

challenges relating to the sheer number of different genomes in a given sample and the degree of homology between members, presenting a complex problem for already laborious procedures.

[0005] The existing techniques for detecting sequence variations are particularly ineffective in detecting fusion gene variations and chromosome rearrangements. Often the 'partner' gene fused with the rearranged gene is not known, which makes the detection challenging. Fusions genes may also be difficult to detect if the junction site is not observed.

SUMMARY

[0006] In view of the foregoing, there is a need for alternative and/or robust methods and compositions of detecting rare sequence variations, particularly rare sequence changes and gene fusion events. The compositions and methods of the present disclosure address this need, and provide additional advantages as well. In particular, the various examples of the disclosure provide amplicons containing multiple copies of a target polynucleotide that can be used for massively parallel sequencing methods. Using amplicons containing multiple copies of a target polynucleotide, a target polynucleotide can be sequenced more than once, decreasing the error in sequencing rare sequence variants and fusion genes.

[0006a] In one aspect, provided here is a method for enriching amplicons comprising a concatemer of at least two or more copies of a target polynucleotide, the method comprising: (a) generating a concatemer comprising a single-stranded polynucleotide from a circular target polynucleotide by multiple cycles of amplification with a first primer, wherein each cycle of the multiple cycles of amplification comprises denaturation and a denaturing temperature, primer annealing at an annealing temperature, and primer elongation at an elongation temperature, and wherein the first primer comprises a first 3' end that specifically hybridizes to the target polynucleotide via sequence complementarity and a first 5' end comprising a first common sequence that does not specifically hybridize to the target polynucleotide via sequence complementarity; (b) generating a plurality of extension products containing one or more copies of the target polynucleotide by extension of a second primer comprising a second 3' end that specifically hybridizes to the concatemer via sequence complementarity and a second 5' end comprising a second common sequence that does not specifically hybridize to the concatemer via sequence complementarity, wherein the first common sequence and the second common sequence each comprise at least 10 contiguous nucleotides at a 5' end and are at least 90% identical when optimally aligned; and

(c) amplifying the plurality of extension products of step (b) under conditions to generate a plurality of amplicons, wherein amplicons comprising at least 2 or more copies of the target polynucleotide are enriched.

[0007] In one example, a method for enriching amplicons comprising a concatemer of at least two or more copies of a target polynucleotide is disclosed. The method comprises (a) generating a concatemer comprising a single-stranded polynucleotide from a circular target polynucleotide by extension of a first primer, the first primer comprising a first 3' end that specifically hybridizes to the target polynucleotide via sequence complementarity and a first 5' end comprising a first common sequence that does not specifically hybridize to the target polynucleotide via sequence complementarity, (b) generating a plurality of extension products containing one or more copies of the target polynucleotide by extension of a second primer comprising a second 3' end that specifically hybridizes to the concatemer via sequence complementarity and a second 5' end comprising a second common sequence that does not specifically hybridize to the concatemer via sequence complementarity, wherein the first common sequence and the second common sequence each comprise at least 10 contiguous nucleotides at a 5' end and are at least 90% identical when optimally aligned, and (c) amplifying the plurality of extension products of step (b) under conditions to generate a plurality of amplicons, wherein amplicons comprising at least 2 or more copies of the target polynucleotide are enriched. In some examples, step (a) is effected by a polymerase

having strand-displacement activity. In some examples, the first common sequence and the second common sequence are identical. In some examples, the amplifying of step (c) comprises primer extension of a third primer, wherein the third primer comprises a sequence that specifically hybridizes to the first common sequence or the second common sequence via sequence complementarity. In some examples, the amplifying step of (c) yields a percentage of amplicons having two or more copies of the target polynucleotide that is greater than a percentage of amplicons having fewer than two copies of the target polynucleotide. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 90%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 80%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 60%. In some examples, the extension products form stem loop structures comprising intramolecular hybridization between (i) the first common sequence and a complement of the second common sequence, or (ii) the second common sequence and a complement of the first common sequence. In some examples, the formation of the stem loop products is effected by performing the amplifying of step (c) with an annealing step held at a temperature within $\pm 5^{\circ}\text{C}$ of a melting temperature of the third primer. In some examples, the formation of the stem loop products is effected by performing the amplifying of step (c) with an annealing step held at a temperature of less than 70°C . In some examples, the stem loop structures comprise intramolecular hybridization of at least 9 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 15 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 20 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 25 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 30 base pairs. In some examples, step (b) comprises no more than 6 cycles of extension of the second primer. In some examples, step (b) comprises no more than 8 cycles of extension of the second primer. In some examples, step (b) comprises no more than 10 cycles of extension of the second primer. In some examples, the first common sequence, the second common sequence, and the hybridizing sequence of the third primer all have melting temperatures (T_m 's) within $\pm 5^{\circ}\text{C}$ of one another. In some examples, the circular target polynucleotide is a circularized cell free DNA. In some examples, the circular target polynucleotide is a circularized fragment of genomic DNA. In some examples, the circular target polynucleotide comprises sequences resulting from a chromosomal

rearrangement. In some examples, the chromosomal rearrangement is at least one of a deletion, duplication, inversion, and translocation. In some examples, the combined length of sequence portions of the target polynucleotide corresponding to, from 5' to 3' along the target polynucleotide, (i) sequence complementary to the first 3' end, (ii) sequence identical to the second 3' end, and (iii) intervening sequence between (i) and (ii), is 75 nucleotides or less. In some examples, at least 50% of concatemers comprise a target polynucleotide of at least 75 nucleotides in length. In some examples, the circular target polynucleotide is single-stranded. In some examples, the method further comprises sequencing the plurality of amplicons produced in step (c). In some examples, the sequencing is performed without selectively purifying amplicons comprising two or more copies of the target polynucleotide from amplicons comprising only one copy of the target polynucleotide. In some examples, the method further comprises purifying amplicons in the plurality of amplicons produced in step (c) that comprise two or more copies of the target polynucleotide. In some examples, the method further comprises sequencing the purified amplicons. In some examples, a plurality of different target polynucleotides is amplified in the same reaction mixture.

[0008] In another example, a reaction mixture for enriching amplicons comprising a concatemer of at least two or more copies of a target polynucleotide is disclosed. In one example, the reaction mixture comprises (a) a circular target polynucleotide, (b) a first primer comprising a first 3' end that specifically hybridizes to the target polynucleotide via sequence complementarity and a first 5' end comprising a first common sequence that does not specifically hybridize to the target polynucleotide via sequence complementarity, and (c) a second primer comprising a second 3' end that specifically hybridizes to the concatemer via

sequence complementarity and a second 5' end comprising a second common sequence that does not specifically hybridize to the concatemer via sequence complementarity, wherein the first common sequence and the second common sequence each comprise at least 10 contiguous nucleotides at a 5' end and are at least 90% identical when optimally aligned, and the concatemer is an extension product of the first primer. In some examples, the first common sequence and the second common sequence are identical. In some examples, the reaction mixture is contained in a container. In some examples, the container is a well, a plate, a tube, a chamber, a flow cell, or a chip. In some examples, the reaction mixture further comprises a third primer having a sequence that specifically hybridizes to the first common sequence or the second common sequence via sequence complementarity. In some examples, the first common sequence, the second common sequence, and the hybridizing

sequence of the third primer all have melting temperatures (T_m 's) within $\pm 5^\circ\text{C}$ of one another. In some examples, the first common sequence and the second common sequence each comprise at least 15 nucleotides. In some examples, the circular target polynucleotide is a circularized cell free DNA. In some examples, the circular target polynucleotide is a circularized fragment of genomic DNA. In some examples, the circular target polynucleotide comprises sequences resulting from a chromosomal rearrangement. In some examples, the chromosomal rearrangement is at least one of a deletion, duplication, inversion, and translocation. In some examples, the combined length of sequence portions of the target polynucleotide corresponding to, from 5' to 3' along the target polynucleotide, (i) sequence complementary to the first 3' end, (ii) sequence identical to the second 3' end, and (iii) intervening sequence between (i) and (ii), is 75 nucleotides or less. In some examples, the circular target polynucleotide is single-stranded.

[0009] In another example, a kit for enriching amplicons comprising a concatemer of at least two or more copies of a target polynucleotide is disclosed. In one example, the kit comprises (a) a first primer comprising a first 3' end that specifically hybridizes to the target polynucleotide via sequence complementarity and a first 5' end comprising a first common sequence that does not specifically hybridize to the target polynucleotide via sequence complementarity; (b) a second primer comprising a second 3' end that specifically hybridizes to the concatemer via sequence complementarity and a second 5' end comprising a second common sequence that does not specifically hybridize to the concatemer via sequence complementarity, wherein the first common sequence and the second common sequence each comprise at least 10 contiguous nucleotides at a 5' end and are at least 90% identical when optimally aligned, and the concatemer is an extension product of the first primer; and (c) a third primer having a sequence that specifically hybridizes to the first common sequence or the second common sequence via sequence complementarity. In some examples, the first common sequence and the second common sequence are identical. In some examples, the first common sequence, the second common sequence, and the hybridizing sequence of the third primer all have melting temperatures (T_m 's) within $\pm 5^\circ\text{C}$ of one another. In some examples, the combined length of sequence portions of the target polynucleotide corresponding to, from 5' to 3' along the target polynucleotide, (i) sequence complementary to the first 3' end, (ii) sequence identical to the second 3' end, and (iii) intervening sequence between (i) and (ii), is 75 nucleotides or less.

[0010] In another example, a system for designing primers for use in enriching amplicons comprising a concatemer of at least two or more copies of a target polynucleotide is disclosed. In one example, the system comprises (a) a computer configured to receive a customer request to design primers for amplifying a specified target sequence; (b) computer readable medium comprising codes that, upon execution by one or more processors, design at least three primers for the amplification of the target sequence, wherein the at least three primers comprise: (i) a first primer comprising a first 3' end that specifically hybridizes to the target polynucleotide via sequence complementarity and a first 5' end comprising a first common sequence that does not specifically hybridize to the target polynucleotide via sequence complementarity; (ii) a second primer comprising a second 3' end that specifically hybridizes to the concatemer via sequence complementarity and a second 5' end comprising a second common sequence that does not specifically hybridize to the concatemer via sequence complementarity, wherein the first common sequence and the second common sequence each comprise at least 10 contiguous nucleotides at a 5' end and are at least 90% identical when optimally aligned, and the concatemer is an extension product of the first primer; and (iii) a third primer having a sequence that specifically hybridizes to the first common sequence or the second common sequence via sequence complementarity; and (c) a report generator that sends a report to a recipient, wherein the report contains sequences of the at least three primers. In some examples, the first common sequence and the second common sequence are identical. In some examples, the first common sequence, the second common sequence, and the hybridizing sequence of the third primer all have melting temperatures (T_m 's) within $\pm 5^\circ\text{C}$ of one another. In some examples, the combined length of sequence portions of the target polynucleotide corresponding to, from 5' to 3' along the target polynucleotide, (i) sequence complementary to the first 3' end, (ii) sequence identical to the second 3' end, and (iii) intervening sequence between (i) and (ii), is 75 nucleotides or less.

[0011] In an example, the present disclosure provides a method of conducting rolling circle amplification. The method comprises (a) providing a circular polynucleotide comprising a target polynucleotide; (b) subjecting an amplification reaction mixture to multiple cycles of rolling circle amplification to generate a plurality of amplification products comprising concatemers, wherein the amplification reaction mixture comprises (i) a polymerase having strand displacement activity, (ii) the circular polynucleotide, and (iii) primers; and wherein each cycle of the multiple cycles of rolling circle amplification comprises denaturation at a denaturing temperature, primer annealing at an annealing temperature, and primer elongation at an elongation temperature for a given elongation time period, to generate the plurality of

amplification products; and wherein the plurality of amplification products generated is characterized in that it contains a higher proportion of concatemers having at least two copies of the target polynucleotide as compared to a plurality of amplification products generated by utilizing one cycle of amplification under comparable conditions for denaturation and primer annealing but with an elongation time period comparable to a sum of the elongation time period of the multiple cycles.

[0012] In an example, the present disclosure provides a method of increasing a proportion of concatemers having at least two copies of a target polynucleotide generated by a rolling circle amplification. The method comprises (a) providing a circular polynucleotide comprising a target polynucleotide; (b) subjecting an amplification reaction mixture to multiple cycles of rolling circle amplification to generate a plurality of amplification products comprising concatemers, wherein the amplification reaction mixture comprises (i) a polymerase having strand displacement activity, (ii) the circular polynucleotide, and (iii) primers; and wherein each cycle of the multiple cycles of rolling circle amplification comprises denaturation at a denaturing temperature, primer annealing at an annealing temperature, and primer elongation at an elongation temperature for a given elongation time period, to generate the plurality of amplification products; thereby increasing a proportion of concatemers having at least two copies of the target polynucleotide. In some examples, the proportion of concatemers in the plurality of amplification products having at least two copies of the target polynucleotide is increased as compared to a plurality of amplification products generated by utilizing one cycle of amplification under comparable conditions for denaturation and primer annealing but an elongation time period comparable to a sum of the elongation time period of the multiple cycles.

[0013] In some examples, the polymerase is selected from the group consisting of: Bsu DNA polymerase, Vent polymerase, Bst DNA polymerase, phi29 DNA polymerase, PyroPhage 3173 polymerase, any variant thereof, and any fragment thereof.

[0014] In some examples, the plurality of amplification products exhibits a mean fragment length of about 180 base pairs when the circular polynucleotide utilized in the reaction mixture comprises a human cell free DNA (cfDNA). In some examples, the plurality of amplification products exhibits a median fragment length of about 170 base pairs when the circular polynucleotide utilized in the reaction mixture comprises a human cell free DNA (cfDNA). In some examples, the plurality of amplification products exhibits a distribution of fragment lengths from about 40 bases to about 450 bases when the circular polynucleotide utilized in the reaction mixture comprises a human cell free DNA (cfDNA). In

some examples, the plurality of amplification products exhibits a distribution of fragment lengths from about 100 bases to about 200 bases when the circular polynucleotide utilized in the reaction mixture comprises a human cell free DNA (cfDNA).

[0015] In some examples, the proportion of concatemers having at least two copies of the target polynucleotide is increased by at least about 1%. In some examples, the method further comprises supplementing the reaction mixture with the polymerase subsequent to at least one cycle of the multiple cycles of rolling circle amplification.

[0016] In some examples, the circular polynucleotide has a length of between about 40 bases and about 500 bases. In some examples, the circular polynucleotide comprises cell free DNA (cfDNA). In some examples, the circular polynucleotide comprises a fragment of genomic DNA. In some examples, the circular polynucleotide comprises a sequence resulting from a chromosomal rearrangement. In some examples, the chromosomal rearrangement is at least one of a deletion, duplication, inversion and translocation. In some examples, the circular polynucleotide is double stranded. In some examples, the circular polynucleotide is single stranded.

[0017] In some examples, a plurality of different circular polynucleotides is amplified in the amplification reaction mixture. In some examples, the multiple cycles comprises at least two cycles. In some examples, each cycle of the multiple cycles comprises denaturation at a denaturing temperature of between about 75 °C and about 95 °C for about 5 seconds to about 60 seconds, (ii) primer annealing at an annealing temperature of between about 45 °C and about 65 °C for about 5 seconds to about 60 seconds, and (iii) primer elongation at an elongation temperature of between about 65 °C and about 75 °C for an elongation time period of about 30 seconds to about 10 minutes. In some examples, each cycle of the multiple cycles comprises denaturation at a denaturing temperature of about 80 °C for about 15 seconds to about 30 seconds, (ii) primer annealing at an annealing temperature of about 50 °C for about 15 seconds to about 45 seconds, and (iii) primer elongation at an elongation temperature of about 70 °C for an elongation time period of about 3 minutes to about 10 minutes.

[0018] In some examples, the primers comprise random sequences capable of randomly hybridizing to and priming various regions of a circular polynucleotide for primer extension. In some examples, the primers comprise a gene specific sequence capable of hybridizing to and priming regions of a circular polynucleotide for primer extension in a sequence specific manner. In some examples, the primers comprise a first primer comprising (i) a first 3' end that specifically hybridizes to the circular polynucleotide via sequence

complementarity and (ii) a first 5' end comprising a first common sequence that does not specifically hybridize to the target polynucleotide via sequence complementarity, wherein a concatemer comprising a single-stranded polynucleotide is generated during the multiple cycles of rolling circle amplification by extension of the first primer using the circular polynucleotide as template. In some examples, the primers comprise a second primer comprising (i) a second 3' end that specifically hybridizes to the concatemer comprising the single-stranded polynucleotide via sequence complementarity and (ii) a second 5' end comprising a second common sequence that does not specifically hybridize to the concatemer via sequence complementarity, wherein a plurality of extension products containing one or more copies of the target polynucleotide are generated during the multiple cycles of rolling circle amplification by extension of the second primer using the concatemer as template. In some examples, the first common sequence and the second common sequence each comprise at least 10 contiguous nucleotides at a 5' end and are at least 90% identical when optimally aligned. In some examples, the first common sequence and the second common sequence are identical.

[0019] In some examples, the method further comprises amplifying the plurality of extension products under conditions to generate a plurality of amplicons, wherein amplicons comprising at least 2 or more copies of the target polynucleotide are enriched. In some examples, amplifying comprises primer extension of a third primer, wherein the third primer comprises a sequence that specifically hybridizes to the first common sequence or the second common sequence via sequence complementarity. In some examples, amplifying yields a percentage of amplicons having two or more copies of the target polynucleotide that is greater than a percentage of amplicons having fewer than two copies of the target polynucleotide. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 5%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 10%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 20%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 30%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 40%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 60%. In some examples, the percentage of amplicons having two or more copies of the target polynucleotide is at least 80%. In some examples,

the percentage of amplicons having two or more copies of the target polynucleotide is at least 90%.

[0020] In some examples, the plurality of extension products form stem loop structures comprising intramolecular hybridization between (i) the first common sequence and a complement of the second common sequence, or (ii) the second common sequence and a complement of the first common sequence. In some examples, formation of the stem loop structures is effected by performing the amplifying with an annealing step held at a temperature within $\pm 5^{\circ}\text{C}$ of a melting temperature of the third primer. In some examples, formation of the stem loop structures is effected by performing the amplifying with an annealing step held at a temperature of less than about 70°C . In some examples, the stem loop structures comprise intramolecular hybridization of at least 9 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 15 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 20 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 25 base pairs. In some examples, the stem loop structures comprise intramolecular hybridization of at least 30 base pairs. In some examples, the first common sequence, the second common sequence, and the hybridizing sequence of the third primer all have melting temperatures (T_m 's) within $\pm 5^{\circ}\text{C}$ of one another.

[0021] In some examples, a combined length of sequence portions of a the target polynucleotide corresponding to, from 5' to 3' along the target polynucleotide, (i) sequence complementary to the first 3' end, (ii) sequence identical to the second 3' end, and (iii) intervening sequence between (i) and (ii), is 75 nucleotides or less.

[0022] In some examples, the method further comprises sequencing the plurality of amplification products comprising concatemers. In some examples, the sequencing is performed without selectively separating concatemers having at least two copies of the target polynucleotide from concatemers comprising less than two copies of the target polynucleotide.

[0023] In some examples, the method further comprises separating concatemers comprising at least two copies of the target polynucleotide from concatemers comprising less than two copies of the target polynucleotide. In some examples, the method further comprises sequencing the concatemers comprising at least two copies of the target polynucleotide.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for enriching amplicons comprising a concatemer of at least two or more copies of a target polynucleotide, the method comprising:

(a) generating a concatemer comprising a single-stranded polynucleotide from a circular target polynucleotide by multiple cycles of amplification with a first primer, wherein each cycle of the multiple cycles of amplification comprises denaturation and a denaturing temperature, primer annealing at an annealing temperature, and primer elongation at an elongation temperature, and wherein the first primer comprises a first 3' end that specifically hybridizes to the target polynucleotide via sequence complementarity and a first 5' end comprising a first common sequence that does not specifically hybridize to the target polynucleotide via sequence complementarity;

(b) generating a plurality of extension products containing one or more copies of the target polynucleotide by extension of a second primer comprising a second 3' end that specifically hybridizes to the concatemer via sequence complementarity and a second 5' end comprising a second common sequence that does not specifically hybridize to the concatemer via sequence complementarity, wherein the first common sequence and the second common sequence each comprise at least 10 contiguous nucleotides at a 5' end and are at least 90% identical when optimally aligned; and

(c) amplifying the plurality of extension products of step (b) under conditions to generate a plurality of amplicons, wherein amplicons comprising at least 2 or more copies of the target polynucleotide are enriched.

2. The method of claim 1, wherein the first common sequence and the second common sequence are identical.

3. The method of claim 1, wherein in the amplifying of step (c) comprises primer extension of a third primer, wherein the third primer comprises a sequence that specifically hybridizes to the first common sequence or the second common sequence via sequence complementarity.

4. The method of claim 1, wherein the amplifying step of (c) yields a percentage of amplicons having two or more copies of the target polynucleotide that is greater than a percentage of amplicons having fewer than two copies of the target polynucleotide.

5. The method of claim 4, wherein the percentage of amplicons having two or more copies of the target polynucleotide is at least 60%.

6. The method of claim 1, wherein the extension products form stem loop structures comprising intramolecular hybridization between (i) the first common sequence and a complement of the second common sequence, or (ii) the second common sequence and a complement of the first common sequence.
7. The method of claim 1, wherein the circular target polynucleotide is a circularized cell free DNA.
8. The method of claim 1, wherein the circular target polynucleotide is a circularized fragment of genomic DNA.
9. The method of claim 1, wherein the circular target polynucleotide is single-stranded.
10. The method of claim 1, further comprising sequencing the plurality of amplicons produced in step (c).
11. The method of claim 1, wherein a plurality of different target polynucleotides are amplified in the same reaction mixture.
12. The method of claim 9, wherein sequencing is performed after selectively purifying amplicons comprising more than one copy of the target polynucleotide from amplicons comprising only one copy of the target polynucleotide.