

METHODS AND COMPOSITIONS FOR SIZE-CONTROLLED HOMOPOLYMER TAILING OF SUBSTRATE POLYNUCLEOTIDES BY A NUCLEIC ACID POLYMERASE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/610,296, filed March 13, 2012, and U.S. Provisional Application No. 61/613,784, filed March 21, 2012, each of which is incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

[0002] The present invention is directed to methods and compositions for adding tails of specific lengths to a substrate polynucleotide. The invention also contemplates methods and compositions for immobilization of tailed substrates to a solid support.

BACKGROUND OF THE INVENTION

[0003] Many current next-generation sequencing (NGS) platforms require special DNA and RNA preparations prior to sequencing. Most commonly used preparations involve addition of adaptor sequences to the ends of double-stranded DNA fragments through a ligation reaction. The reaction typically involves blunt-ended DNA or DNA with a single deoxyadenosine (dA) nucleotide at the 3' end and a high concentration of DNA ligase, and the reaction results in formation of a significant number of chimeric templates. Template-independent polymerases such as DNA-specific terminal deoxynucleotidyl transferase (TdT), and RNA-specific poly(A) and poly(U) polymerases potentially represent an attractive alternative approach for preparation of DNA and RNA for NGS analysis with the challenging caveat that the length of polymeric tails produced by these enzymes varies in a wide range (from 20 to 500 nucleotides), depends on many factors, and is not easy to control, thus reducing their utility for NGS.

SUMMARY

[0003a] In one aspect, the present disclosure refers to a kit comprising:

- a template-independent nucleic acid polymerase;
- an attenuator polynucleotide comprising an attenuator sequence and a sequence W positioned adjacent to the attenuator sequence, wherein the attenuator sequence is from about 10 nucleotides to about 100 nucleotides in length, and wherein the attenuator polynucleotide

comprises a 3' blocking group selected from the group consisting of at least one ribonucleotide, at least one deoxynucleotide, a C3 spacer, a phosphate, a dideoxynucleotide, an amino group, and an inverted deoxythymidine;

an adaptor polynucleotide, wherein the adaptor polynucleotide comprises a sequence X which is complementary to sequence W of the attenuator polynucleotide;

nucleotides complementary to the attenuator sequence; and

a ligase.

[0004] Accordingly, the present disclosure provides a composition comprising a nucleic acid polymerase and an attenuator molecule. The disclosure contemplates that the attenuator molecule is any biomolecule that associates with a tail sequence added to a substrate polynucleotide and controls the addition of a tail sequence to the 3' end of the substrate polynucleotide. The sequence that is added to the substrate polynucleotide is referred to herein

as a tail sequence, or simply a tail, and the process of adding a nucleotide to a substrate polynucleotide is referred to herein as tailing. An attenuator molecule, as used herein, is a polynucleotide, a polypeptide, a polysaccharide, and combinations thereof. In examples where the attenuator molecule is a polynucleotide, it is further contemplated that the polynucleotide is a circular molecule, or that the polynucleotide comprises a peptide nucleic acid, a Schizophyllan polysaccharide, a locked nucleic acid and combinations thereof.

[0005] As described above, the attenuator molecule associates with a tail sequence added to the substrate polynucleotide and controls the addition of nucleotides thereto. In some embodimentsexamples, the attenuator molecule is a polynucleotide that hybridizes to a sequence added to a substrate polynucleotide, wherein the number of nucleotides added to the substrate polynucleotide is essentially equal to the number of nucleotides in the portion of the attenuator molecule that associates with the tail sequence. In some examples, the number of nucleotides added to the substrate polynucleotide is essentially equal to a multiple of the number of nucleotides in the attenuator molecule that associates with the tail sequence. As used herein, the terms "essentially" and "essentially equal" are understood to mean approximately or approximately equal.

[0006] In some examples, the nucleic acid polymerase is a template-independent polymerase. In one example, the nucleic acid polymerase is a DNA polymerase, and in a further example the DNA polymerase is terminal deoxynucleotidyl transferase (TdT). In related examples, the nucleic acid polymerase is a RNA polymerase, which in various examples is selected from the group consisting of poly(A) polymerase, RNA-specific nucleotidyl transferase and poly(U) polymerase.

[0007] It is contemplated by the disclosure that, in some examples, the attenuator molecule comprises a nucleotide selected from the group consisting of 2'-deoxythymidine 5'-monophosphate (dTMP), 2'-deoxyguanosine 5'-monophosphate (dGMP), 2'-deoxyadenosine 5'-monophosphate (dAMP), 2'-deoxycytidine 5'-monophosphate (dCMP), 2'-deoxyuridine 5'-monophosphate (dUMP), thymidine monophosphate (TMP), guanosine monophosphate (GMP), adenosine monophosphate (AMP), cytidine monophosphate (CMP), uridine monophosphate (UMP), a base analog, and combinations thereof. Thus, in certain examples the attenuator molecule comprises an attenuator sequence that is a heteropolymeric sequence or a

homopolymeric sequence, wherein the sequence is either a dinucleotide sequence or a homopolymer sequence.

[0008] In various examples, the attenuator molecule comprises 1 nucleotide, 2 nucleotides, 3 nucleotides, 4 nucleotides, 5 nucleotides, 10 nucleotides, 20 nucleotides, 30 nucleotides, 50 nucleotides, 100 nucleotides or more.

[0009] The attenuator molecule, in some examples, comprises a blocking group and in one example, the blocking group is on the 3' end of the molecule. The blocking group prevents extension of the attenuator molecule by the nucleic acid polymerase. Thus, in various examples the blocking group is selected from the group consisting of at least one ribonucleotide, at least one deoxynucleotide, a C3 spacer, a phosphate, a dideoxynucleotide, an amino group, and an inverted deoxythymidine.

[0010] In some examples of the disclosure, the attenuator molecule further comprises an adaptor sequence, an identifier tag sequence, or both, located 5' to the attenuator sequence homopolymer sequence or dinucleotide sequence. In one example, the attenuator molecule is immobilized. In further examples, a ligase is present in the composition. In another example, the composition further comprises a ligase enzyme.

[0011] In another example, the attenuator molecule is an attenuator-adaptor molecule which comprises an attenuator sequence and further comprises a sequence W positioned adjacent the attenuator sequence and is complementary to a sequence X on a separate polynucleotide; the composition further comprising an adaptor molecule comprising a sequence Y complementary to a sequence V, wherein sequence V is the same length as Y or is less than the same length as sequence Y, the adaptor molecule being a separate molecule from the attenuator-adaptor molecule.

[0012] In some examples of the disclosure, the attenuator molecule further comprises a next generation sequencing (NGS) adaptor sequence. An NGS adaptor sequence differs from an adaptor sequence in that the NGS adaptor sequence is useful in a sequencing platform. In some examples of the disclosure, the attenuator molecule further comprises a next generation sequencing (NGS) adaptor sequence comprises sequence X and sequence Y (for further description of the various sequences (*e.g.*, sequence X, sequence Y, etc.) discussed herein, see Figures and discussion, below), an identifier tag sequence, or a combination thereof, located 5' to

a homopolymer sequence or dinucleotide sequence. In examples wherein the attenuator molecule further comprises an adaptor sequence, it is referred to herein as an attenuator-adaptor molecule. In one example, the attenuator molecule and/or attenuator-adaptor molecule is immobilized. In related examples, sequence X and sequence Y are NGS adaptor sequences that are compatible with Illumina, Ion Torrent, Roche 454 or SOLiD sequencing platforms.

[0013] In further examples, sequence X and sequence Y are on separate attenuator-adaptor molecules, while in other examples sequence X and sequence Y are adjacent to each other on the same attenuator-adaptor molecule.

[0014] The disclosure also provides, in some examples, compositions wherein an adaptor sequence further comprises a cleavable sequence (Z) that is located between sequence X and sequence Y. In some examples, the cleavage site in sequence Z is at least one dU base or RNA base, or a restriction endonuclease site.

[0015] Also provided by the disclosure are compositions wherein the attenuator molecule is single stranded or is at least partially double stranded. By "partially double stranded" is meant that the attenuator molecule comprises a single stranded portion and a double stranded portion. In some examples wherein the attenuator molecule is at least partially double stranded, the partially double stranded attenuator molecule is produced by annealing a portion of the attenuator molecule to an adaptor molecule (which comprises an adaptor sequence that, in some examples, is an NGS adaptor sequence) to which it is complementary. In some examples, annealing is (a) between an attenuator molecule and a separate adaptor molecule, or (b) annealing occurs in a single attenuator molecule that forms a hairpin structure, thus the attenuator molecule comprises both a homopolymeric sequence or dinucleotide sequence and an adaptor sequence.

[0016] In still further examples, the attenuator molecule comprises a sequence W that is fully complementary to adaptor sequence X. In some examples, sequence W is also all or partially complementary to adaptor sequence Y.

[0017] In various examples, the attenuator molecule comprises a homopolymeric sequence selected from the group consisting of poly (dA), poly (dT), poly (dC), poly (dG), poly (dU), poly (rA), poly (U), poly (rC), poly (rG) and a heteropolymeric, or a dinucleotide, sequence

comprising combinations of: (i) dA and rA bases, (ii) dT, dU and U bases, (iii) dC and rC bases, or (iv) dG and rG bases.

[0018] In further examples, the attenuator molecule comprises deoxyribonucleotides and is degradable with a DNA-specific nuclease. In some of these examples, the DNA-specific nuclease is DNase I. In further examples, a composition provided by the disclosure comprises a single strand circularization ligase, including but not limited to CircLigase and/or CircLigase II.

[0019] The disclosure also provides, in some examples, a composition that comprises a DNA polymerase which lacks proofreading activity, and Kapa HiFi Polymerase, which possesses proofreading activity. In additional examples, a composition of the disclosure comprises a ligase enzyme.

[0020] Further examples of the disclosure provide a composition that comprises a restriction endonuclease capable of cleaving sequence Z, which is located between sequence X and sequence Y and comprises a restriction endonuclease site, when hybridized to a complementary X'Z'Y' polynucleotide.

[0021] In some examples, a composition is provided wherein a partially double stranded adaptor sequence comprised of sequence V and sequence Y wherein V is a truncated complement of Y and comprises a blocked 3' end, such that the partially double stranded adaptor can be blunt ligated to a double stranded substrate molecule. In another example, sequence V is fully complementary to sequence Y.

[0022] The disclosure further contemplates compositions wherein the attenuator molecule is degradable. In some examples, the attenuator molecule comprises dU bases and is degradable by incubation with a dU-glycosylase (which creates abasic sites) followed by incubation at a temperature that is above 80° C (introduces breaks within abasic sites), or a mixture of dU-glycosylase and an apurinic/apyrimidinic endonuclease. Thus, the disclosure provides compositions, in various examples, wherein the attenuator molecule comprises dU bases and incubation with a dU-glycosylase destabilizes the attenuator molecule, or incubation with a dU-glycosylase and subsequent incubation at a temperature that is above 80° C degrades the attenuator molecule, or the attenuator molecule is incubated with a mixture of dU-glycosylase and an apurinic/apyrimidinic endonuclease. In further examples, the attenuator molecule comprises a ribonucleotide and is degradable with a ribonuclease under conditions sufficient for

ribonuclease activity. In related examples, the ribonuclease is selected from the group consisting of RNase H, RNase HII, RNase A, and RNase T1.

[0023] In further examples, the attenuator molecule comprises deoxyribonucleotides and is degradable with a DNA-specific nuclease. In some of these examples, the DNA-specific nuclease is DNase I.

[0024] The disclosure also provides a method of extending a substrate polynucleotide comprising incubating the substrate polynucleotide with a composition as described herein under conditions sufficient to allow addition of a tail sequence to the 3' end of the substrate polynucleotide, and wherein the addition of the tail sequence allows association between the tail sequence and the attenuator molecule to form a complex. In another example, the method further comprises degrading the attenuator molecule following extension of the substrate polynucleotide. In a further example, the method further comprises isolating the extended substrate polynucleotide. Other examples of the methods further comprise mixing a composition as described herein with the substrate polynucleotide and a nucleotide that is complementary to the homopolymeric portion of the attenuator molecule.

[0025] According to various examples of the disclosure, the substrate polynucleotide is a single stranded polynucleotide or is a double stranded polynucleotide. The double stranded polynucleotide, in some examples, has a blunt end, a 3' overhanging end, a 3' recessed end, or a free 3' hydroxyl group. The present disclosure provides methods wherein the substrate polynucleotide is double stranded, and in certain examples, the double stranded substrate polynucleotide is produced by annealing a first substrate polynucleotide to a second substrate polynucleotide under conditions sufficient to allow the first substrate polynucleotide to associate with the second substrate polynucleotide. According to further examples of the disclosure, the substrate polynucleotide comprises a free 3' hydroxyl group. The single stranded polynucleotide, in various examples, is prepared by denaturation of fragmented double stranded DNA or from reverse transcription of RNA. The double stranded polynucleotide, in some examples, has a blunt end or a 3' overhanging end with a free 3' hydroxyl group.

[0026] In various examples of the methods of the disclosure, a multiplicity of nucleotides are added to the substrate polynucleotide. The number of nucleotides added to the substrate polynucleotide comprises, in various examples, at least about 1 nucleotide and up to about 10, 20,

50 or 100 nucleotides, at least about 3 nucleotides and up to about 10, 20, 50 or 100 nucleotides, at least about 10 nucleotides and up to about 20, 30, 50 or 100 nucleotides, at least about 5 nucleotides and up to about 10, 20, 50 or 100 nucleotides, at least about 10 nucleotides and up to about 20, 30, 50 or 100 nucleotides, at least about 1 nucleotide and up to about 5, 10, or 20 nucleotides, at least about 3 nucleotides and up to about 5, 10, or 20 nucleotides, at least about 5 nucleotides and up to about 20, 40 or 50 nucleotides, at least 1 nucleotide, at least 2 nucleotides, at least 3 nucleotides, at least 4 nucleotides, at least 5 nucleotides, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50, at least 51, at least 52, at least 53, at least 54, at least 55, at least 56, at least 57, at least 58, at least 59, at least 60, at least 61, at least 62, at least 63, at least 64, at least 65, at least 66, at least 67, at least 68, at least 69, at least 70, at least 71, at least 72, at least 73, at least 74, at least 75, at least 76, at least 77, at least 78, at least 79, at least 80, at least 81, at least 82, at least 83, at least 84, at least 85, at least 86, at least 87, at least 88, at least 89, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, at least 99, at least 100 nucleotides or more.

[0027] Further examples provided by the disclosure include those wherein the attenuator molecule associates with the tail sequence over all or a portion of the attenuator molecule length. In some examples, the attenuator molecule associates with the tail sequence during the process of adding the tail sequence. Association of the attenuator molecule to the tail sequence, in further examples, regulates addition of nucleotides to the substrate polynucleotide. The attenuator molecule additionally comprises an adaptor sequence, in some examples, and the adaptor sequence is ligated by a ligase enzyme to the substrate polynucleotide during addition of the tail sequence to the substrate polynucleotide, and in various examples the ligase is a DNA ligase or a RNA ligase.

[0028] It is also contemplated by the disclosure that the conditions of the method, in some examples, regulate addition of the tail sequence to the substrate polynucleotide. With respect to the conditions that are contemplated to regulate addition of the tail sequence to the substrate

polynucleotide, it is contemplated that, in some examples, the addition of the tail sequence to the substrate polynucleotide is temperature sensitive. Conditions wherein the temperature is between about 4° C and about 50° C are contemplated. In examples wherein a thermostable polymerase is used, the temperature can be above 50° C. Accordingly, in further examples the temperature is between about 50° C and about 90° C.

[0029] In another example, the addition of the tail sequence to the substrate polynucleotide is time sensitive, and in various examples the incubation step is allowed to progress for a length of time in the range of about 0.5 minutes to about 120 minutes. In further examples, the addition of the tail sequence to the substrate polynucleotide is pH sensitive. In some of these examples, the addition of the tail sequence to the substrate polynucleotide is performed under conditions wherein pH is in the range of about pH 5.0 to about pH 9.0.

[0030] In various examples, the substrate polynucleotide is DNA or RNA.

[0031] Methods provided herein also include those wherein the attenuator-adaptor molecule is immobilized. In some examples, the immobilized attenuator-adaptor molecule is ligated by a DNA or RNA ligase to a substrate polynucleotide during addition of a tail sequence to the substrate polynucleotide resulting in immobilization of the substrate polynucleotide. In further examples, the amount of ligase enzyme added to a reaction is from about 0.1 to about 1000 units (U).

[0032] In certain examples, the methods described herein further comprise magnesium in an amount of about 1 mM to about 100 mM. In further examples, the methods further comprise potassium or sodium in an amount of about 1 mM to about 1 M.

[0033] In a specific example of the disclosure, a method of extending a DNA substrate polynucleotide is provided comprising mixing the DNA substrate polynucleotide with TdT enzyme, a degradable attenuator polynucleotide comprising a 3' phosphate and nucleotides that are complementary to the homopolymeric portion of the attenuator polynucleotide, incubating the mixture at 37° C for about 30 minutes, followed by an additional incubation at 70° C for about 10 minutes, degrading the attenuator molecule by adding a DNA glycosylase and incubating the mixture at 37° C for about 5 minutes, and optionally isolating the extended DNA substrate polynucleotide.

[0034] In another example, the disclosure provides a method of extending a DNA substrate polynucleotide comprising mixing a substrate polynucleotide with TdT enzyme, an attenuator polynucleotide comprising two ribonucleotides at the 3' end, and nucleotides that are complementary to the homopolymeric portion of the attenuator polynucleotide, incubating the mixture at 30°C for 30 minutes, followed by an additional incubation at 70°C for about 10 minutes to inactivate the TdT enzyme, and optionally isolating the extended DNA substrate polynucleotide.

[0035] The disclosure further provides, in one example, a method of extending a substrate RNA polynucleotide comprising mixing the substrate RNA polynucleotide with an RNA polymerase, a degradable attenuator polynucleotide and ribonucleotides that are complementary to the homopolymeric portion of the attenuator polynucleotide, incubating the mixture at 30° C for about 30 minutes, followed by an additional incubation at 95° C for about 10 minutes, degrading the attenuator molecule by adding a DNA glycosylase and incubating the mixture at 37° C for about 10 minutes, and optionally isolating the extended substrate polynucleotide.

[0036] In another example, the disclosure provides a method of extending a DNA substrate polynucleotide comprising annealing attenuator and adaptor molecules that are partially complementary to each other by heating a mixture of the attenuator and the adaptor molecules in a suitable buffer to about 100° C and then cooling to about 25° C, wherein the annealing results in a partially double stranded attenuator-adaptor molecule, mixing the DNA substrate polynucleotide with TdT enzyme, a ligase enzyme, the partially double stranded attenuator-adaptor molecule and nucleotides that are complementary to the homopolymeric portion of the attenuator-adaptor molecule, incubating at about 37° C for about 15 to about 30 minutes, and optionally isolating the extended DNA substrate polynucleotide ligated to the attenuator-adaptor molecule.

[0037] The disclosure further provides, in one example, a method of extending a DNA substrate polynucleotide comprising annealing one attenuator-adaptor molecule to a second, biotinylated adaptor molecule that are at least partially complementary to each other by heating a mixture of the two molecules in a suitable buffer to about 100° C and then cooling to about 25° C, wherein the annealing results in a double stranded biotinylated attenuator-adaptor molecule, immobilizing the double stranded biotinylated attenuator-adaptor molecule by mixing the double stranded

biotinylated attenuator-adaptor molecule with a solution comprising a streptavidin-coated magnetic bead at about 25° C for about 30 to about 60 minutes, resulting in immobilization of the double stranded biotinylated attenuator-adaptor molecule to the streptavidin-coated magnetic bead, incubating the immobilized double stranded biotinylated attenuator-adaptor molecule attached to the streptavidin-coated magnetic bead with the DNA substrate polynucleotide, TdT enzyme, a ligase enzyme and nucleotides that are complementary to the homopolymeric portion of the double stranded attenuator molecule at about 37°C for 15 to about 30 minutes, washing the solution with NaOH to remove non-biotinylated single stranded DNA from the beads, and optionally isolating the extended DNA substrate polynucleotide ligated to the double stranded biotinylated attenuator-adaptor molecule.

[0038] In another example, a method of extending a RNA substrate polynucleotide is provided comprising annealing an attenuator molecule and an adaptor molecule that are at least partially complementary to each other by heating a mixture of the two molecules in a suitable buffer to about 100° C and then cooling to about 25° C, wherein the annealing results in a partially double stranded attenuator-adaptor molecule; mixing an RNA substrate polynucleotide with poly (A) or poly(U) enzyme, a ligase enzyme, the partially double stranded attenuator-adaptor molecule and ribonucleotides that are complementary to the single-stranded homopolymeric portion of the partially double stranded attenuator molecule; incubating at about 30° C to about 37° C for about 15 – 30 minutes; and optionally isolating the extended RNA substrate polynucleotide ligated to the attenuator-adaptor molecule.

[0039] In a further example, the disclosure provides a method of extending and immobilizing an RNA substrate polynucleotide comprising annealing an attenuator-adaptor molecule to a biotinylated adaptor molecule that are at least partially complementary to each other by heating a mixture of the two molecules in a suitable buffer to about 100° C and then cooling to about 25° C, wherein the annealing results in a partially double stranded biotinylated attenuator-adaptor molecule; immobilizing the partially double stranded biotinylated attenuator-adaptor molecule by mixing the partially double stranded biotinylated attenuator-adaptor molecule with a solution comprising a streptavidin-coated magnetic bead at about 25° C for about two hours, resulting in immobilization of the partially double stranded biotinylated attenuator-adaptor molecule to the streptavidin-coated magnetic bead; incubating the immobilized partially double stranded

biotinylated attenuator-adaptor molecule attached to the streptavidin-coated magnetic bead with the RNA substrate polynucleotide, poly(A) or poly(U) polymerase, a ligase enzyme and ribonucleotides that are complementary to the single stranded homopolymeric portion of the partially double stranded attenuator-adaptor molecule at about 30° C to about 37° C for about 15 - 30 minutes; washing the solution with NaOH to remove non-biotinylated single stranded polynucleotide from the beads; and optionally isolating the extended and immobilized RNA substrate polynucleotide ligated to the double stranded biotinylated attenuator-adaptor molecule.

[0040] The disclosure also provides, in various examples, methods wherein a DNA polymerase and dNTPs are mixed to perform a polymerase extension of a substrate polynucleotide, said polymerase extension occurring subsequent to controlled homopolymer tailing and leading to incorporation of NGS adaptor sequence(s) sequence X, sequence Y or sequences X and Y 3' to the substrate homopolymer that are complementary to the additional sequence X' and sequence Y' that are 5' to the homopolymer of the attenuator molecule.

[0041] In further examples, NGS adaptor sequence X, sequence Y or sequences X and Y are optionally ligated by a ligase enzyme to the substrate polynucleotide during addition of nucleotides to the substrate polynucleotide. In other examples, NGS adaptor sequence X, sequence Y or sequences X and Y are optionally ligated by a ligase enzyme to the substrate polynucleotide after addition of nucleotides to the substrate polynucleotide. In related examples, the ligase is a DNA ligase or a RNA ligase.

[0042] In still further examples, an attenuator molecule sequence W is optionally truncated with respect to sequences X' and Y' to allow a full-length X'Y' polynucleotide primer to displace the truncated attenuator and enable polymerase extension to create a double stranded adapted substrate molecule. As used herein, an "adapted molecule" is a substrate molecule that has undergone a tailing and ligation reaction.

[0043] The disclosure further provides examples wherein the substrate molecule, following homopolymer addition and polymerase extension, is optionally incubated with a single stranded DNA circularization ligase that results in circularization of the adapted single stranded DNA molecule. In one example, circularization of the attenuator molecule comprising sequence X and sequence Y is prevented by degradation.

[0044] In another example, the substrate molecule, following homopolymer addition and ligation, is optionally incubated with a single stranded DNA circularization ligase which results in circularization of the adapted single stranded DNA molecule.

[0045] In a further example of the disclosure, circularization of the XZY adaptor molecule is prevented by formation of a double-stranded or partially double-stranded attenuator-adaptor molecule.

[0046] Examples of the disclosure contemplate cleavage of the circular DNA molecule at sequence Z, said cleavage resulting from incubation with, for example and without limitation, dU glycosylase and an apurinic/apyrimidinic endonuclease in examples wherein Z comprises dU bases.

[0047] Further examples of the disclosure include cleavage of the circular DNA molecule at sequence Z, said cleavage resulting from incubation with RNase H, RNase H II (in examples wherein Z comprises RNA bases) or by a restriction enzyme, following hybridization of an oligonucleotide complementary to the XZY junction.

[0048] In further examples, the disclosure provides a method of extending a DNA substrate polynucleotide comprising: mixing the DNA substrate polynucleotide with a polymerase enzyme and a ligase enzyme, an adaptor polynucleotide comprising NGS adaptor sequences X and Y and cleavable sequence Z, wherein the adaptor optionally comprises a 3' ribonucleotide and a 5' phosphate, and is annealed to an attenuator with a truncated NGS adaptor sequence W and 3' block, and deoxynucleotides that are complementary to the homopolymeric portion of the attenuator polynucleotide; performing the tailing and ligation simultaneous reaction, heat inactivating the polymerase and ligase enzymes, followed by incubation with a single strand specific circularization ligase enzyme; optionally including a cleavage reaction at the Z sequence or an amplification reaction with reverse X and Y primers, either of which is performed to resolve the circular molecule into a completed linear NGS library molecule.

[0049] In another example, the disclosure provides a method of extending a DNA substrate polynucleotide comprising mixing a DNA substrate polynucleotide with a polymerase enzyme, an attenuator with NGS adaptor sequences X' and Y' and comprising a 3' extension block and a cleavage site as described herein, and deoxynucleotides that are complementary to the homopolymeric portion of the attenuator polynucleotide; performing the tailing reaction, heat

inactivating the polymerase enzyme, followed by addition of a DNA polymerase and dNTP mix to perform a polymerase extension to the tailed substrate polynucleotide to incorporate NGS adaptor sequences X and Y 3' of the homopolymer addition on the substrate; the attenuator-template polynucleotide is then degraded with RNase or dU-glycosylase, followed by incubation with a single strand specific circularization ligase enzyme; optionally including a cleavage reaction at a Z sequence or an amplification reaction with reverse X and Y primers, either of which is performed to resolve the circular molecule into a completed linear NGS library molecule.

[0050] In another example, the disclosure provides a method of extending a DNA substrate polynucleotide comprising mixing the DNA substrate polynucleotide with a polymerase enzyme and a ligase enzyme, an adaptor polynucleotide comprising the NGS adaptor sequence X, wherein the adaptor optionally comprises a 3' extension block and a 5' phosphate, and is annealed to an attenuator with a truncated NGS adaptor sequence W and 3' block, and deoxynucleotides that are complementary to the homopolymeric portion of the attenuator polynucleotide; performing the tailing and ligation simultaneous reaction, heat inactivating the polymerase and ligase enzymes, followed by incubation with a primer complementary to full-length NGS adaptor sequence X' that is capable of displacing the attenuator molecule, a DNA polymerase and dNTPs optionally including dUTP to perform an extension reaction to generate second strand and a double stranded substrate molecule with a blunt end; performing a ligation with T4 DNA ligase and a blunt-end adaptor which is formed by annealing two polynucleotides comprising NGS adaptor sequence Y and a truncated complement (sequence V) and a 3' phosphate, wherein the Y polynucleotide is ligated to the 5' phosphate of the substrate molecule to complete a linear NGS library molecule; optionally the synthesized strand is degraded using dU glycosylase and heat to 95° C.

[0051] A further example of the disclosure provides a method of extending a DNA substrate polynucleotide comprising: mixing the DNA substrate polynucleotide with a polymerase enzyme, an attenuator with NGS adaptor sequences X' and comprising a 3' extension block and a cleavage site, and deoxynucleotides that are complementary to a homopolymeric portion of the attenuator polynucleotide; performing a tailing reaction, heat inactivating the polymerase enzyme, followed by addition of a proofreading DNA polymerase and dNTP mix to perform a polymerase extension to incorporate NGS adaptor sequence X 3' of the homopolymer addition

on the substrate and to remove non-complementary bases from the attenuator 3' end to enable polymerase extension to generate a blunt end double stranded substrate; performing a ligation with T4 DNA ligase and a blunt-end adaptor molecule which is formed by annealing two polynucleotides comprising NGS adaptor sequence Y and a truncated complement (sequence V) and a 3' phosphate, where the Y polynucleotide is ligated to the 5' phosphate of the substrate molecule to complete a linear NGS library molecule; optionally the synthesized strand is degraded using dU glycosylase and heat to between about 90°C and 100°C. In various examples, the mixture is heated to between about 90°C and 95°C, or between about 95°C and 100°C, or is about 90°C, or about 91°C, or about 92°C, or about 93°C, or about 94°C, or about 95°C, or about 96°C, or about 97°C, or about 98°C, or about 99°C, or about 100°C.

[0052] In still another example, a method of extending an RNA substrate polynucleotide is provided comprising: mixing the RNA substrate polynucleotide with a Poly(A) or Poly(U) enzyme and T4 DNA ligase, a DNA adaptor polynucleotide comprising the NGS adaptor sequence X, wherein the adaptor optionally comprises a 3' extension block and a 5' phosphate, and is annealed to an RNA attenuator with a truncated NGS adaptor sequence W and 3' block, and ribonucleotides that are complementary to the homopolymeric portion of the attenuator polynucleotide; performing a tailing and ligation simultaneous reaction, heat inactivating the Poly(A) or Poly(U) enzyme, followed by incubation with a primer complementary to full-length NGS adaptor sequence X' that is capable of displacing the attenuator molecule truncated for sequence X', a reverse transcriptase and dNTPs to perform an extension reaction to generate a second strand and a double stranded substrate molecule; performing a magnetic bead DNA purification step followed by strand denaturation at 95° C; a second simultaneous tailing and ligation is then performed using a polymerase enzyme and a ligase enzyme and a DNA attenuator-adaptor molecule that will add a homopolymer and Y NGS adaptor sequence to the free 3' end of the substrate molecule, thus completing a linear NGS library molecule where an optional DNA purification step is required.

[0053] The disclosure also provides a method of NGS library synthesis using controlled homopolymer tailing and ligation followed by circularization of the substrate polynucleotide (Figure 25), where fragmented single stranded DNA is prepared by denaturation of fragmented double stranded DNA or by reverse transcription of RNA, wherein controlled tailing and ligation is performed in the presence of the polymerase enzyme, a ligase enzyme, nucleotide D (where D

= dATP, dTTP or dGTP) and an attenuator-adaptor molecule, wherein the attenuator-adaptor molecule is formed by annealing two polynucleotides: polynucleotide XZY and polynucleotide W(H)_n.

[0054] Polynucleotide XZY comprises a 5' phosphate, and a ribonucleotide base or other blocking group at the 3' end which prevents addition of a homopolymer tail. Sequences X and Y represent adaptor sequences of an NGS library and optional ID tag. Optional sequence Z is used for cleavage and is comprised of a dU base, an RNA base or a restriction endonuclease site. Polynucleotide W(H)_n comprises two sequences: a 5' sequence W that is complementary to the 5' portion of the polynucleotide XZY and a homopolymeric attenuator sequence (H)_n where H is A, T or C base, and n = 10-30. Polynucleotide W(H)_n comprises a 3' blocking group including but not limited to a 3' phosphate, dideoxynucleotide, C3-spacer, inverted thymidine or one or more ribonucleotides that specifically inhibit addition of bases by the polymerase enzyme. In the reaction, the polymerase enzyme will add a limited number of bases to the 3' end of DNA substrates followed by ligation of the XZY adaptor molecule by a ligase enzyme. In certain examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is between about 1-50 dA or dT bases or between about 1-50 dG bases. In further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is from about 1 nucleotide and up to about 5, 10, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 5 and up to about 10, 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 10 and up to about 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 9 to about 12 dA, dT or dG nucleotides, or from about 6 to about 8 dA, dT or dG nucleotides. In additional examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 21, at least about 22, at least about 23, at least about 24, at least about 25, at least about 26, at least about 27, at least about 28, at least about 29, at least about 30, at least about 31, at least about 32, at least about 33, at least about 34, at least about 35, at least about 36, at least about 37, at least about 38, at least about 39, at least about 40, at least about 41, at least about 42, at least about 43, at least about 44, at least about 45, at least about 46, at least about 47, at least about 48, at least about 49 or at least about 50 dA, dT or dG

nucleotides. In further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more dA, dT or dG nucleotides.

[0055] Following optional heat inactivation of the polymerase enzyme and the ligase enzyme, incubation with single stranded circularization ligase (Epicentre /Illumina) results in circularization of the adapted single stranded DNA molecule. Circularization of the XZY polynucleotide is prevented by formation of a double-stranded attenuator-adaptor complex between the XZY and W(H)_n polynucleotides. Circularized NGS libraries can be directly used for cluster formation (emulsion PCR in the case of Ion Torrent, 454 and Solid platforms, and bridge amplification in the case of Illumina platforms) or directly for sequencing (PacBio). Optionally, cleavage of the circular DNA molecule is achieved by dU glycosylase and an apurinic/apyrimidinic endonuclease (in the case when Z sequence comprises a dU base), by RNase H or RNase H II (in the case when Z sequence comprises an RNA base) or by a restriction endonuclease. In the latter two cases, hybridization of a polynucleotide complementary to the XZY junction is necessary to provide a template for RNase H or restriction endonuclease. Alternatively, an amplification reaction is performed to resolve the circular to linear form.

[0056] The disclosure also provides an alternative method for NGS library synthesis using controlled homopolymer tailing followed by polymerase extension and circularization (Figure 26), wherein tailing of single stranded substrate polynucleotides is performed in the presence of a polymerase enzyme, nucleotide D (where D = dATP, dTTP or dGTP) and an attenuator-template polynucleotide, where the attenuator-template polynucleotide comprises three sequences: a 5' sequence Y' that is complementary to adaptor sequence Y, sequence X' that is complementary to adaptor sequence X and a homopolymeric attenuator sequence (H)_n where H is A, T or C base, and n = 10-30. The attenuator-template polynucleotide additionally comprises a phosphate group, ribonucleotide or other blocking group at the 3' end. Sequences X and Y represent adaptor sequences for an NGS library, and optional ID tag. Both the attenuator sequence and X',Y' sequences have degradable bases such as ribonucleotides or dU. In the presence of the attenuator molecule, a polymerase enzyme adds a limited number of bases to the 3' end of DNA. In certain examples, the number of bases added by the polymerase to the 3' end of a DNA

substrate is between about 1-50 dA or dT bases or between about 1-50 dG bases. In further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is from about 1 nucleotide and up to about 5, 10, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 5 and up to about 10, 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 10 and up to about 12, 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 10 to about 13 dA, dT or dG nucleotides. In additional examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 21, at least about 22, at least about 23, at least about 24, at least about 25, at least about 26, at least about 27, at least about 28, at least about 29, at least about 30, at least about 31, at least about 32, at least about 33, at least about 34, at least about 35, at least about 36, at least about 37, at least about 38, at least about 39, at least about 40, at least about 41, at least about 42, at least about 43, at least about 44, at least about 45, at least about 46, at least about 47, at least about 48, at least about 49 or at least about 50 dA, dT or dG nucleotides. In further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more dA, dT or dG nucleotides.

[0057] After optional heat inactivation of the polymerase enzyme and the ligase enzyme, a DNA polymerase and dNTP mix are added to perform polymerase extension which results in addition of X and Y sequences to the 3' end of the homopolymeric sequence (D)_n. Following attenuator-template degradation by addition of dU-glycosylase or RNase, incubation with single strand circularization ligase (Epicentre /Illumina) results in circularization of the adapted single stranded DNA molecule. If necessary, an optional amplification is performed to resolve the circular to linear form. Alternatively an optional sequence Z between X and Y is used to create linear form following hybridization of a complementary polynucleotide to the XZY domain and a cleavage reaction (as described herein above). In certain examples, the optional amplification that is performed to resolve the circular to linear form is a technique including, without limitation, inverse PCR.

[0058] Also contemplated is a method of NGS library synthesis comprising controlled homopolymer tailing and ligation followed by reverse strand synthesis and blunt adaptor ligation (Figure 27), wherein tailing and ligation are performed in the presence of TdT enzyme, E.coli DNA ligase, nucleotide D (where D = dATP, dTTP or dGTP) and an attenuator-adaptor molecule that is formed by annealing two polynucleotides: polynucleotide X and polynucleotide W(H)_n. Polynucleotide X comprises a 5' phosphate and a 3' blocking group, where sequence X comprises an NGS library adaptor sequence and optional ID tag. Polynucleotide W(H)_n consists of two sequences: a 5' sequence W that is complementary to the 5' portion of polynucleotide X and a homopolymeric attenuator sequence (H)_n where H is A, T or C base, and n = 10-30, and additionally comprises a 3' blocking group. In the reaction, the polymerase enzyme adds a limited number of bases to the 3' end of DNA substrates followed by ligation of the attenuator-adaptor molecule by a ligase enzyme. In certain examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is between about 1-50 dA or dT bases or between about 1-50 dG bases. In further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is from about 1 nucleotide and up to about 5, 10, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 5 and up to about 10, 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 10 and up to about 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 9 to about 12 dA, dT or dG nucleotides, or from about 6 to about 8 dA, dT or dG nucleotides. In additional examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 21, at least about 22, at least about 23, at least about 24, at least about 25, at least about 26, at least about 27, at least about 28, at least about 29, at least about 30, at least about 31, at least about 32, at least about 33, at least about 34, at least about 35, at least about 36, at least about 37, at least about 38, at least about 39, at least about 40, at least about 41, at least about 42, at least about 43, at least about 44, at least about 45, at least about 46, at least about 47, at least about 48, at least about 49 or at least about 50 dA, dT or dG nucleotides. In further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,

12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more dA, dT or dG nucleotides.

[0059] Following optional heat inactivation of the polymerase enzyme and the ligase enzyme, addition of a DNA polymerase and primer containing 5' sequence X', 1-10 H bases (or, in further embodiments, from about 1 to about 5, 7, or 10 H bases, or from about 5 to about 6, 8 or 10 H bases, or from about 6 to about 8 H bases) at the 3' end and optional dU bases leads to reverse strand replication, thus forming either a double-stranded blunt end (in the presence of proofreading DNA polymerase) or double-stranded end with 3' dA base (in the case when DNA polymerase lacks the proofreading activity), and wherein the polymerization mix optionally comprises dUTP. Ligation of a blunt-end or dT-adaptor is achieved by a ligase enzyme, wherein the adaptor to be ligated is formed by two polynucleotides: polynucleotide Y, where Y is a second NGS adaptor and polynucleotide V that is complementary to the 3' portion of polynucleotide Y. The 3' end of polynucleotide V has a phosphate blocking group. Ligation results in covalent attachment of the 3' end of polynucleotide Y to the 5' phosphate of the original DNA fragment, whereas no ligation is formed between the 5' end of polynucleotide V and the 3' end of the primer-extension product. Optionally the replicated DNA strand and primer X' is degraded by incubation with dU glycosylase and 95°C incubation. In some examples, the incubation takes place between about 90°C and 100°C. In further examples, the mixture is heated to between about 90°C and 95°C, or between about 95°C and 100°C, or is about 90°C, or about 91°C, or about 92°C, or about 93°C, or about 94°C, or about 95°C, or about 96°C, or about 97°C, or about 98°C, or about 99°C, or about 100°C.

[0060] The disclosure also provides an alternative method for NGS library construction comprising controlled tailing and polymerization followed by reverse strand synthesis and blunt ligation (Figure 28), wherein tailing is performed in the presence of the polymerase enzyme, nucleotide D (where D = dATP, dTTP or dGTP) and attenuator-template polynucleotide comprising two sequences: a 5' sequence X' that is complementary to NGS adaptor X, and a homopolymeric attenuator sequence (H)_n where H is A, T or C base, and n = 10-30, and additionally comprises a 3' ribonucleotide and internal degradable bases such as ribonucleotide or dU. In the presence of the attenuator molecule, the polymerase enzyme adds a limited number of bases to the 3' end of DNA substrates and following optional heat inactivation, the inclusion of a DNA polymerase with proofreading activity and dNTP mix extends the DNA substrate to

include sequence X and also removes excessive non-complementary bases from the attenuator polynucleotide 3' terminus and then leads to reverse strand replication which results in a double-stranded blunt end. The polymerization mix can optionally comprise dUTP. Ligation of a blunt-end adaptor is achieved by T4 DNA ligase, where the blunt-end adaptor is formed by two polynucleotides: polynucleotide Y, where Y is a sequence of second NGS adaptor and polynucleotide V that is complementary to the 3' portion of the polynucleotide Y, and where the 3' end of polynucleotide V comprises a phosphate or other blocking group. Blunt ligation results in covalent attachment of the 3' end of polynucleotide Y to the 5' phosphate of the original DNA fragment, whereas no ligation occurs between the 5' end of the polynucleotide V and the 3' end of the primer-extension product. Optionally, the replicated DNA strand and primer X' is degraded by incubation with dU glycosylase and 95°C incubation. In some examples, the incubation takes place between about 90°C and 100°C. In further examples, the mixture is heated to between about 90°C and 95°C, or between about 95°C and 100°C, or is about 90°C, or about 91°C, or about 92°C, or about 93°C, or about 94°C, or about 95°C, or about 96°C, or about 97°C, or about 98°C, or about 99°C, or about 100°C.

[0061] Another method of the disclosure for NGS library preparation comprises two sequential tailing and ligation reactions (Figure 29), wherein the first tailing and ligation reaction is performed in the presence of a polymerase enzyme, a ligase enzyme, nucleotide D (where D = dATP, dTTP or dGTP) and attenuator-adaptor molecule, which is formed by annealing two polynucleotides: polynucleotide X and polynucleotide W(H)_n, where polynucleotide X comprises a 5' phosphate, 3' blocking group and NGS adaptor sequence and optional ID tag; and polynucleotide W(H)_n which comprises two sequences: 5' sequence W that is complementary to the 5' portion of polynucleotide X and a homopolymeric attenuator sequence (H)_n where H is A, T or C base, and n = 10-30, and additionally comprises a 3' blocking group.

[0062] In the reaction, a polymerase enzyme adds a limited number of bases to the 3' end of DNA substrates (1-50 dA or dT bases and 1-50 dG bases), followed by ligation of the first attenuator-adaptor molecule by a ligase enzyme. Following optional heat inactivation of the polymerase and the ligase, addition of a primer containing a 5' sequence X' complementary to sequence X and 1-10 H bases at the 3' end results in primer annealing to the adaptor sequence X and displacement of the attenuator polynucleotide. Additionally, primer X' can comprise an rH blocking base at the 3' end to prevent polymerase-mediated primer tailing with the second tailing

and ligation reaction. Addition of a DNA polymerase will extend the primer and replicate the reverse strand of the substrate, where the reaction is optionally stopped by EDTA and purified by magnetic bead-based DNA purification before a second addition of polymerase enzyme adds a limited number of bases to the 3' end of DNA extension product (1-50 dA or dT bases and 1-50 dG bases) in the presence of the second attenuator that is annealed to a second NGS adaptor Y, followed by ligation of the second attenuator-adaptor molecule comprising the second NGS adaptor Y by a ligase enzyme. Optionally, the polymerase and the ligase are heat inactivated, followed by magnetic bead based DNA purification. As above, and in further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is from about 1 nucleotide and up to about 5, 10, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 5 and up to about 10, 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 10 and up to about 15, 20, 30, 40 or 50 dA, dT or dG nucleotides, or from about 9 to about 12 dA, dT or dG nucleotides, or from about 6 to about 8 dA, dT or dG nucleotides. In additional examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 21, at least about 22, at least about 23, at least about 24, at least about 25, at least about 26, at least about 27, at least about 28, at least about 29, at least about 30, at least about 31, at least about 32, at least about 33, at least about 34, at least about 35, at least about 36, at least about 37, at least about 38, at least about 39, at least about 40, at least about 41, at least about 42, at least about 43, at least about 44, at least about 45, at least about 46, at least about 47, at least about 48, at least about 49 or at least about 50 dA, dT or dG nucleotides. In further examples, the number of bases added by the polymerase to the 3' end of a DNA substrate is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more dA, dT or dG nucleotides.

[0063] The disclosure also provides methods for NGS library synthesis using a combination of 5' tailed random primer extension on a substrate polynucleotide followed by controlled tailing and ligation (Figure 30), where intact or fragmented single stranded nucleic acid is contacted under conditions that allow hybridization to a random primer comprising two domains, a 5'

domain X where sequence X comprises in various examples an NGS adaptor sequence and optional identification tag and a 3' domain comprising a random sequence n, thereby enabling primer hybridization to any region within a nucleic acid sample, and in the presence of a polymerase and nucleotides under appropriate reaction conditions, primer extension and incorporation of adaptor X at the 5' terminus of the extension products is achieved. The primer in various examples comprise a 5' label, including but not limited to biotin. Following an optional purification or nucleotide degradation step, tailing and ligation reactions are performed on the extension product in the presence of a polymerase, a ligase enzyme, nucleotide D (where D = A, T or G) and an attenuator-adaptor molecule that is formed by annealing two polynucleotides: polynucleotide Y and polynucleotide V(H)_n. Polynucleotide Y comprises a 5' phosphate and a 3' blocking group, where sequence Y comprises a second NGS library adaptor sequence and optional identification tag. Polynucleotide V(H)_n consists of two sequences: a 5' sequence V that is complementary to the 5' portion of polynucleotide Y and a homopolymeric attenuator sequence (H)_n where H is A, T or C base, and n = 10-30, and additionally comprises a 3' blocking group. In the reaction, the polymerase enzyme adds a limited number of bases (*i.e.*, from about 1 to about 50 nucleotides) to the 3' end of primer extension products followed by ligation of the Y/V(H)_n attenuator-adaptor molecule by a ligase enzyme, thus completing the addition of both the first and second NGS adaptors.

[0064] The disclosure also contemplates a method of targeted NGS library synthesis using a combination of 5' tailed target-specific primer extension on a substrate polynucleotide followed by controlled tailing and ligation (Figure 31), where intact or fragmented single stranded nucleic acid is subject to hybridization by an plurality of target-specific primers comprising two domains, a 5' domain X common to all primers of the plurality where sequence X comprises an NGS adaptor sequence and optional identification tag, and a 3' domain unique to each primer of the plurality, each comprising a target-specific sequence, thereby enabling primer hybridization to any desired plurality of targets within a nucleic acid sample, and in the presence of a polymerase and nucleotides under appropriate reaction conditions, primer extension and incorporation of adaptor X at the 5' terminus of the extension products is achieved. The primer plurality can additionally comprise a 5' label including but not limited to biotin. Following an optional purification or nucleotide degradation step, tailing and ligation reactions are performed in the presence of a polymerase enzyme, a ligase enzyme, nucleotide D (where D = A, T or G)

is complementary to the 3' portion of polynucleotide Y. The 3' end of polynucleotide V has a phosphate blocking group. Ligation results in covalent attachment of the 3' end of polynucleotide Y to the 5' phosphate of the plurality of target-specific fragments, whereas no ligation is formed between the 5' end of polynucleotide V and the 3' end of the target specific primer-extension products. Optionally the completed plurality of target-specific library products are amplified by PCR using NGS adaptor-specific primers.

[0066] In Figure 33, a summary of target-specific NGS library preparation methods involving a controlled tailing and ligation step are depicted. Method 1 summarizes the method described in Figure 32 with optional targeted library bead capture in Method 2. Method 3 summarizes a method in which a whole genome NGS library is constructed and then followed by target-specific primer extension and targeted library bead capture and amplification. Methods 4 and 5 depict alternate workflows to the method presented in Figure 31. In Method 4, a plurality of biotinylated target-specific primers extend select regions from a fragmented nucleic acid sample, followed by blunt or TA ligation of the first NGS adaptor, bead capture, then controlled tailing and ligation to add the second NGS adaptor. Method 5 is similar to 4 except that in the first step target-specific primers comprise 5' tails with an NGS adaptor sequence. In Method 6, following controlled tailing and ligation to introduce a first NGS adaptor and adaptor-specific primer extension, denaturation followed by second NGS adaptor 5' tailed target-specific primer extension completes the NGS library that can be further amplified. In any of the methods for target-specific NGS library preparation, it is contemplated that use of the attenuator molecules described herein enables one to multiplex. In additional examples, use of the attenuator molecules described herein enables one to immobilize the library products to a surface.

[0067] For any of the various examples of whole genome or targeted library construction using the disclosed method of controlled tailing and ligation to introduce an NGS adaptor sequence, a second adaptor sequence is introduced by either a second controlled tailing and ligation or is introduced by blunt or TA ligation to a double-stranded substrate following a primer extension reaction. As shown in Figure 37, various methods are contemplated for blunt and TA adaptor ligation that either ligate one strand selectively or ligates both strands. Thus, the adaptor sequence comprises, in various examples, a blunt end or a T-overhanging end (thus allowing TA ligation to occur). In additional examples, the adaptor sequence can be blunt-

ended, have a T-base 3'-overhang, 5'-phosphate, or 3'-group blocking ligation (for example and without limitation, a dideoxynucleotide) or a combination thereof.

[0068] The disclosure further provides a method of extending a substrate polynucleotide comprising: (1) incubating a mixture comprising the substrate polynucleotide with (i) a polymerase enzyme; (ii) a composition comprising an attenuator molecule comprising an attenuator sequence and further comprises a sequence W positioned adjacent the attenuator sequence and is complementary to an adaptor sequence X on a separate polynucleotide; the composition further comprising an adaptor molecule comprising a sequence Y complementary to a sequence V, wherein sequence V is the same length as Y or is less than the same length as sequence Y, the adaptor molecule being a separate molecule from the attenuator-adaptor molecule; and (iii) deoxynucleotides that are complementary to the attenuator sequence of the attenuator molecule, under conditions that allow extension of the substrate polynucleotide to tail the substrate; (2) ligating the adaptor sequence X to the substrate polynucleotide and dissociating the attenuator molecule from the separate polynucleotide; (3) adding a primer complementary to a sequence in the substrate polynucleotide under conditions wherein the primer hybridizes to the substrate polynucleotide; (4) adding a polymerase and deoxynucleotides to perform polymerase extension from the primer to produce a second strand polynucleotide complementary to the substrate polynucleotide and create a double stranded substrate molecule; (5) ligating the adaptor molecule to the double stranded substrate molecule; (6) optionally degrading the second strand polynucleotide. In some examples, the primer is sufficiently complementary to a sequence in the substrate polynucleotide to hybridize under appropriate conditions to sequence X. In further examples, the primer is a target-specific primer sufficiently complementary to hybridize under appropriate conditions to a sequence in the substrate molecule other than sequence X. In additional examples, the substrate polynucleotide is a single strand DNA polynucleotide, and in still further examples the substrate polynucleotide is a ribonucleic acid (RNA).

[0069] A kit comprising any of the compositions disclosed herein is also provided.

BRIEF DESCRIPTION OF THE FIGURES

WHAT IS CLAIMED IS:

1. A kit comprising:

a template-independent nucleic acid polymerase;

an attenuator polynucleotide comprising an attenuator sequence and a sequence W positioned adjacent to the attenuator sequence, wherein the attenuator sequence is from about 10 nucleotides to about 100 nucleotides in length, and wherein the attenuator polynucleotide comprises a 3' blocking group selected from the group consisting of at least one ribonucleotide, at least one deoxynucleotide, a C3 spacer, a phosphate, a dideoxynucleotide, an amino group, and an inverted deoxythymidine;

an adaptor polynucleotide, wherein the adaptor polynucleotide comprises a sequence X which is complementary to sequence W of the attenuator polynucleotide;

nucleotides complementary to the attenuator sequence; and

a ligase.

2. The kit of claim 1, wherein the sequence W is positioned 5' adjacent to the attenuator sequence.

3. The kit of claim 1, wherein the template-independent nucleic acid polymerase is terminal deoxynucleotidyl transferase (TdT).

4. The kit of claim 1, wherein the template-independent nucleic acid polymerase is an RNA-specific nucleotidyl transferase selected from the group consisting of poly(A) polymerase and poly(U) polymerase.

5. The kit of claim 1, wherein the attenuator polynucleotide comprises a degradable base.

6. The kit of claim 1, wherein the adaptor polynucleotide further comprises a 5' phosphate.

7. The kit of claim 1, wherein the ligase is a DNA ligase or an RNA ligase.

8. The kit of claim 1 further comprising a primer, a polymerase, and a second adaptor polynucleotide comprising a sequence Y and a sequence V, wherein sequence V is complementary to sequence Y when sequence V is the same length as sequence Y, or wherein sequence V is complementary to a portion of sequence Y when sequence V is less than the length of sequence Y, the second adaptor polynucleotide being a separate molecule from the attenuator polynucleotide.
9. The kit of claim 1, wherein the attenuator polynucleotide comprises an affinity label for immobilization.
10. The kit of claim 1 wherein the attenuator sequence of the attenuator polynucleotide comprises a homopolymeric sequence selected from the group consisting of poly (dA), poly (dT), poly (dC), poly (dG), and poly (dU).
11. The kit of claim 1, wherein the attenuator sequence of the attenuator polynucleotide comprises a homopolymeric sequence selected from the group consisting of poly (rA), poly (U), poly (rC), and poly (rG).
12. The kit of claim 1, wherein the attenuator sequence of the attenuator polynucleotide comprises a heteropolymeric sequence selected from the group consisting of (i) dA and rA bases, (ii) dT, dU and U bases, (iii) dC and rC bases, and (iv) dG and rG bases.
13. The kit of claim 1, wherein the attenuator sequence of the attenuator polynucleotide comprises a dinucleotide sequence comprising a plurality of random sequences comprised of the following dinucleotide combinations: (i) dG and dC; (ii) dA and dT; (iii) dG and dT; (iv) dG and dA; (v) dA and dC; or (vi) dC and dT.
14. The kit of claim 1, wherein the attenuator sequence of the attenuator polynucleotide comprises a dinucleotide sequence comprising a mixture of a ribonucleotide and a deoxyribonucleotide.
15. The kit of claim 1, wherein sequence X is a sequence complementary to any one of SEQ ID NOS: 44-51.

16. The kit of claim 1, wherein the attenuator sequence is from about 10 nucleotides to about 20 nucleotides in length.
17. The kit of claim 1, wherein the attenuator polynucleotide and the adaptor polynucleotide are part of the same polynucleotide.
18. The kit of claim 17, wherein the attenuator polynucleotide and adaptor polynucleotide form a hairpin structure.