[0056] Fig. 10 shows a pre-PCR amplification sequencing count for each of 1116 IGH VJ bias control molecules and 243 IGH DJ bias control molecules.

[0057] Fig. 11 shows TCRB-primer iterations for synthetic TCRB VJ templates graphed against relative amplification bias.

[0058] Fig. 12 shows IGH primer iterations for synthetic IGH VJ templates graphed against relative amplification bias.

[0059] Fig. 13 shows the relative amplification bias for 27 synthetic IGH DJ templates of the V gene.

[0060] Figs. 14a-d show TCRG-primer iterations for 55 synthetic TCRG VJ templates. Relative amplification bias was determined for the TCRG VJ primers prior to chemical bias control correction (Fig. 14a), 1st iteration of chemical correction (Fig. 14b), 2nd iteration of chemical correction (Fig. 14c), and final iteration of chemical correction (Fig. 14d).

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DETAILED DESCRIPTION OF THE INVENTION

[0061] The present invention provides, in certain embodiments and as described herein, compositions and methods that are useful for reliably quantifying large and structurally diverse populations of rearranged genes encoding adaptive immune receptors, such as immunoglobulins (Ig) and/or T cell receptors (TCR). These rearranged genes may be present in a biological sample containing DNA from lymphoid cells of a subject or biological source, including a human subject.

[0062] A "rearranged nucleic acid molecule," as used herein, can include any genomic DNA, cDNA, or mRNA obtained directly or indirectly from a lymphoid cell line that includes sequences that encode a rearranged adaptive immune receptor.

[0063] Disclosed herein are unexpectedly advantageous approaches for the standardization and calibration of complex oligonucleotide primer sets that are used in multiplexed nucleic acid amplification reactions to generate a population of amplified rearranged DNA molecules from a biological sample containing rearranged genes encoding adaptive immune receptors, prior to quantitative high throughput sequencing of such amplified products. Multiplexed amplification and high throughput sequencing of rearranged TCR and BCR (IG) encoding DNA sequences are described, for example, in Robins et al., 2009 *Blood* 114, 4099; Robins et al., 2010 *Sci. Translat. Med.* 2:47ra64; Robins et al., 2011 *J. Immunol. Meth.* doi:10.1016/j.jim.2011.09. 001; Sherwood et al. 2011 *Sci. Translat. Med.* 3:90ra61; U.S.A.N. 13/217,126 (US Pub. No. 2012/0058902), U.S.A.N. 12/794,507 (US Pub. No. 2010/0330571), WO/2010/151416, WO/2011/106738 (PCT/US2011/026373),

The present invention also provides any one of the embodiments disclosed below and indicated with the numbers 1 to 46.

1. A composition for standardizing the amplification efficiency of an oligonucleotide primer set that is capable of amplifying rearranged nucleic acid molecules encoding one or more adaptive immune receptors in a biological sample that comprises rearranged nucleic acid molecules from lymphoid cells of a mammalian subject, each adaptive immune receptor comprising a variable region and a joining region, the composition comprising:

a plurality of template oligonucleotides having a plurality of oligonucleotide sequences of general formula:

5'-U1-B1-V-B2-R-B3-J-B4-U2-3' [I]

wherein:

(a) V is a polynucleotide comprising at least 20 and not more than 1000 contiguous nucleotides of an adaptive immune receptor variable (V) region encoding gene sequence, or the complement thereof, and each V polynucleotide comprising a unique oligonucleotide sequence;

(b) J is a polynucleotide comprising at least 15 and not more than 600 contiguous nucleotides of an adaptive immune receptor joining (J) region encoding gene sequence, or the complement thereof, and each J polynucleotide comprising a unique oligonucleotide sequence;

(c) U1 is either nothing or comprises an oligonucleotide sequence that is selected from (i) a first universal adaptor oligonucleotide sequence and (ii) a first sequencing platform-specific oligonucleotide sequence that is linked to and positioned 5' to a first universal adaptor oligonucleotide sequence;

(d) U2 is either nothing or comprises an oligonucleotide sequence that is selected from (i) a second universal adaptor oligonucleotide sequence, and (ii) a second sequencing platform-specific oligonucleotide sequence that is linked to and positioned 5' to a second universal adaptor oligonucleotide sequence;

(e) B1, B2, B3, and B4 are each independently either nothing or each comprises an oligonucleotide B that comprises a barcode sequence of 3-25 contiguous nucleotides, wherein each B1, B2, B3 and B4 comprises an oligonucleotide sequence that uniquely identifies, as a paired combination, (i) the unique V oligonucleotide sequence of (a) and (ii) the unique J oligonucleotide sequence of (b);

(f) R is either nothing or comprises a restriction enzyme recognition site that comprises an oligonucleotide sequence that is absent from (a)-(e),

and wherein:

(g) the plurality of template oligonucleotides comprises at least *a* or at least *b* unique oligonucleotide sequences, whichever is larger, wherein *a* is the number of unique adaptive immune receptor V region-encoding gene segments in the subject and *b* is the number of unique adaptive immune receptor J region-encoding gene segments in the subject, and the composition comprises at least one template oligonucleotide for each unique V polynucleotide and at least one template oligonucleotide for each unique J polynucleotide.

2. The composition of claim 1, wherein *a* ranges from 1 to a number of maximum V gene segments in the mammalian genome of the subject.

3. The composition as in any one of claims 1 or 2, wherein *b* ranges from 1 to a number of maximum J gene segments in the mammalian genome of the subject.

4. The composition as in any one of claims 1-3, wherein *a* is 1 or *b* is 1.

5. The composition of claim 1, wherein the plurality of template oligonucleotides comprises at least (*a* x *b*) unique oligonucleotide sequences, where *a* is the number of unique adaptive immune receptor V region-encoding gene segments in the mammalian subject and *b* is the number of unique adaptive immune receptor J region-encoding gene segments in the mammalian subject, and the composition comprises at least one template oligonucleotide for every possible combination of a V region-encoding gene segment and a J region-encoding gene segment.

6. The composition as in any one of claims 1-5, wherein J comprises a constant region of the adaptive immune receptor J region encoding gene sequence.

7. The composition as in any one of claims 1-6, wherein the adaptive immune receptor is selected from the group consisting of TCRB, TCRG, TCRA, TCRD, IGH, IGK, and IGL.

8. The composition as in any one of claims 1-7, wherein the V polynucleotide of (a) encodes a TCRB, TCRG, TCRA, TCRD, IGH, IGK, or IGL receptor V-region polypeptide.

9. The composition as in any one of claims 1-8, wherein the J polynucleotide of (b) encodes a TCRB, TCRG, TCRA, TCRD, IGH, IGK, or IGL receptor J-region polypeptide.

10. The composition as in any one of claims 1-9, further comprising a stop codon between V and B2.

11. The composition as in any one of claims 1-10, wherein each template oligonucleotide in the plurality of template oligonucleotides is present in a substantially equimolar amount.

12. The composition as in any one of claims 1-11, wherein the plurality of template oligonucleotides have a plurality of sequences of general formula (I) that is selected from:

(1) the plurality of oligonucleotide sequences of general formula (I) in which the V and J polynucleotides have the TCRB V and J sequences set forth in at least one set of 68 TCRB V and J SEQ ID NOs. in Figures 5a-5l as TCRB V/J set 1, TCRB V/J set 2, TCRB V/J set 3, TCRB V/J set 4, TCRB V/J set 5, TCRB V/J set 6, TCRB V/J set 7, TCRB V/J set 8, TCRB V/J set 9, TCRB V/J set 10, TCRB V/J set 11, TCRB V/J set 12 and TCRB V/J set 13;

(2) the plurality of oligonucleotide sequences of general formula (I) in which the V and J polynucleotides have the TCRG V and J sequences set forth in at least one set of 14 TCRG V and J SEQ ID NOs. in Figures 6a and 6b as TCRG V/J set 1, TCRG V/J set 2, TCRG V/J set 3, TCRG V/J set 4 and TCRG V/J set 5;

(3) the plurality of oligonucleotide sequences of general formula (I) in which the V and J polynucleotides have the IGH V and J sequences set forth in at least one set of 127 IGH V and J SEQ ID NOs. in Figures 7a-7m as IGH V/J set 1, IGH V/J set 2, IGH V/J set 3, IGH V/J set 4, IGH V/J set 5, IGH V/J set 6, IGH V/J set 7, IGH V/J set 8 and IGH V/J set 9;

(4) the plurality of oligonucleotide sequences of general formula (I) as set forth in SEQ ID NOS:3157-4014;

(5) the plurality of oligonucleotide sequences of general formula (I) as set forth in SEQ ID NOS:4015-4084;

(6) the plurality of oligonucleotide sequences of general formula (I) as set forth in SEQ ID NOS:4085-5200;

(7) the plurality of oligonucleotide sequences of general formula (I) as set forth in SEQ ID NOS:5579-5821;

(8) the plurality of oligonucleotide sequences of general formula (I) as set forth in SEQ ID NOS: 5822-6066; and

(9) the plurality of oligonucleotide sequences of general formula (I) as set forth in SEQ ID NOS: 6067-6191.

13. The composition as in any one of claims 1-12, wherein V is a polynucleotide comprising at least 30, 60, 90, 120, 150, 180, or 210 contiguous nucleotides of the adaptive immune receptor V region encoding gene sequence, or the complement thereof.

14. The composition as in any one of claims 1-13, wherein V is a polynucleotide comprising not more than 900, 800, 700, 600 or 500 contiguous nucleotides of an adaptive immune receptor V region encoding gene sequence, or the complement thereof.

15. The composition as in any one of claims 1-14, wherein J is a polynucleotide comprising at least 16-30, 31-60, 61-90, 91-120, or 120-150 contiguous nucleotides of an adaptive immune receptor J region encoding gene sequence, or the complement thereof.

16. The composition as in any one of claims 1-15, wherein J is a polynucleotide comprising not more than 500, 400, 300 or 200 contiguous nucleotides of an adaptive immune receptor J region encoding gene sequence, or the complement thereof.

17. The composition as in any one of claims 1-16, wherein each template oligonucleotide is less than 1000, 900, 800, 700, 600, 500, 400, 300 or 200 nucleotides in length.

18. The composition as in any one of claims 1-17, further comprising: a set of oligonucleotide primers that is capable of amplifying rearranged nucleic acid molecules encoding one or more adaptive immune receptors comprising a plurality *a'* of unique V-segment oligonucleotide primers and a plurality *b'* of unique J-segment oligonucleotide primers.

19. The composition of claim 18, wherein *a'* ranges from 1 to a number of maximum V gene segments in the mammalian genome, and *b'* ranges from 1 to a number of maximum number of J gene segments in the mammalian genome.

20. The composition of claim 19, wherein a' is a.

21. The composition of claim 19, wherein b' is b.

22. The composition as in any one of claims 18-21, wherein each V-segment oligonucleotide primer and each J-segment oligonucleotide primer in the oligonucleotide primer set is capable of specifically hybridizing to at least one template oligonucleotide in the plurality of template oligonucleotides.

23. The composition as in any one of claims 18-22, wherein each V-segment oligonucleotide primer comprises a nucleotide sequence of at least 15 contiguous nucleotides

that is complementary to at least one adaptive immune receptor V region-encoding gene segment.

24. The composition as in any one of claims 18-23, wherein each J-segment oligonucleotide primer comprises a nucleotide sequence of at least 15 contiguous nucleotides that is complementary to at least one adaptive immune receptor J region-encoding gene segment.

25. The composition as in any one of claims 18-24, wherein the composition comprises at least one template oligonucleotide having an oligonucleotide sequence of general formula (I) to which each V-segment oligonucleotide primer can specifically hybridize, and at least one template oligonucleotide having an oligonucleotide sequence of general formula (I) to which each J-segment oligonucleotide primer can specifically hybridize.

26. A method for determining non-uniform nucleic acid amplification potential among members of a set of oligonucleotide primers that is capable of amplifying rearranged nucleic acid molecules encoding one or more adaptive immune receptors in a biological sample that comprises rearranged nucleic acid molecules from lymphoid cells of a mammalian subject, the method comprising:

(a) amplifying the composition as in any one of claims 18-25 in a multiplex PCR reaction to obtain a plurality of amplified template oligonucleotides;

(b) sequencing said plurality of amplified template oligonucleotides to determine, for each unique template oligonucleotide comprising said plurality, (i) a template oligonucleotide sequence and (ii) a frequency of occurrence of said template oligonucleotide sequence; and

(c) comparing a frequency of occurrence of each of said template oligonucleotide sequences to an expected distribution, wherein said expected distribution is based on predetermined molar ratios of said plurality of template oligonucleotides comprising said composition, and wherein a deviation between said frequency of occurrence of said template oligonucleotide sequences and said expected distribution indicates a non-uniform nucleic acid amplification potential among members of the set of oligonucleotide amplification primers.

27. The method of claim 26, wherein said predetermined molar ratios are equimolar.

28. The method of claim 27, wherein said expected distribution comprises a uniform amplification level for said set of template oligonucleotides amplified by said set of oligonucleotide primers.

29. The method of claim 26, wherein each amplified template nucleic acid molecule is less than 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80 or 70 nucleotides in length.

30. The method as in any one of claims 26-29, further comprising: for each member of the set of oligonucleotide primers that exhibits non-uniform amplification potential relative to the expected distribution, adjusting the relative representation of the oligonucleotide primer member in the set of oligonucleotide amplification primers.

31. The method of claim 30, wherein adjusting comprises increasing the relative representation of the member in the set of oligonucleotide primers, thereby correcting non-uniform nucleic acid amplification potential among members of the set of oligonucleotide primers.

32. The method of claim 30, wherein adjusting comprises decreasing the relative representation of the member in the set of oligonucleotide primers, thereby correcting non-uniform nucleic acid amplification potential among members of the set of oligonucleotide primers.

33. The method as in any one of claims 26-32, wherein said set of oligonucleotide primers does not include oligonucleotide primers that specifically hybridize to a V-region pseudogene or orphon or to a J-region pseudogene or orphon.

34. The method as in any one of claims 26-33, further comprising: for each member of the set of oligonucleotide amplification primers that exhibits non-uniform amplification potential relative to the expected distribution, calculating a proportionately increased or decreased frequency of occurrence of the amplified template nucleic acid molecules, the amplification of which is promoted by said member, thereby correcting for non-uniform nucleic acid amplification potential among members of the set of oligonucleotide primers.

35. A method for quantifying a plurality of rearranged nucleic acid molecules encoding one or a plurality of adaptive immune receptors in a biological sample that comprises rearranged nucleic acid molecules from lymphoid cells of a mammalian subject, each adaptive immune receptor comprising a variable (V) region and a joining (J) region, the method comprising:

(A) amplifying rearranged nucleic acid molecules in a multiplex polymerase chain reaction (PCR) that comprises:

(1) rearranged nucleic acid molecules from the biological sample that comprises lymphoid cells of the mammalian subject,

(2) the composition as in any one claims 1-17, wherein a known number of each of the plurality of template oligonucleotides having a unique oligonucleotide sequence is present,

(3) an oligonucleotide amplification primer set that is capable of amplifying rearranged nucleic acid molecules encoding one or a plurality of adaptive immune receptors from the biological sample, the primer set comprising:

(a) in substantially equimolar amounts, a plurality of V-segment oligonucleotide primers that are each independently capable of specifically hybridizing to at least one polynucleotide encoding an adaptive immune receptor V-region polypeptide or to the complement thereof, wherein each V-segment primer comprises a nucleotide sequence of at least 15 contiguous nucleotides that is complementary to at least one functional adaptive immune receptor V region-encoding gene segment and wherein the plurality of V-segment primers specifically hybridize to substantially all functional adaptive immune receptor V region-encoding gene segments that are present in the composition, and

(b) in substantially equimolar amounts, a plurality of J-segment oligonucleotide primers that are each independently capable of specifically hybridizing to at least one polynucleotide encoding an adaptive immune receptor J-region polypeptide or to the complement thereof, wherein each J-segment primer comprises a nucleotide sequence of at least 15 contiguous nucleotides that is complementary to at least one functional adaptive immune receptor J region-encoding gene segment and wherein the plurality of J-segment primers specifically hybridize to substantially all functional adaptive immune receptor J region-encoding gene segments that are present in the composition,

wherein the V-segment and J-segment oligonucleotide primers are capable of promoting amplification in said multiplex polymerase chain reaction (PCR) of (i) substantially all template oligonucleotides in the composition to produce a multiplicity of amplified template oligonucleotides, said multiplicity of amplified template nucleic acid molecules being sufficient to quantify diversity of the template oligonucleotides in the composition, and (ii) substantially all rearranged nucleic acid molecules encoding adaptive immune receptors in the biological sample to produce a multiplicity of amplified rearranged

DNA molecules, said multiplicity of amplified rearranged nucleic acid molecules being sufficient to quantify diversity of the rearranged nucleic acid molecules in the DNA from the biological sample,

and wherein each amplified nucleic acid molecule in the plurality of amplified template oligonucleotides and in the plurality of amplified rearranged nucleic acid molecules is less than 1000 nucleotides in length;

(B) quantitatively sequencing said amplified template oligonucleotides and said amplified rearranged nucleic acid molecules to quantify (i) a template product number of amplified template oligonucleotides which contain at least one oligonucleotide barcode sequence, and (ii) a rearranged product number of amplified rearranged nucleic acid molecules which lack an oligonucleotide barcode sequence;

(C) calculating an amplification factor by dividing the template product number of (B)(i) by the known number of each of the plurality of template oligonucleotides having a unique oligonucleotide sequence of (A)(2); and

(D) dividing the rearranged product number of (B)(ii) by the amplification factor calculated in (C) to quantify the number of unique adaptive immune receptor encoding rearranged nucleic acid molecules in the sample.

36. The method of claim 35, wherein said the quantified number of unique adaptive immune receptor encoding rearranged nucleic acid molecules in the sample is the number of unique B cell or unique T cell genome templates in the sample.

37. A method for calculating an average amplification factor in a multiplex PCR assay, comprising:

obtaining a biological sample that comprises rearranged nucleic acid molecules from lymphoid cells of a mammalian subject;

contacting said sample with a known quantity of template oligonucleotides comprising a composition as in any one of claim 18-25;

amplifying the template oligonucleotides and the rearranged nucleic acid molecules from lymphoid cells of the mammalian subject in a multiplex PCR reaction to obtain a plurality of amplified template oligonucleotides and a plurality of amplified rearranged nucleic acid molecules;

sequencing said plurality of amplified template oligonucleotides to determine, for each unique template oligonucleotide comprising said plurality, (i) a template

oligonucleotide sequence and (ii) a frequency of occurrence of said template oligonucleotide sequence; and

determining said average amplification factor for said multiplex PCR reaction based on an average number of copies of said plurality of amplified template oligonucleotides and said known quantity of said template oligonucleotides.

38. The method of claim 37, further comprising:

sequencing said plurality of amplified rearranged nucleic acid molecules from lymphoid cells of the mammalian subject to determine for each unique rearranged nucleic acid molecule comprising said plurality, i) a rearranged nucleic acid molecule sequence and (ii) a number of occurrences of said rearranged nucleic acid molecule sequence; and

determining the number of lymphoid cells in said sample, based on the average amplification factor for said multiplex PCR reaction and said number of occurrences of said rearranged nucleic acid molecules.

39. The method of claim 38, wherein determining the number of lymphoid cells in said sample comprises generating a sum of the number of occurrences of each of said amplified rearranged nucleic acid sequences and dividing said sum by said average amplification factor.

40. The method of claim 37, wherein said known quantity is one copy each of said template oligonucleotides.

41. The method of claim 40, wherein $100 \leq a \leq 500$.

42. The method of claim 40, wherein $100 \leq b \leq 500$.

43. A method for correcting for amplification bias in an multiplex PCR amplification reaction to quantify rearranged nucleic acid molecules encoding one or a plurality of adaptive immune receptors in a biological sample that comprises rearranged nucleic acid molecules from lymphoid cells of a mammalian subject, comprising:

(a) contacting said sample with a composition of any one of claims 18-25 to generate a template-spiked sample, wherein said templates and said rearranged nucleic acid molecules comprise corresponding V and J region sequences;

(b) amplifying said template-spiked sample in a multiplex PCR reaction to obtain a plurality of amplified template oligonucleotides and a plurality of amplified rearranged nucleic acid molecules encoding a plurality of adaptive immune receptors;

(c) sequencing said plurality of amplified template oligonucleotides to determine, for each unique template oligonucleotide comprising said plurality, (i) a template

oligonucleotide sequence and (ii) a frequency of occurrence of said template oligonucleotide sequence;

(d) sequencing said plurality of amplified rearranged nucleic acid molecules encoding one or a plurality of adaptive immune receptors, for each unique rearranged nucleic acid molecules encoding said plurality of adaptive immune receptors comprising said plurality, (i) a rearranged nucleic acid molecule sequence and (ii) a frequency of occurrence of said rearranged nucleic acid molecule sequence;

(e) comparing said frequency of occurrence of said template oligonucleotide sequences to an expected distribution, wherein said expected distribution is based on predetermined molar ratios of said plurality of template oligonucleotides comprising said composition, and wherein a deviation between said frequency of occurrence of said template oligonucleotide sequences and said expected distribution indicates non-uniform nucleic acid amplification potential among members of the set of oligonucleotide amplification primers;

(f) generating a set of correction values for a set of template molecules and rearranged nucleic acid molecule sequences amplified by said members of the set of oligonucleotide amplification primers having said indicated non-uniform nucleic acid amplification potential, wherein said set of correction values corrects for amplification bias in said multiplex PCR reaction; and

(g) optionally applying said set of correction values to said frequency of occurrence of said rearranged nucleic acid molecule sequences to correct for amplification bias in said multiplex PCR reaction.

44. A kit, comprising:

reagents comprising: a composition comprising a plurality of template oligonucleotides and a set of oligonucleotide primers as in any one of claims 18-25;

instructions for determining a non-uniform nucleic acid amplification potential among members of the set of oligonucleotide primers that are capable of amplifying rearranged nucleic acid molecules encoding one or more adaptive immune receptors in a biological sample that comprises rearranged nucleic acid molecules from lymphoid cells of a mammalian subject.

45. The kit of claim 44, further comprising instructions for correcting for one or more members of the set of oligonucleotide primers having a non-uniform nucleic acid amplification potential.

46. The kit of claim 44, further comprising instructions for quantifying the number of unique adaptive immune receptor encoding rearranged nucleic acid molecules in the sample

DETAILED DESCRIPTION OF THE INVENTION

[0061] The present invention provides, in certain embodiments and as described herein, compositions and methods that are useful for reliably quantifying large and structurally diverse populations of rearranged genes encoding adaptive immune receptors, such as immunoglobulins (Ig) and/or T cell receptors (TCR). These rearranged genes may be present in a biological sample containing DNA from lymphoid cells of a subject or biological source, including a human subject.

[0062] A "rearranged nucleic acid molecule," as used herein, can include any genomic DNA, cDNA, or mRNA obtained directly or indirectly from a lymphoid cell line that includes sequences that encode a rearranged adaptive immune receptor.

[0063] Disclosed herein are unexpectedly advantageous approaches for the standardization and calibration of complex oligonucleotide primer sets that are used in multiplexed nucleic acid amplification reactions to generate a population of amplified rearranged DNA molecules from a biological sample containing rearranged genes encoding adaptive immune receptors, prior to quantitative high throughput sequencing of such amplified products. Multiplexed amplification and high throughput sequencing of rearranged TCR and BCR (IG) encoding DNA sequences are described, for example, in Robins et al., 2009 *Blood* 114, 4099; Robins et al., 2010 *Sci. Translat. Med.* 2:47ra64; Robins et al., 2011 *J. Immunol. Meth.* doi:10.1016/j.jim.2011.09. 001; Sherwood et al. 2011 *Sci. Translat. Med.* 3:90ra61; U.S.A.N. 13/217,126 (US Pub. No. 2012/0058902), U.S.A.N. 12/794,507 (US Pub. No. 2010/0330571), WO/2010/151416, WO/2011/106738 (PCT/US2011/026373),