotide sequence, such that when the kit includes a plurality of heterogeneous splint oligonucleotides having various random SSB-bound ssNA hybridization regions, the collection is capable of acting as splint oligonucleotides for a heterogeneous population of SSB-bound ssNAs irrespective of the sequences of the terminal regions of the SSB-bound ssNAs of interest.

In certain aspects, a splint oligonucleotide provided in a kit of the present disclosure includes an SSB-bound ssNA hybridization region that includes one or more universal bases. Universal bases that may be incorporated into the SSB-bound ssNA hybridization region include, but are not limited to, 2′-deoxyinosine (dI, dInosine) and 5-nitroindole.

In some embodiments, the length of the SSB-bound ssNA hybridization region of a splint oligonucleotide provided in a kit of the present disclosure is 10 nucleotides or less. In certain aspects, the SSB-bound ssNA hybridization region is from 4 to 20 nucleotides in length, e.g., from 5 to 15, 5 to 10, 5 to 9, 5 to 8, or 5 to 7 (e.g., 6 or 7) nucleotides in length.

Components of the subject kits may be present in separate containers, or multiple components may be present in a single container. A suitable container includes a single tube (e.g., vial), one or more wells of a plate (e.g., a 96-well plate, a 384-well plate, etc.), or the like.

The instructions for using the SSB, one or more adapter oligonucleotides, and one or more splint oligonucleotides to produce a nucleic acid library may be recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging), etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer-readable storage medium, e.g., portable flash drive, DVD, CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining

the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, the means for obtaining the instructions is recorded on a suitable substrate.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1

Disclosed herein is an approach for fast, efficient, and targeted ligation of adapters to single stranded DNA in one reaction. In the following examples, sequencing adapter oligonucleotides containing Illumina P7 or P5 adapter sequences are hybridized to a splint oligonucleotide containing a string of 3-prime Ns for the P7 splint and a string of 5-prime Ns for the P5 splint. The splint creates an opportunity for ligases that can only perform double strand ligation with high efficiency to ligate the adapters to the target single-stranded DNA. All oligonucleotide DNA ends that are not needed to participate in ligation are blocked by oligonucleotide modifications (e.g., amino modifications) that prevent ligation.

The addition of single-stranded binding proteins (SSBs) to the assay increases the efficiency of the reaction. The concentration and length of the target DNA is used to calculate appropriate amounts of SSB to achieve optimal ligation efficiency. The SSBs may prevent single-stranded DNA from re-annealing while preventing secondary structures.

This method can be favorably compared to a single-stranded library preparation described by Gansauge et al. (2017) *Nucleic Acids Research* 45(10):e79, known as SS2.0. Compared to SS2.0, the present method requires significantly less time and exhibits significantly increased efficiency of conversion of DNA into proper adapter-ligated DNA molecules that can be sequenced. In addition, the present approach reduces reagent costs compared to SS2.0.

The adapter and splint oligonucleotides are engineered to carry a ligation-blocking modification on all ends that should not participate in proper adapter ligation. This includes blocking the 5-prime end for the P5 adapter and the 3-prime end of the P7 adapter and all ends of the splint. These ligation-blocking modifications may be amino modifiers, carbon spacers, dideoxy bases, or any other suitable modifications that prevent access of a ligase to the 3-prime hydroxyl group of the 3-prime end or the 5-prime phosphate of the 5-prime end. Oligonucleotides may be synthesized, e.g., by Integrated DNA Technologies (IDT). Example oligonucleotides are shown in Table 1 below.

Table 1 – Example Oligonucleotides

P5 Adapter (5' → 3') SEQ ID NO:1	/5AmMC12/ACACTCTTTCCCTACACGACGCTCTTCCGATCT
P5 Splint (5' → 3') SEQ ID NO:2	/5AmMC6/NNNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/3AmMO/
P7 Adapter (5' → 3') SEQ ID NO:3	/5Phos/AGATCGGAAGAGCACACGTCTGAACTCCAGTCA/3ddC/
P7 Splint (5' → 3') SEQ ID NO:4	/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNN/3AmMO/

/5AmMC12/ = 5' Amino Modifier C12
/5AmMC6/ = 5' Amino Modifier C6
/3AmMO/ = 3' Amino Modifier
/5Phos/ = 5' Phosphate
/3ddC/ = 3' Dideoxy Cytosine

In the protocol employed in this Example, template DNA was combined with SSBs and heat denatured. After denaturation, the reactions were placed on ice or a PCR cooler at 4°C. After cooling, adapters were added to each reaction. Then, the reaction master mix is added, followed by mixing. Incubation at 37°C allows for ligation to begin immediately with most ligation occurring before 45 minutes. Reactions can be cleaned up with established methods and downstream applications such as amplification and sequencing remain unchanged.

To prepare the adapters, combined were P5 adapter to a final concentration of 10uM and P5 splint oligonucleotide to a final concentration of 20 µM with 1X final concentration of T4 RNA Ligase Buffer (Cat# B0216L). A similar, separate mixture was prepared using the P7 adapter and P7 splint oligonucleotide. Adapters were hybridized by heating to 95°C for 10 seconds and then ramped down to 10°C at a rate of 0.1°C/s.

An example protocol is provided below.

1. Sample input (36uL)
    a. Combine sheared DNA and ET SSB (Cat# M2401S) to a volume of 36uL
        i. 1uL of ET SSB promotes ligation without inhibiting for most sample types tested

ii. Fill remaining volume with buffer EBT (10mM Tris-HCl, pH 8.0 and 0.05% Tween 20)

2. Denature sample
   a. Incubate samples in a thermocycler with a lid pre-heated to 95°C for 3 minutes
   b. Immediately place denatured sample on ice or a PCR cooler for 30 seconds.
3. Add 2uL of pooled adapter mixes (equal volume P5 and P7 adapters)
   a. Adapter input will depend on the molarity of the input. A molar ratio between 6 and 10 to 1, adapters to template, is preferred.
4. Add reaction master mix and mix thoroughly by pipetting
   a. 8uL of T4 DNA Ligase Buffer (Cat# M0202M)
   b. 32uL of 50% PEG 8000 (Cat# B0216L)
   c. 1uL of T4 Polynucleotide Kinase – 10,000 U/mL (Cat# M0201L)
   d. 1uL of T4 DNA Ligase – 2,000,000 U/mL (Cat# M0202M)
5. Incubate at 37°C for up to 60 minutes
   a. Most ligation occurs in the first 15 minutes but the plateau isn't achieved until around 45 minutes.
6. Clean up reaction
   a. Column cleanup (e.g., for degraded DNA) or SPRI (e.g., for modern samples).

Comparison and Optimization Assay

After clean up, qPCR was performed on a dilution of pre-amplified libraries to determine ligation efficiency. A lower CT value indicates greater ligation efficiency relative to another sample on the same run with a higher CT value. A difference of one is roughly equal to a two-fold difference in library efficiency. An aliquot of the pre-amplified libraries was also amplified with an index PCR reaction. Post-indexing. the libraries were cleaned with SPRI and visualized on an Agilent TapeStation 2200 system to estimate the proportion of adapter artifacts in each library.

Single-Stranded Binding Proteins

It was observed that single-stranded binding protein (SSB) such as ET SSB protein supplied by NEB enhances the ligation efficiency of single-stranded ligation. Given equal molarity inputs, samples with higher average fragment lengths required a greater input of SSB to achieve peak ligation. The molarity of DNA in the reaction also affects the amount of SSB required. In vast excess ET SSB has the potential to inhibit ligation.

Protocol Comparison

The efficiency of the present protocol was compared to the NEB Ultra 2 kit (dsDNA), SS2.0 (Gansauge et al. (2017) *Nucleic Acids Research* 45(10):e79), and the blunt end single tube (BEST) method described in Caroe et al. (2017) *Methods in Ecology and Evolution* 9(2):410-419; and Mak et al. (2017) *GigaScience* 6:1-13. Comparison results were obtained using modern human DNA with an average fragment length of about 350bp and an ancient bison sample that is heavily degraded with an average fragment length of about 35-40bp.

The NEB Ultra 2 kit is recognized as a highly efficient library preparation method for modern samples while SS2.0 is recognized as a highly efficient library preparation method for degraded samples. The BEST protocol involves blunt end repair using T4 DNA Polymerase and T4 PNK to blunt end the DNA (no tailing) and phosphorylate 5' ends. Next, blunt end dsDNA adapters are ligated to the blunt ends using T4 DNA Ligase, followed by a fill-in reaction using Bst 2.0 Warmstart polymerase and clean-up using SPRI beads or a column.

Comparison results are provided in FIG. 2. From left to right on the top panel are: the method described in this example (asterisk), ss2.0, and BEST. From left to right on the bottom panel are: the method described in this example (asterisk), the NEB Ultra 2 kit, ss2.0, and BEST. The method described in this example exhibits far greater ligation efficiency for ancient samples compared to ss2.0. For modern samples, the method described in this example is between 0.3 and 0.5 qPCR cycles behind the NEB Ultra 2 Kit.

Example 2 – Hair DNA

DNA was collected from hair using a standard Proteinase K treatment at high temperature. 6 nanograms of DNA was used as template for making the library. The protocol was followed as described above. Two sequencing libraries were produced from the adapter-ligated product. One used 1 µL of the 50 µL total ligation product (SRL3). The other used 2.5 µL of this product (SRL4). Both libraries were sequenced on the Illumina MiSeq sequencing platform to assess the library characteristics and complexity (number of unique library molecules).

After 2x75 paired-end sequencing, the SeqPrep program was used to combine the forward and reverse read pairs that overlap with one another. This occurs when the original DNA template is short enough such that the forward read and the reverse read cover some of the same sequence (referred to herein as "merged reads"). After merging, merged and unmerged reads were mapped to the reference human genome sequence. Shown in FIG. 3 is the observed original template length distribution of the merged & mapped, merged and

unmapped, and merged and unmapped reads for both SRL3 and SRL4 libraries. Note that for unmerged and unmapped, it is not possible to infer the length of the template DNA.

The Preseq software program was used to estimate the number of unique library molecules in both libraries. This program counts the number of observed duplicate molecules to model the complexity of the nucleic acid library from a large sample of observed reads, as produced here. This program shows an estimate for the fraction of observed reads that are predicted to be unique at various depths of library sequencing. As shown in FIG. 4, both libraries are predicted to have complexity of over 250,000,000 unique molecules. SRL4, which was made from 2.5 µL of the adapter-ligated template has more unique molecules than SRL3.

Example 3 – Ancient DNA from Bison Bone

The efficiency of conversion from template DNA molecules to sequencing library was compared using DNA extracted from an ancient bison bone. Libraries were generated from the same amount of DNA from the same extract using four different protocols, including the protocol as described above.

The complexity of the libraries was measured using two approaches: qPCR of the adapter-ligated product and direct sequencing. The qPCR estimates were done in triplicate. Both approaches, shown in FIG. 5, demonstrate that the approach described herein is more efficient at converting DNA into sequencing libraries.

Accordingly, the preceding merely illustrates the principles of the present disclosure. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein.

WHAT IS CLAIMED IS:

1. A method of producing a nucleic acid library, comprising:
contacting single-stranded nucleic acid (ssNA) with single-stranded nucleic acid binding protein (SSB) to produce SSB-bound ssNA; and
combining:
the SSB-bound ssNA,
a first adapter oligonucleotide, and
a first splint oligonucleotide comprising an SSB-bound ssNA hybridization region and a first adapter oligonucleotide hybridization region;
to form complexes comprising the first splint oligonucleotide hybridized to a terminal region of the SSB-bound ssNA via the SSB-bound ssNA hybridization region, and the first splint oligonucleotide hybridized to the first adapter oligonucleotide via the first adapter oligonucleotide hybridization region, such that an end of the first adapter oligonucleotide is adjacent to an end of the terminal region of the SSB-bound ssNA.

2. The method according to Claim 1, further comprising covalently linking the adjacent ends of the first adapter oligonucleotide and SSB-bound ssNA.

3. The method according to Claim 2, wherein the total duration of the contacting, combining, and covalently linking steps is 3 hours or less.

4. The method according to any one of Claims 1 to 3, wherein the complexes are formed from 80% or more of the ssNA contacted with the SSB.

5. The method according to any one of Claims 1 to 4, wherein the ssNA is from a degraded nucleic acid sample.

6. The method according to Claim 5, wherein the ssNA is from an ancient nucleic acid sample.

7. The method according to any one of Claims 1 to 6, wherein the ssNA is from a forensic nucleic acid sample.

8. The method according to any one of Claims 1 to 7, wherein the ssNA is single-stranded DNA (ssDNA).

9. The method according to Claim 8, wherein the ssDNA is derived from double-stranded DNA (dsDNA).

10. The method according to Claim 9, further comprising, prior to contacting the ssDNA with SSB, producing the ssDNA by denaturing the dsDNA.

11. The method according to Claim 9 or Claim 10, further comprising, after formation of the complexes, rehybridizing the ssDNA to produce dsDNA.

12. The method according to Claim 11, further comprising sequencing the produced dsDNA.

13. The method according to Claim 12, wherein a sub-sample of the produced dsDNA is sequenced.

14. The method according to any one of Claims 1 to 6, wherein the ssNA is single-stranded RNA (ssRNA).

15. The method according to any one of Claims 1 to 14, wherein the combining comprises:

combining:

a complex comprising the first splint oligonucleotide hybridized to the first adapter oligonucleotide via the first adapter oligonucleotide hybridization region, and

the SSB-bound ssNA.

16. The method according to any one of Claims 1 to 14, wherein the combining comprises:

combining:

a complex comprising the first splint oligonucleotide hybridized to the SSB-bound ssNA via the SSB-bound ssNA hybridization region, and

the first adapter oligonucleotide.

17. The method according to any one of Claims 1 to 16, wherein the combining further comprises:

combining:

the SSB-bound ssNA,

a second adapter oligonucleotide, and

a second splint oligonucleotide comprising an SSB-bound ssNA hybridization region and a second adapter oligonucleotide hybridization region,

wherein the formed complexes further comprise the second splint oligonucleotide hybridized via the SSB-bound ssNA hybridization region to the terminal region of the SSB-bound ssNA opposite the terminal region hybridized to the first splint oligonucleotide, and the second splint oligonucleotide hybridized to the second adapter oligonucleotide via the second adapter oligonucleotide hybridization region, such that an end of the second adapter oligonucleotide is adjacent to the end of the SSB-bound ssNA opposite the end adjacent to the first adapter oligonucleotide.

18. The method according to Claim 17, further comprising covalently linking the adjacent ends of the second adapter oligonucleotide and SSB-bound ssNA.

19. The method according to any one of Claims 1 to 18, wherein the covalently linking comprises ligating the adjacent ends.

20. The method according to Claim 19, wherein the ligating is by enzymatic ligation.

21. The method according to any one of Claims 1 to 20, wherein the end of an adapter oligonucleotide not adjacent to the SSB-bound ssNA comprises a blocking modification.

22. The method according to Claim 21, wherein the blocking modification is a ligation-blocking modification.

23. The method according to Claim 21 or Claim 22, wherein the blocking modification is selected from the group consisting of: the absence of a 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA; and an inaccessible 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA.

24. The method according to Claim 23, wherein the blocking modification is an inaccessible 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA, and wherein the blocking modification is selected from the group consisting of: an amino modifier, a spacer, a dideoxy base, an inverted dideoxy base, and a 3' phosphate.

25. The method according to any one of Claims 1 to 24, wherein the SSB-bound ssNA hybridization region comprises a random sequence.

26. The method according to any one of Claims 1 to 25, wherein the SSB-bound ssNA hybridization region comprises a universal base.

27. The method according to any one of Claims 1 to 26, wherein the length of the SSB-bound ssNA hybridization region is 10 nucleotides or less.

28. The method according to any one of Claims 1 to 27, wherein the adapter oligonucleotide comprises an adapter for PCR amplification or a complement thereof.

29. The method according to any one of Claims 1 to 28, wherein the adapter oligonucleotide comprises a partial or complete sequencing adapter or a complement thereof.

30. The method according to any one of Claims 1 to 29, further comprising sequencing at least a portion of the ssNA or a derivative thereof.

31. A composition, comprising:
single-stranded nucleic acid binding protein-bound single-stranded nucleic acid (SSB-bound ssNA);
a first adapter oligonucleotide; and
a first splint oligonucleotide comprising an SSB-bound ssNA hybridization region and a first adapter oligonucleotide hybridization region.

32. The composition of Claim 31, further comprising:
a second adapter oligonucleotide; and
a second splint oligonucleotide comprising an SSB-bound ssNA hybridization region and a second adapter oligonucleotide hybridization region.

33. The composition of Claim 31 or Claim 32, wherein the ssNA is from a degraded nucleic acid sample.

34. The composition of Claim 33, wherein the ssNA is from an ancient nucleic acid sample.

35. The composition of any one of Claims 31 to 33, wherein the ssNA is from a forensic nucleic acid sample.

36. The composition of any one of Claims 31 to 35, wherein the ssNA is single-stranded DNA (ssDNA).

37. The composition of Claim 36, wherein the ssDNA is derived from double-stranded DNA (dsDNA).

38. The composition of any one of Claims 31 to 35, wherein the ssNA is single-stranded RNA (ssRNA).

39. The composition of any one of Claims 31 to 38, further comprising a reagent for covalently linking an adapter oligonucleotide end to an end of the SSB-bound ssNA.

40. The composition of Claim 39, wherein the reagent is a ligase.

41. A kit, comprising:
single-stranded nucleic acid binding protein (SSB);
a first adapter oligonucleotide;
a first splint oligonucleotide comprising an SSB-bound ssNA hybridization region and a first adapter oligonucleotide hybridization region; and
instructions for using the SSB, first adapter oligonucleotide, and first splint oligonucleotide to produce a nucleic acid library.

42. The kit of Claim 41, further comprising:
a second adapter oligonucleotide; and
a second splint oligonucleotide comprising an SSB-bound ssNA hybridization region and a second adapter oligonucleotide hybridization region,

wherein the instructions are for using the SSB, first adapter oligonucleotide, first splint oligonucleotide, second adapter oligonucleotide, and second splint oligonucleotide to produce a nucleic acid library.

43. The kit of Claim 41 or 42, wherein the SSB is a single-stranded DNA binding protein.

44. The kit of Claim 41 or 42, wherein the SSB is a single-stranded RNA binding protein.

45. The kit of any one of Claims 41 to 44, further comprising a reagent for linking an end of an adapter oligonucleotide to an end of a single-stranded nucleic acid binding protein-bound single-stranded nucleic acid (SSB-bound ssNA).

46. The kit of Claim 45, wherein the reagent is a ligase.

47. The kit of any one of Claims 41 to 46, wherein an end of an adapter oligonucleotide comprises a blocking modification.

48. The kit of Claim 47, wherein the blocking modification is a ligation-blocking modification.

49. The kit of Claim 47 or Claim 48, wherein the blocking modification is selected from the group consisting of: the absence of a 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA; and an inaccessible 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA.

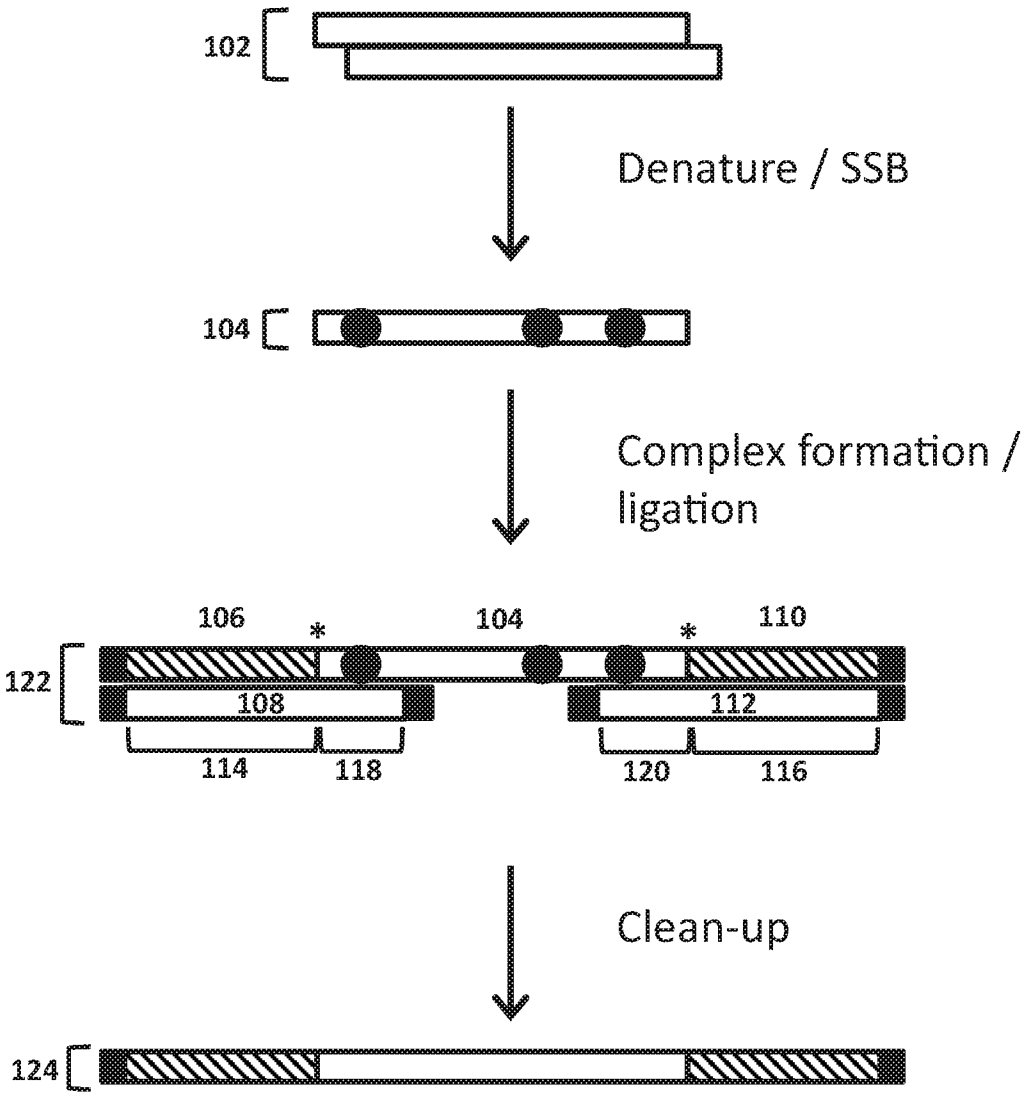
50. The kit of Claim 47 or Claim 48, wherein the blocking modification is an inaccessible 3' OH at the end of the adapter oligonucleotide not adjacent to the SSB-bound ssNA, and wherein the blocking modification is selected from the group consisting of: an amino modifier, a spacer, a dideoxy base, an inverted dideoxy base, and a 3' phosphate.

51. The kit of Claims 41 to 50, wherein the SSB-bound ssNA hybridization region comprises a random sequence.

52. The kit of Claims 41 to 51, wherein the SSB-bound ssNA hybridization region comprises a universal base.

53. The kit of Claims 41 to 52, wherein the length of the SSB-bound ssNA hybridization region is 10 nucleotides or less.

FIG. 1


102
Denature / SSB
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Complex formation / ligation
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104
110
122
108
112
114
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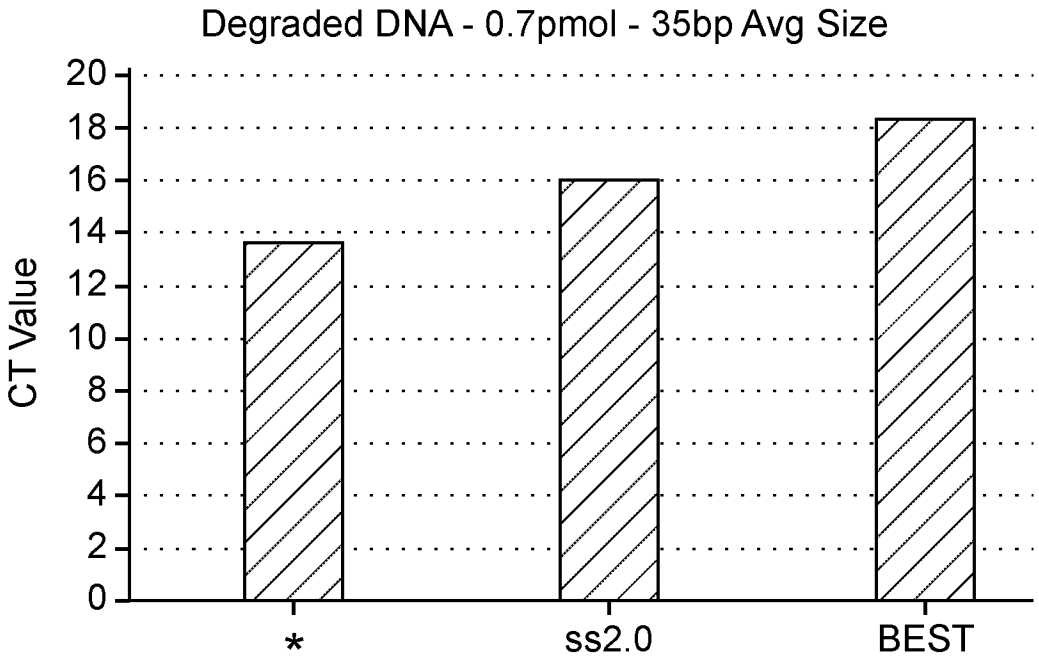
120
116
Clean-up
124

FIG. 2


Degraded DNA - 0.7pmol - 35bp Avg Size
CT Value
20
18
16
14
12
10
8
6
4
2
0
*
ss2.0
BEST

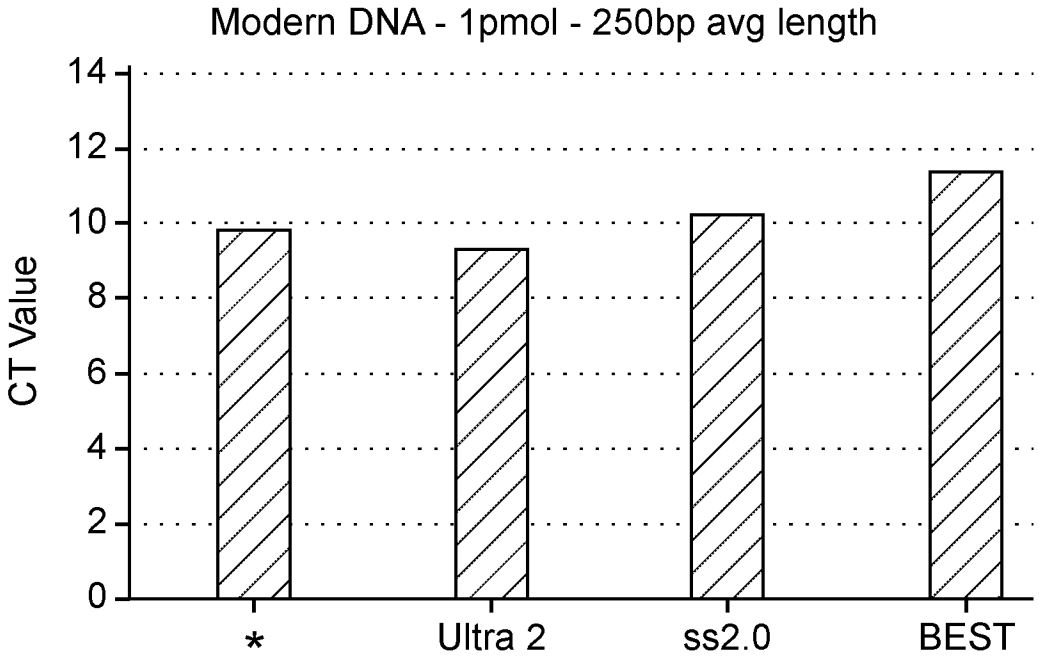
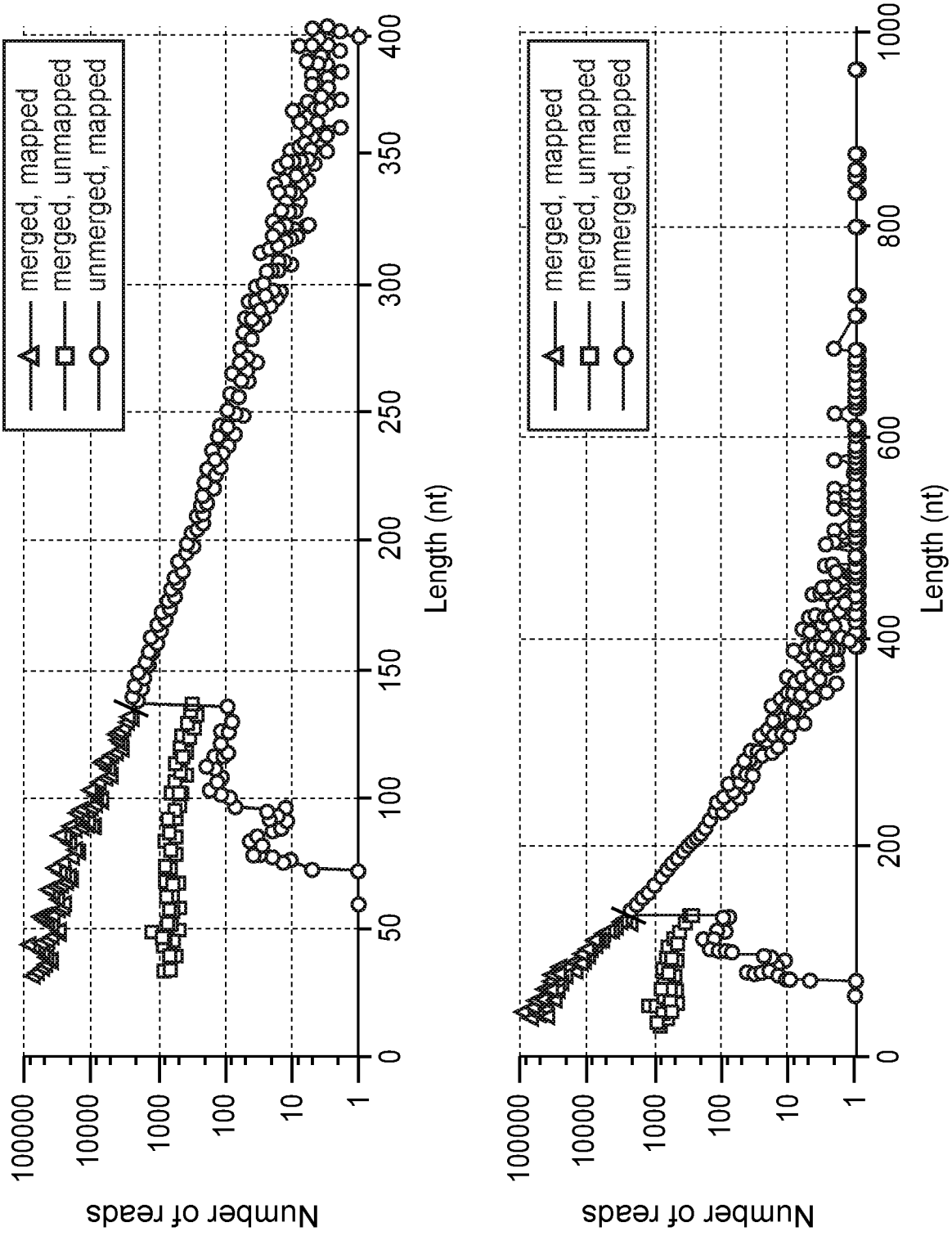

Modern DNA - 1pmol - 250bp avg length
CT Value
14
12
10
8
6
4
2
0
*
Ultra 2
ss2.0
BEST

FIG. 3


merged, mapped
merged, unmapped
unmerged, mapped
Number of reads
Length (nt)
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10000
1000
100
10
1
0
50
100
150
200
250
300
350
400
600
800
1000

FIG. 3 (Cont.)

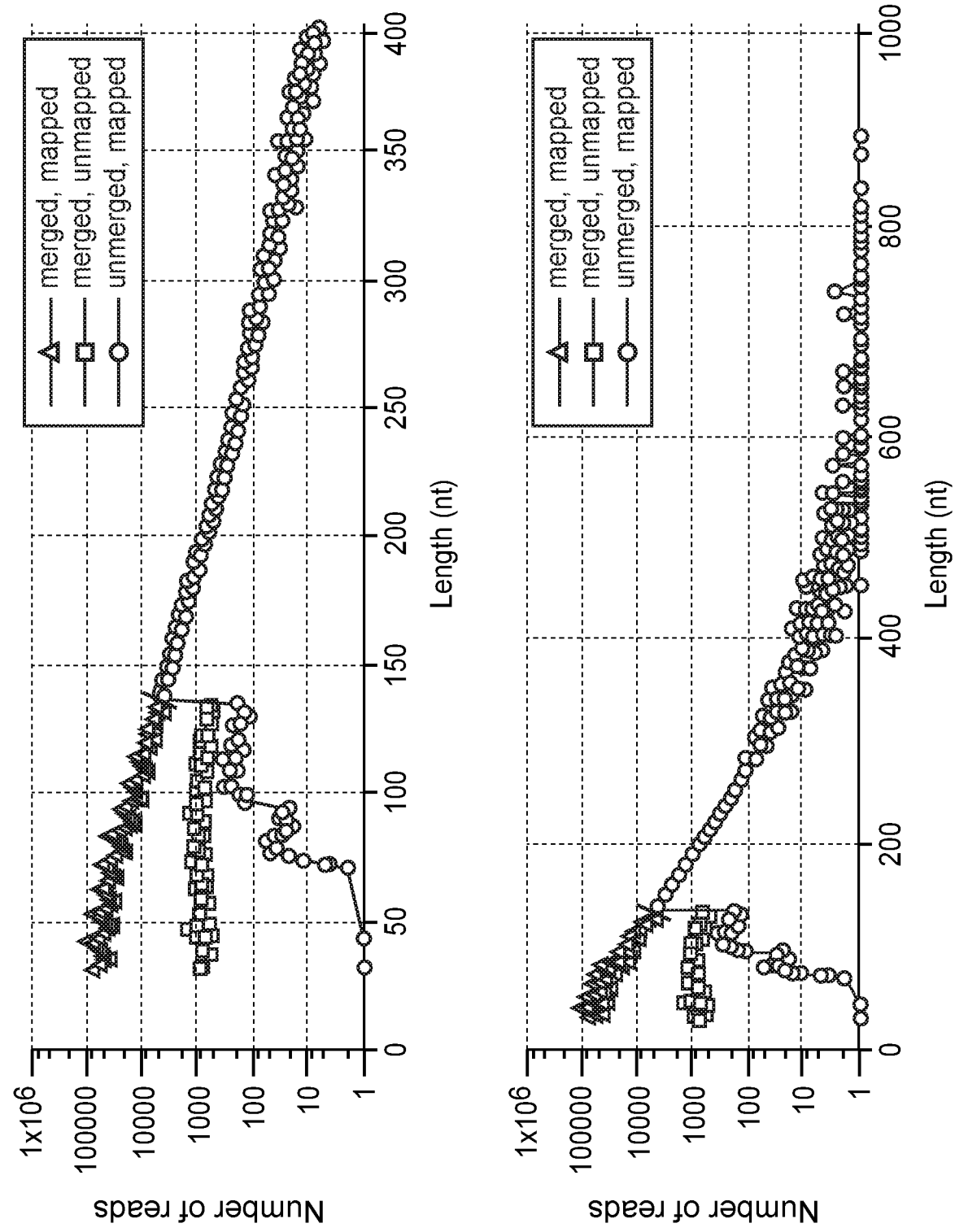
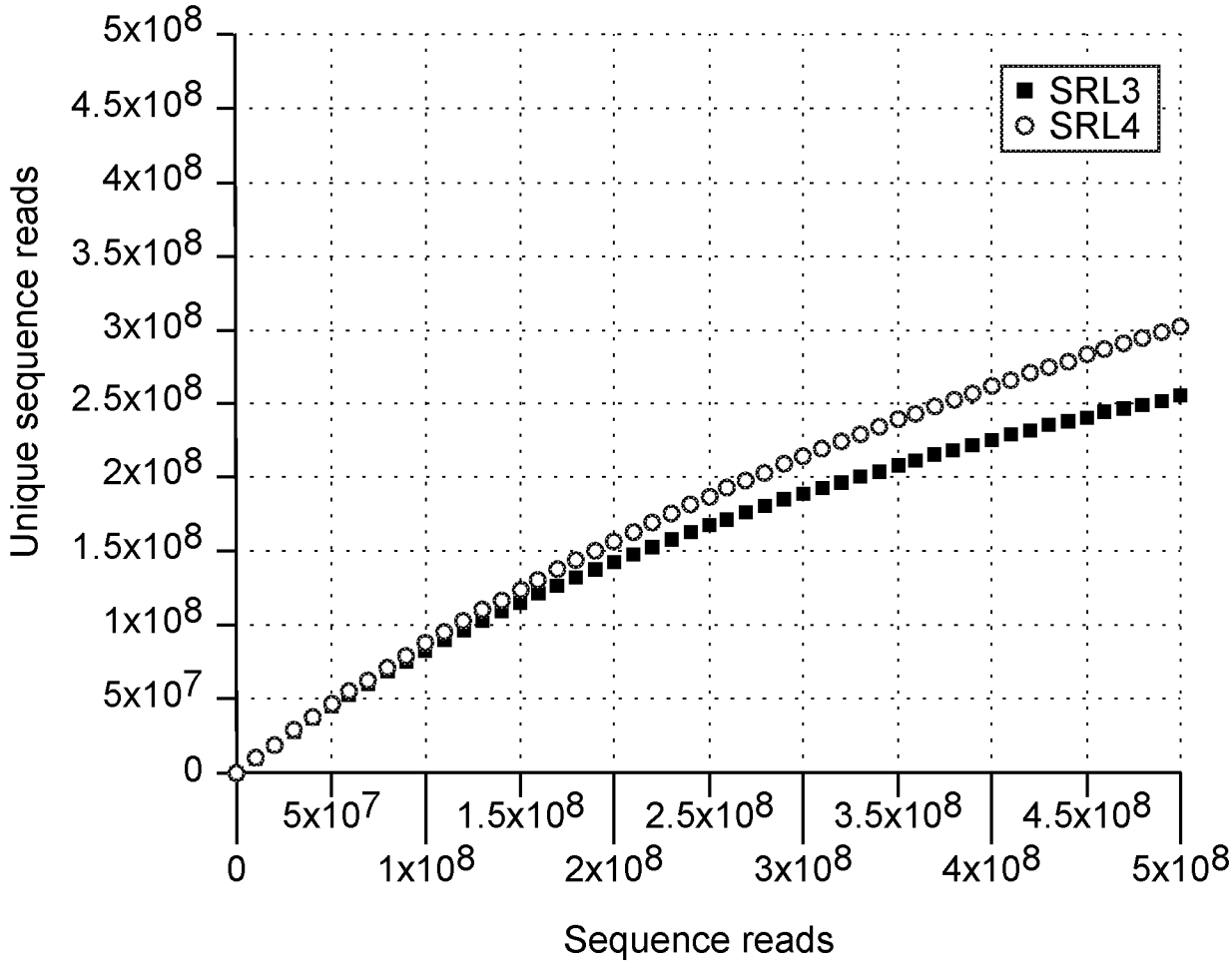

merged, mapped
merged, unmapped
unmerged, mapped
Number of reads
Length (nt)
1x10^6
100000
10000
1000
100
10
1
0
50
100
150
200
250
300
350
400
merged, mapped
merged, unmapped
unmerged, mapped
Number of reads
Length (nt)
1x10^6
100000
10000
1000
100
10
1
0
200
400
600
800
1000

FIG. 4


Unique sequence reads
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4.5x10^8
4x10^8
3.5x10^8
3x10^8
2.5x10^8
2x10^8
1.5x10^8
1x10^8
5x10^7
0
SRL3
SRL4
5x10^7
1.5x10^8
2.5x10^8
3.5x10^8
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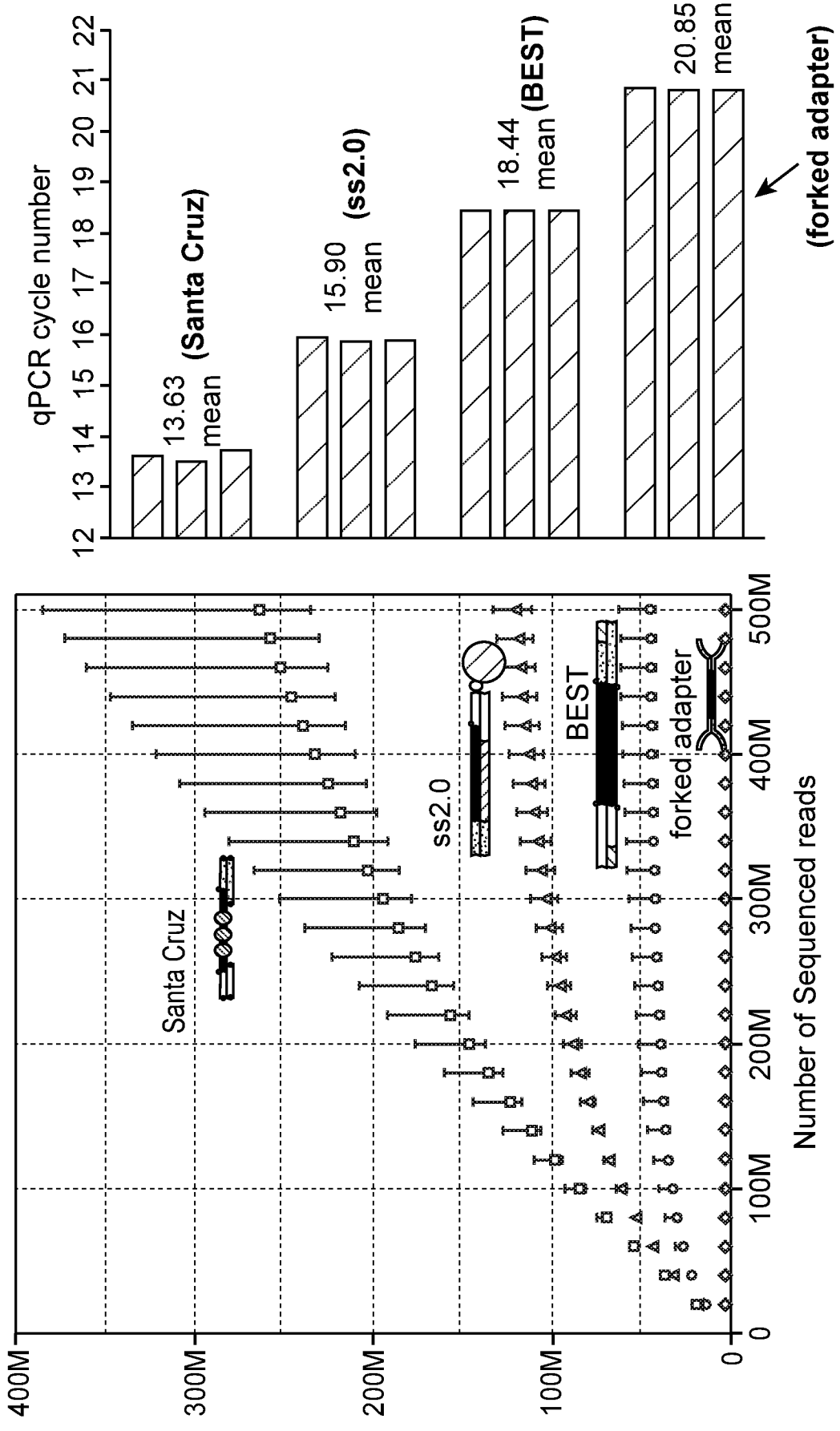
3x10^8
4x10^8
5x10^8
Sequence reads

FIG. 5


qPCR cycle number
12 13 14 15 16 17 18 19 20 21 22
13.63 mean (Santa Cruz)
15.90 mean (ss2.0)
18.44 mean (BEST)
20.85 mean (forked adapter)
Estimated number of unique reads
400M
300M
200M
100M
0
Santa Cruz
ss2.0
BEST
forked adapter
0 100M 200M 300M 400M 500M
Number of Sequenced reads

SEQUENCE LISTING

<110> The Regents of the University of California
Green, Richard
Kapp, Joshua

<120> METHODS OF PRODUCING NUCLEIC ACID LIBRARIES AND COMPOSITIONS AND KITS FOR PRACTICING SAME

<130> UCSC-376WO

<150> 62/681,524
<151> 2018-06-06

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