Electronic Acknowledgement Receipt

EFS ID:	20691707
Application Number:	14540917
International Application Number:	
Confirmation Number:	7076
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Customer Number:	58249
Filer:	Angie S.Y. Mah
Filer Authorized By:	
Attorney Docket Number:	NUGN-001/01US
Receipt Date:	13-NOV-2014
Filing Date:	
Time Stamp:	18:19:01
Application Type:	Utility under 35 USC 111(a)

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Deposit Account	501283
Authorized User	

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal of New Application	NUGN-001_01US_UtilityTransmittal.pdf	172100 3346403fd2f14f20763898f70c724a1778f746db	no	1
Warnings:					
Information:					
2	TrackOne Request	NUGN-001_01US_TrackOneTransmittal.pdf	135963 91825e519d517729ef1f6d96a2504778db586b4a	no	1
Warnings:					
Information:					
3	Application Data Sheet	NUGN-001_01US_ADS.pdf	111444 c7f45f621f9dd878c7ce4f71890e4e52371fca9a	no	6
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
4		NUGN-001_01US_Specifcation.pdf	254432 5c13358ecaa123e6469567a44e06e8d48c925771	yes	44

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	40
Claims	41	43
Abstract	44	44

Warnings:					
Information:					
5	Drawings-other than black and white line drawings	NUGN-001_01US_Drawings.pdf	116178 40d64004a908d38384740853fb9d29e73a77b4fc	no	6
Warnings:					
Information:					
6	Sequence Listing (Text File)	NUGN_001_01US_SeqList_ST25.txt	2809	no	0

Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	41712 7927943e0c25baae0ae7bdbef708110e664e020e	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	834638	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NUGN-001/01US
	Application Number	
Title of Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Douglas		AMORESE	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Los Altos	State/Province	CA	Country of Residence	US

Mailing Address of Inventor:

Address 1	357 South El Monte Avenue		
Address 2			
City	Los Altos	State/Province	CA
Postal Code		Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Jonathan		SCOLNICK	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

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Address 2			
City	San Francisco	State/Province	CA
Postal Code		Country	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Ben		SCHROEDER	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NUGN-001/01US
	Application Number	
Title of Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ	

City	San Mateo	State/Province	CA	Country of Residence	US

Mailing Address of Inventor:

Address 1	1964 Shoreview Avenue		
Address 2			
City	San Mateo	State/Province	CA
Postal Code		Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	58249		
Email Address	zpatdcdocketing@cooley.com	Add Email	Remove Email

Application Information:

Title of the Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ		
Attorney Docket Number	NUGN-001/01US	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	6	Suggested Figure for Publication (if any)	

Filing By Reference :

Only compete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NUGN-001/01US
	Application Number	
Title of Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ	

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	(●) Customer Number	( ) US Patent Practitioner	( ) Limited Recognition (37 CFR 11.9)
Customer Number	58249		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the application number blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Claims benefit of provisional	61903826	2013-11-13

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

			Remove
Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NUGN-001/01US
	Application Number	
Title of Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ	

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization to Permit Access:

☒ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date o f filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NUGN-001/01US
	Application Number	
Title of Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ	

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

○ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
◉ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name	NUGEN TECHNOLOGIES, INC.

Mailing Address Information For Applicant:

Address 1	201 Industrial Road		
Address 2	Suite 310		
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Country	US	Postal Code	94070
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

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Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NUGN-001/01US
	Application Number	
Title of Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ	

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.

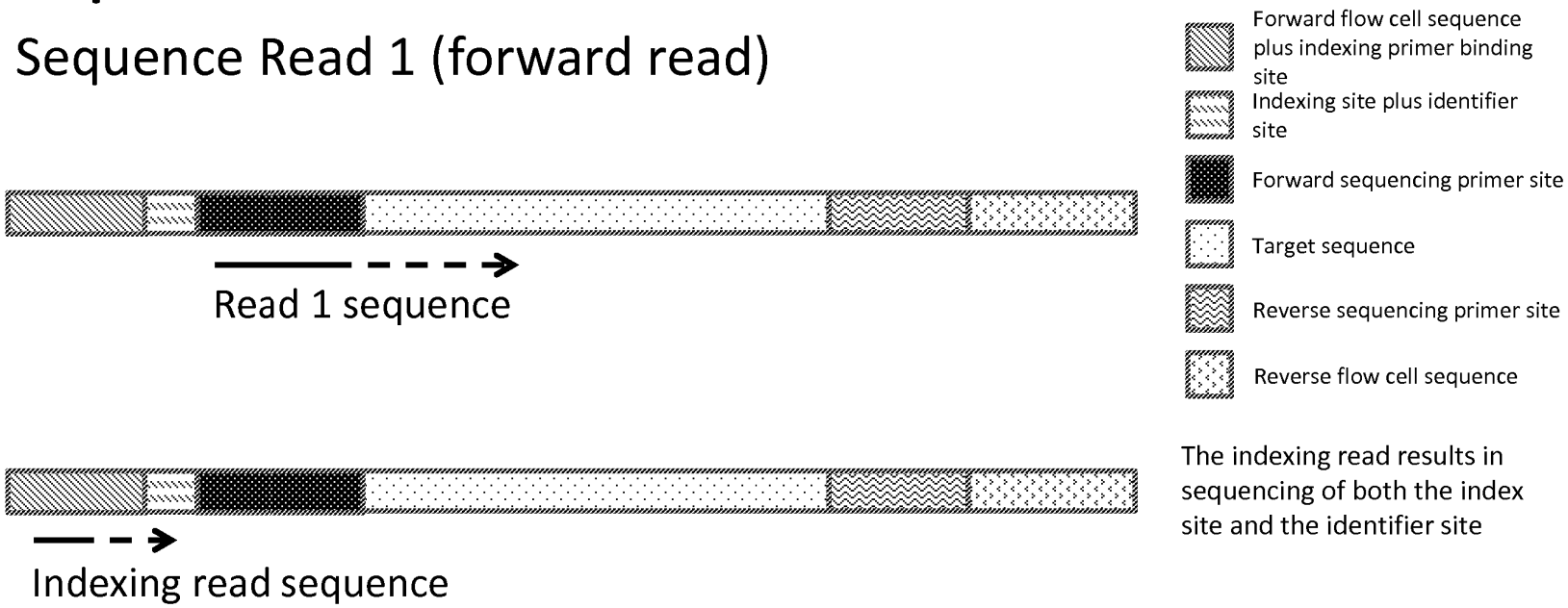
Signature	/Angie Mah/			Date (YYYY-MM-DD)	2014-11-13
First Name	Angie	Last Name	Mah	Registration Number	64902

Additional Signature may be generated within this form by selecting the Add button.

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Standard DNA Sequencing With Duplication Counter

- Sequence Read 1 (forward read)

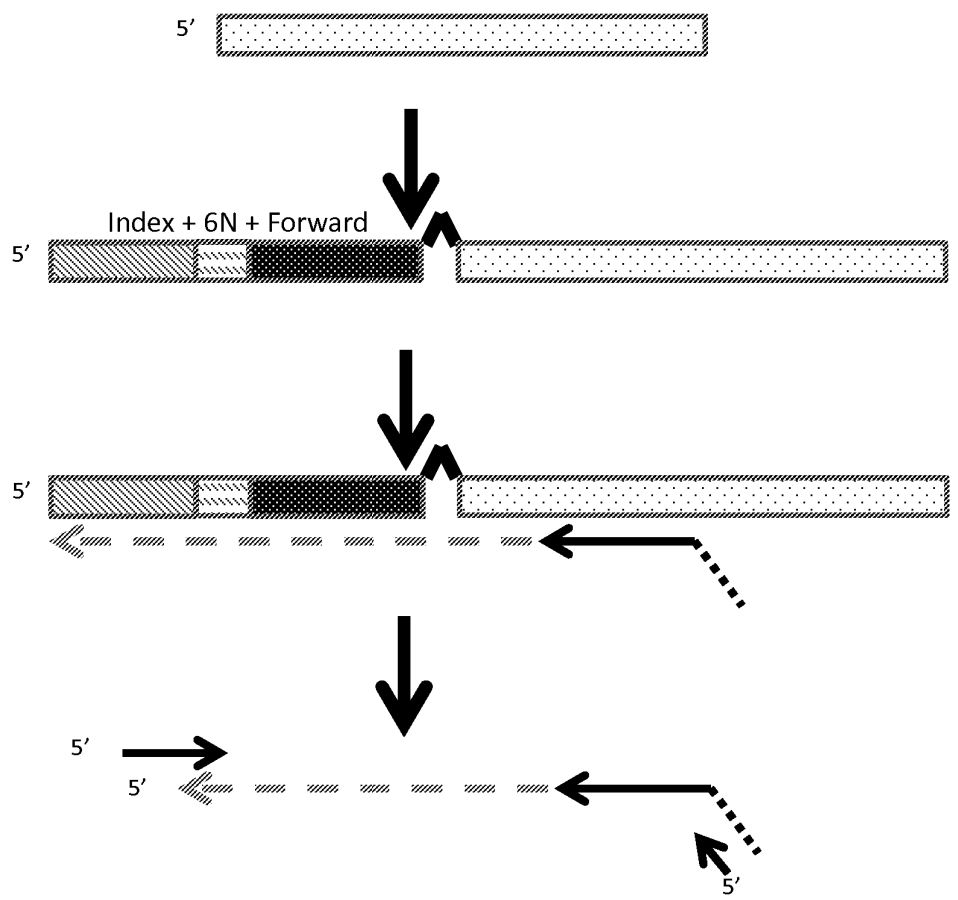


- Extension of the indexing read beyond the indexing site and into the nucleotides of the identifier site provides data on which nucleic acid molecule is being sequenced.

Figure 1

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QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

Single Primer Extension (SPET) Reaction



gDNA is sheared

Adapters are ligated on to end-repaired DNA. The adapters contain at least a partial indexing sequence priming site, an indexing site, an identifier site, and the forward sequencing priming site. Each gDNA is now labeled with a unique identifier sequence

Targeting oligos are hybridized to gDNA and extended through the adapter containing an identifier site (6N) sequence

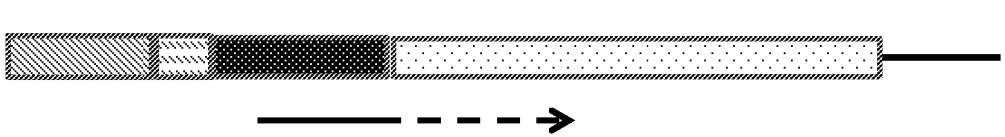
Probes that have extended through an adapter are PCR amplified for sequencing

Figure 2A

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QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

SPET Sequencing

- Sequence from random end toward the probe



PCR adds sequence specific primer binding sites and Read 1 of sequencing is performed

Read 1 sequence



Indexing Read performed sequences both the indexing site as well as the identifier site (N6)

Indexing read sequence

- Extending the indexing read past the 6 base indexing site sequence and into the random Ns of the identifier site provides data on which gDNA molecule the extension reaction occurred upon.

Figure 2B

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QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

Example Adaptor

An adaptor sequence with necessary priming sites, the index site, and the identifier site

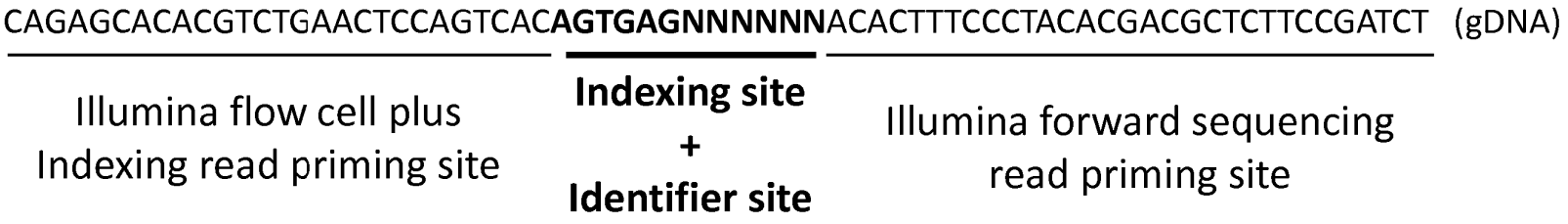
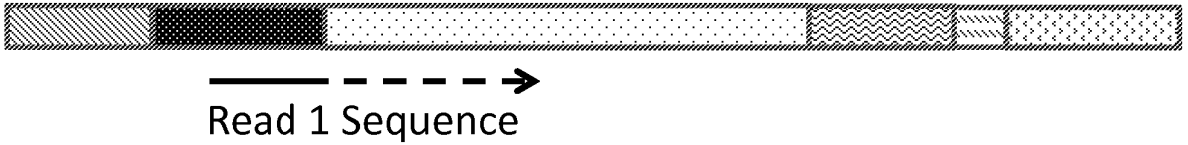


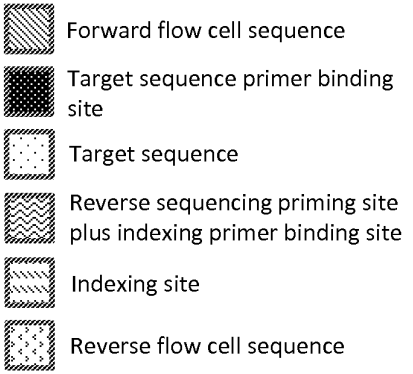
Figure 3

Tecan Ex. 2021, Page 13 of 1314
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

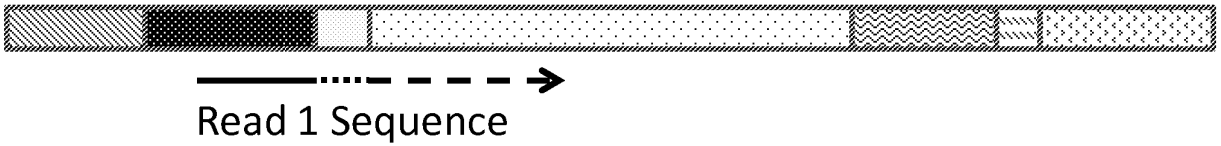
DNA Sequencing With Duplicate Recognition at the insert junction

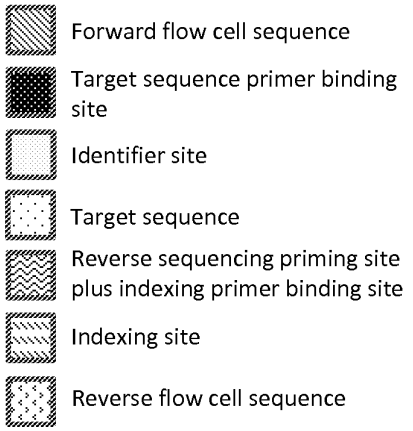
- Traditional library, sequence read 1 (forward read)





- Duplicate recognition library, sequence read 1 (forward read)





Read 1 initially records the approximately 8 random bases of the identifier site appended to the library sequences, followed by the target sequence

Figure 4

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Resolution of apparent duplicate reads by identifier sequence ligation

Libraries were generated from 100ng genomic DNA Covaris fragmented to ~500bp in length. These were enriched with the 344 gene cancer panel, ~12k probes covering 969kb of genomic space and sequenced on the ILMN Miseq. Different numbers of reads were randomly selected for this analysis

Total reads randomly selected	Aligned read	Fold Target Coverage	Total duplicates after identifier analysis (true rate)	Percentage duplicates after identifier analysis (true rate)	Total apparent duplicates with no identifier	Percentage apparent duplicates with no identifier
100K	97,919	2X	1854	1.89	3256	3.33
500K	488,423	7X	39731	8.13	68741	14.07
1M	976,662	13X	145589	14.91	237882	24.36
2M	1,954,673	21X	514848	26.34	764825	39.13
10M	9,771,462	43X	6493577	66.45	7515496	76.91

"Apparent" Duplicates – individual targets within libraries consist of one random end (result of fragmentation) and one common end (initiated with the sequence specific probe). Apparent duplicates are derived from sequencing libraries and using conventional measures of duplicate reads ie, reads were mapped using bowtie and all reads with the same start and end genomic coordinates were counted as duplicates.

"True" duplicates - An identifier sequence is introduced through ligation to differentiate between fragments of DNA that randomly have the same start and end mapping coordinates. The analysis method is described in the specification.

Figure 5

QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026
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COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 61/903,826, filed November 13, 2013, which is herein incorporated by reference in its entirety.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

[0002] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: NUGN_001_01US_SeqList_ST25.txt, date recorded: November 12, 2014, file size 3 kilobytes).

FIELD OF THE PRESENT INVENTION

[0003] The present invention relates generally to the field of high throughput sequencing reactions and the ability to discern artifacts arising through sequence duplications from nucleotide molecules that are unique molecules.

BACKGROUND OF THE PRESENT INVENTION

[0004] In RNA sequencing applications, accurate gene expression measurements may be hampered by PCR duplicate artifacts that occur during library amplification. When analyzing RNA sequencing data, when two or more identical sequences are found, it can be difficult to know if these represent unique cDNA molecules derived independently from different RNA molecules, or if they are PCR duplicates derived from a single RNA molecule. In genotyping by

sequencing, duplicate reads can be considered non-informative and may be collapsed down to a single read, thus reducing the number of sequencing reads used in final analysis. Generally, sequencing reads may be determined to be duplicates if both forward and reverse reads have identical starting positions, even though two independently generated molecules can have identical starting positions by random chance. Single primer extension based targeted re-sequencing suffers from an issue in that only one end of a sequencing read is randomly generated, while the other (reverse read) end is generated by a specific probe. This may make it difficult to determine if two reads are duplicates because they have been duplicated by PCR or because by chance they happened to start at the same position.

[0005] In expression analysis studies there may be limited value in doing paired end sequencing since the goal of the experiment is to determine amounts of transcript present as opposed to studying exon usage. In these studies, paired end sequencing adds costs while the only value is in helping distinguish PCR duplicates. The probability of two reads starting in the same position on only one end is higher than the probability of two reads having the same starting position on two ends (forward and reverse read). There is a need for improved methods that allow for low-cost, high throughput sequencing of regions of interest, genotyping or simple detection of RNA transcripts without inherent instrument inefficiencies that drive up sequencing costs due to the generation of unusable or non-desired data reads. The invention described herein fulfills this need. Here, we describe an adaptor approach that allows for the identification of true PCR duplicates and their removal.

[0006] The methods of the present invention provide novel methods for identifying true duplicate reads during sequencing, such as to improve data analysis of sequencing data, and other related advantages.

SUMMARY OF THE PRESENT INVENTION

[0007] The present invention is based, in part, on compositions and methods for discerning duplicate sequencing reads from a population of sequencing reads. The detection and/or removal of duplicate sequencing reads presented herein is a novel approach to increasing the efficacy of evaluating data generated from high throughput sequence reactions, including complex multiplex sequence reactions.

[0008] Accordingly, the present invention provides a method of detecting a duplicate sequencing read from a population of sample sequencing reads, the method comprising ligating an adaptor to a 5' end of each nucleic acid fragment of a plurality of nucleic acid fragments from one or more samples, wherein the adaptor comprises an indexing primer binding site, an indexing site, an identifier site, and a target sequence primer binding site. The ligated adaptor-nucleic acid fragment products can be amplified, thus generating a population of sequencing reads from the amplified adaptor-nucleic acid ligation products. The sequencing reads with a duplicate identifier site and target sequence can then be detected from the population of sequencing reads. The methods can further include the removal of the sequencing reads with the duplicate identifier site and target sequence from the population of sequence reads.

[0009] In some embodiments, the identifier site is sequenced with the indexing site or the target sequence. In further embodiments, the identifier site is sequenced separately from the indexing site or the target sequence.

[0010] In some embodiments, the adaptor comprises from 5' to 3' the indexing primer binding site; the indexing site; the identifier site; and the target sequence primer binding site. In further embodiments, the adaptor comprises from 5' to 3' the indexing primer binding site; the indexing site; the target sequence primer binding site; and the identifier site.

[0011] In some embodiments, the plurality of nucleic acid fragments is generated from more than one sample. In some embodiments, the nucleic acid fragments from each sample have the same indexing site. In some embodiments, the sequencing reads are separated based on the indexing site. In yet other embodiments, the separation of sequencing reads is performed prior to detecting sequence reads with a duplicate identifier site and target sequence.

[0012] In some embodiments, the nucleic acid fragments are DNA fragments, RNA fragments, or DNA/RNA fragments. In further embodiments, the nucleic acid fragments are genomic DNA fragments or cDNA fragments.

[0013] In some embodiments, the indexing site is between 2 and 8 nucleotides in length. In further embodiments, the indexing site is about 6 nucleotides in length. In some embodiments, the identifier site is between 1 and 8 nucleotides in length. In further embodiments, the identifier site is about 8 nucleotides in length.

[0014] In some embodiments, the indexing primer binding site is a universal indexing primer binding site; and in some embodiments, the target sequence primer binding site is a universal target sequence primer binding site.

[0015] The present invention also encompasses embodiments that include a kit comprising a plurality of adaptors, wherein each adaptor comprise an indexing primer binding site; an indexing site, and identifier site, and a target sequencing primer binding site.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] A better understanding of the novel features of the invention and advantages of the present invention will be obtained by reference to the following description that sets forth

illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0017] Figure 1 depicts a schematic of generating sequencing reads of a library including where an indexing primer and a target sequence primer anneal.

[0018] Figure 2A depicts a mechanism of the Single Primer Enrichment Technology (SPET) and how an identifier site is carried over into the final library and how sequencing through the indexing site into the identifier site provides data on which nucleic acid molecule is being identified.

[0019] Figure 2B is a continuation of Figure 2A.

[0020] Figure 3 offers a detailed view of the sequence of an example adaptor, among many envisioned embodiments, and the position of the indexing and identifier sites (SEQ ID NO: 1). "N" refers to any nucleic acid.

[0021] Figure 4 depicts a schematic of two separate sequence libraries, indicating where the indexing primers and the target primers anneal in a traditional library as compared to a library using an identifier site.

[0022] Figure 5 depicts a data table demonstrating the accuracy of resolving true duplicates versus apparent or perceived duplicates using an identifier site in the adaptors.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0023] The present invention is based, in part, on compositions and methods for discerning duplicate sequencing reads from a population of sequencing reads. The present invention encompasses methods of detecting sequences duplicated in sequencing applications, and further removal of the duplicated sequence reads. The present invention further encompasses kits

comprising components that would allow for customized applications of the method of detecting and removing duplicated sequence reads in high throughput sequencing reactions. The compositions and methods can be used with various applications for genetic sample analysis, such as RNA sequence analysis, copy number variation analysis, methylation sequencing analysis, genotyping and whole genome amplification.

[0024] Reference will now be made in detail to exemplary embodiments of the invention. While the disclosed methods and compositions will be described in conjunction with the exemplary embodiments, it will be understood that these exemplary embodiments are not intended to limit the present invention. On the contrary, the present disclosure is intended to encompass alternatives, modifications and equivalents, which may be included in the spirit and scope of the present invention.

[0025] Unless otherwise specified, terms and symbols of genetics, molecular biology, biochemistry and nucleic acid used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, *DNA Replication,* Second Edition (W.H. Freeman, New York, 1992); Lehninger, *Biochemistry,* Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human *Molecular Genetics,* Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, *Oligonucleotides and Analogs: A Practical Approach* (Oxford University Press, New York, 1991); Gait, editor, *Oligonucleotide Synthesis: A Practical Approach* (IRL Press, Oxford,1984); and the like.

[0026] In some embodiments, the methods disclosed herein is for detecting from a population of sequencing reads a sequencing read, such as a duplicate sequencing read with a duplicate identifier site and target sequence. A duplicate sequencing read can be a sequencing read with

the same identifier site and target sequence as another sequencing read in the population of sequencing reads.

Adaptor

[0027] The present invention provides compositions of adaptors and methods comprising use of an adaptor. An adapter refers to an oligonucleotide sequence, the ligation of which to a target polynucleotide or a target polynucleotide strand of interest enables the generation of amplification-ready products of the target polynucleotide or the target polynucleotide strand of interest. The target polynucleotide molecules may be fragmented or not prior to the addition of adaptors. In some embodiments, a method disclosed herein comprises ligating an adaptor to a 5' end of each nucleic acid fragment of a plurality of nucleic acid fragments from one or more samples.

[0028] Various adaptor designs are envisioned which are suitable for generation of amplification-ready products of target sequence regions/strands of interest. For example, the two strands of the adaptor may be self-complementary, non-complementary or partially complementary. In some embodiments, the adaptor can comprise an indexing primer binding site, an indexing site, an identifier site, and a target sequence primer binding site.

[0029] An indexing primer binding site is a nucleotide sequence for binding a primer for an indexing site. An indexing site is a nucleic acid sequence that acts as an index for multiple polynucleotide samples, thus allowing for the samples to be pooled together into a single sequencing run, which is known as multiplexing. In some embodiments, the indexing site is at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In some embodiments, the indexing site is between 2 and 8 nucleotides in length. In some embodiments, the indexing site is about 6 nucleotides in length.

[0030] An identifier site is a nucleic acid sequence that comprises random bases and is used to identify duplicate sequencing reads. In some embodiments, the identifier site is at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In some embodiments, the identifier site is between 1 and 8 nucleotides in length. In some embodiments, the identifier site is about 8 nucleotides in length. This identifier site can be designed as a set of sequences, or it can be semi-random, or it can be completely random. In addition, this identifier site can be a fixed length, or it can be a variable length. In some embodiments, the identifier sites in a plurality of adaptors are of a fixed length. For example, the identifier sites can all be eight random bases. In another embodiment, the identifier sites in a plurality of adaptors are of a variable length. For example, the identifier sites can range in size from 1 to 8 bases. In yet another embodiment, the identifier sites can be of a defined set of defined sequence. For example, the identifier site of a plurality of adaptors can be one of 96 defined six-base nucleotide sequences.

[0031] A target sequence primer binding site nucleotide sequence for binding a primer for a target sequence. The primer can be used to amplify the target sequence (e.g., a nucleic acid fragment from a sample). Accordingly, in some embodiments, the adaptor comprises an indexing primer binding site and a target sequence primer binding site.

[0032] A primer is a polynucleotide chain, typically less than 200 residues long, most typically between 15 and 100 nucleotides long, but can encompass longer polynucleotide chains. A primer targeting the primer binding sites are typically designed to hybridize to single-stranded nucleic acid strands. In some embodiments, the primers targeting the primer binding sites are designed to hybridize to single-stranded DNA targets. In the case where the sample comprises genomic DNA or other double-stranded DNA, the sample can be first denatured to render the target single stranded and enable hybridization of the primers to the desired sequence regions of

interest. In these embodiments, the methods and compositions described herein can allow for region-specific enrichment and amplification of sequence regions of interest. In some embodiments, the other double-stranded DNA can be double-stranded cDNA generated by first and second strand synthesis of one or more target RNAs.

[0033] In other embodiments, the primers targeting the primer binding sites are designed to hybridize to double-stranded nucleic acid targets, without denaturation of the double stranded nucleic acids. In other embodiments, the primers targeting the primer binding sites are designed to hybridize to a double-stranded DNA target, without denaturation of the dsDNA. In these embodiments, the primers targeting the selected sequence regions of interest are designed to form a triple helix (triplex) at the selected sequence regions of interest. The hybridization of the primers to the double-stranded DNA sequence regions of interest can be carried out without prior denaturation of the double stranded nucleic acid sample. In such embodiments, the methods and compositions described herein can allow for region-specific enrichment as well as strand-specific enrichment and amplification of sequence regions of interest. This method can be useful for generation of copies of strand specific sequence regions of interest from complex nucleic acid without the need to denature the dsDNA input DNA, thus enabling enrichment and analysis of multiplicity of sequence regions of interest in the native complex nucleic acid sample. The method can find use for studies and analyses carried out in situ, enable studies and analysis of complex genomic DNA in single cells or collection of very small well defined cell population, as well as permit the analysis of complex genomic DNA without disruption of chromatin structures.

[0034] The primers of the invention are generally oligonucleotides that are employed in an extension reaction by a polymerase along a polynucleotide template, such as for amplification of a target sequence (e.g., in PCR). The oligonucleotide primer can be a synthetic polynucleotide

that is single stranded, containing a sequence at its 3'-end that is capable of hybridizing with a sequence of the target polynucleotide. In some embodiments, the 3' region of the primer that hybridizes with the target nucleic acid has at least 80%, preferably 90%, more preferably 95%, most preferably 100%, complementarity to a primer binding site.

[0035] In some embodiments, the primer binding site is a binding site for a universal primer. A universal primer is a primer that can be used for amplifying a number of different sequences. In some embodiments, a universal primer is used to amplify different libraries. In some embodiments, an indexing primer binding site is a binding site for a universal indexing primer (i.e., the indexing primer binding site is a universal indexing primer binding site). In some embodiments, the adaptors used for ligating a plurality of nucleic acid fragments have a universal indexing primer binding site. In some embodiments, the universal indexing primer can be used to amplify and/or sequence a number of different indexing sites.

[0036] In some embodiments, a target sequence primer binding site is a binding site for a universal target sequence primer (i.e., the target sequence primer binding site is a universal target sequence primer binding site). In some embodiments, the adaptors used for ligating a plurality of nucleic acid fragments have a universal target sequence primer binding site. In some embodiments, the universal target sequence primer can be used to amplify and/or sequence a number of different target sequences.

[0037] In some embodiments, the adaptor comprises an identifier site 3' to an indexing site. In some embodiments, the adaptor comprises an identifier site 5' to an indexing site. In some embodiments, the adaptor comprises from 5' to 3' an indexing primer binding site, indexing site, identifier site, and target sequence primer binding site. In other embodiments, the adaptor comprises from 5' to 3' an indexing primer binding site, indexing site, identifier site, and target

sequence primer binding site. In yet other embodiments, the adaptor comprises from 5' to 3' an indexing primer binding site, identifier site, indexing site, and target sequence primer binding site.

Samples

[0038] In some embodiments, an adaptor is ligated to a nucleic acid fragment (e.g., the 5' end of the nucleic acid fragment). The nucleic acid fragment can be from a plurality of nucleic acid fragments from one or more samples. The nucleic acid fragment can be RNA, DNA, or complex DNA, for example genomic DNA and PNA, in which case one might use a modified nucleic acid. The nucleic acid fragment may also be cDNA. The cDNA can be generated from RNA, e.g., mRNA.

[0039] The sample can be a biological sample. For example, the sample can be an animal, plant, bacterial, algal, or viral sample. In some embodiments, the sample is a human, rat, or mouse sample. The sample can be from a mixture of genomes of different species such as host-pathogen, bacterial populations and the like. The sample can be cDNA made from a mixture of genomes of different species. In some embodiments, the sample can be from a synthetic source. The sample can be mitochondrial DNA. The sample can be cell-free DNA. The cell-free DNA can be obtained from sources such as a serum or a plasma sample. The sample can comprise one or more chromosomes. For example, if the sample is from a human, the sample can comprise one or more of chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, or Y. In some embodiments, the sample comprises a linear or circular genome. The sample can be plasmid DNA, cosmid DNA, bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC). The sample can be from more than one individual or organism. The sample

can be double-stranded or single-stranded. The sample can be part of chromatin. The sample can be associated with histones.

[0040] In some embodiments, adaptors are ligated to a plurality of nucleic acid fragments from more than one sample, such as 2, 3, 4, 5, or more samples. In some embodiments, the nucleic acid fragments from each sample have the same indexing site. In some embodiments, a plurality of nucleic acid fragments is generated from a first sample and a second sample and adaptors are ligated to each nucleic acid fragment, in which adaptors ligated to each nucleic acid fragment from the first sample have the same first indexing site and adaptors ligated to nucleic acid fragments from the second sample has the same second indexing site. In some embodiments, the nucleic acid fragments or data associated with the nucleic acid fragments (e.g., sequencing reads) are separated based on the indexing site.

[0041] In some embodiments, a population of nucleic acid fragments generated from a sample is of one or more specific size range(s). In some embodiments, the fragments have an average length from about 10 to about 10,000 nucleotides. In some embodiments, the fragments have an average length from about 50 to about 2,000 nucleotides. In some embodiments, the fragments have an average length from about 100-2,500, 10-1,000, 10-800, 10-500, 50-500, 50-250, or 50-150 nucleotides. In some embodiments, the fragments have an average length less than 10,000 nucleotide, such as less than 5,000 nucleotides, less than 2,500 nucleotides, less than 2,500 nucleotides, less than 1,000 nucleotides, less than 500 nucleotides, such as less than 400 nucleotides, less than 300 nucleotides, less than 200 nucleotides, or less than 150 nucleotides.

[0042] In some embodiments, fragmentation of the nucleic acids can be achieved through methods known in the art. Fragmentation can be achieved through physical fragmentation methods and/or enzymatic fragmentation methods. Physical fragmentation methods can include

nebulization, sonication, and/or hydrodynamic shearing. In some embodiments, the fragmentation can be accomplished mechanically comprising subjecting the nucleic acids in the input sample to acoustic sonication. In some embodiments, the fragmentation comprises treating the nucleic acids in the input sample with one or more enzymes under conditions suitable for the one or more enzymes to generate double-stranded nucleic acid breaks. Examples of enzymes useful in the generation of nucleic acid or polynucleotide fragments include sequence specific and non-sequence specific nucleases. Non-limiting examples of nucleases include DNase I, Fragmentase, restriction endonucleases, variants thereof, and combinations thereof. Reagents for carrying out enzymatic fragmentation reactions are commercially available (e.g., from New England Biolabs). For example, digestion with DNase I can induce random double-stranded breaks in DNA in the absence of Mg^{++} and in the presence of Mn^{++}. In some embodiments, fragmentation comprises treating the nucleic acids in the input sample with one or more restriction endonucleases. Fragmentation can produce fragments having 5′ overhangs, 3′ overhangs, blunt ends, or a combination thereof. In some embodiments, such as when fragmentation comprises the use of one or more restriction endonucleases, cleavage of sample polynucleotides leaves overhangs having a predictable sequence. In some embodiments, the method includes the step of size selecting the fragments via standard methods known in the art such as column purification or isolation from an agarose gel.

[0043] In some embodiments, fragmentation of the nucleic acids is followed by end repair of the nucleic acid fragments. End repair can include the generation of blunt ends, non-blunt ends (i.e sticky or cohesive ends), or single base overhangs such as the addition of a single dA nucleotide to the 3′-end of the nucleic acid fragments, by a polymerase lacking 3′-exonuclease activity. End repair can be performed using any number of enzymes and/or methods known in the art

including, but not limited to, commercially available kits such as the Encore™ Ultra Low Input NGS Library System I. In some embodiments, end repair can be performed on double stranded DNA fragments to produce blunt ends wherein the double stranded DNA fragments contain 5′ phosphates and 3′ hydroxyls. In some embodiments, the double-stranded DNA fragments can be blunt-end polished (or "end-repaired") to produce DNA fragments having blunt ends, prior to being joined to adapters. Generation of the blunt ends on the double stranded fragments can be generated by the use of a single strand specific DNA exonuclease such as for example exonuclease 1, exonuclease 7 or a combination thereof to degrade overhanging single stranded ends of the double stranded products. Alternatively, the double stranded DNA fragments can be blunt ended by the use of a single stranded specific DNA endonuclease, for example, but not limited to, mung bean endonuclease or S1 endonuclease. Alternatively, the double stranded products can be blunt ended by the use of a polymerase that comprises single stranded exonuclease activity such as for example T4 DNA polymerase, or any other polymerase comprising single stranded exonuclease activity or a combination thereof to degrade the overhanging single stranded ends of the double stranded products. In some cases, the polymerase comprising single stranded exonuclease activity can be incubated in a reaction mixture that does or does not comprise one or more dNTPs. In other cases, a combination of single stranded nucleic acid specific exonucleases and one or more polymerases can be used to blunt end the double stranded fragments generated by fragmenting the sample comprising nucleic acids. In still other cases, the nucleic acid fragments can be made blunt ended by filling in the overhanging single stranded ends of the double stranded fragments. For example, the fragments may be incubated with a polymerase such as T4 DNA polymerase or Klenow polymerase or a combination thereof in the presence of one or more dNTPs to fill in the single stranded portions

of the double stranded fragments. Alternatively, the double stranded DNA fragments can be made blunt by a combination of a single stranded overhang degradation reaction using exonucleases and/or polymerases, and a fill-in reaction using one or more polymerases in the presence of one or more dNTPs.

[0044] U.S. Patent Publication Nos. 2013-0231253 A1 and 2014-0274729 A1 further describe methods of generating nucleic acid fragments, methods of modifying the fragments and analysis of the fragments, and are incorporated by reference in their entirety herein.

Ligation of Adaptors

[0045] Ligation of adaptors at the desired end of the sequence regions of interest (e.g., at the 5' or 3' end of a nucleic acid fragment generated form a sample) is suitable for carrying out the methods of the invention. Various ligation modalities are envisioned, dependent on the choice of nucleic acid, nucleic acid modifying enzymes and the resulting ligatable end of the nucleic acid. For example, when a blunt end product comprising the target region/sequence of interest is generated, blunt end ligation can be suitable. Alternatively, where the cleavage is carried out using a restriction enzyme of known sequence specificity, leading to the generation of cleavage sites with known sequence overhangs, suitable ends of the adaptors can be designed to enable hybridization of the adaptor to the cleavage site of the sequence region of interest and subsequent ligation. Ligation also refers to any joining of two nucleic acid molecules that results in a single nucleic acid sequence that can be further modified to obtain the sequence of the nucleic acids in question. Reagents and methods for efficient and rapid ligation of adaptors are commercially available, and are known in the art.

[0046] In some embodiments, the 5′ and/or 3′ end nucleotide sequences of fragmented nucleic acids are not modified or end-repaired prior to ligation with the adapter oligonucleotides of the present invention. For example, fragmentation by a restriction endonuclease can be used to leave

a predictable overhang, followed by ligation with one or more adapter oligonucleotides comprising an overhang complementary to the predictable overhang on a nucleic acid fragment. In another example, cleavage by an enzyme that leaves a predictable blunt end can be followed by ligation of blunt-ended nucleic acid fragments to adapter oligonucleotides comprising a blunt end. In some embodiments, end repair can be followed by an addition of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides, such as one or more adenine, one or more thymine, one or more guanine, or one or more cytosine, to produce an overhang. Nucleic acid fragments having an overhang can be joined to one or more adapter oligonucleotides having a complementary overhang, such as in a ligation reaction. For example, a single adenine can be added to the 3′ ends of end-repaired DNA fragments using a template independent polymerase, followed by ligation to one or more adapters each having a thymine at a 3′ end. In some embodiments, adapter oligonucleotides can be joined to blunt end double-stranded nucleic acid fragments which have been modified by extension of the 3′ end with one or more nucleotides followed by 5′ phosphorylation. In some cases, extension of the 3′ end can be performed with a polymerase such as for example Klenow polymerase or any of the suitable polymerases provided herein, or by use of a terminal deoxynucleotide transferase, in the presence of one or more dNTPs in a suitable buffer containing magnesium. In some embodiments, nucleic acid fragments having blunt ends can be joined to one or more adapters comprising a blunt end. Phosphorylation of 5′ ends of nucleic acid fragments can be performed for example with T4 polynucleotide kinase in a suitable buffer containing ATP and magnesium. The fragmented nucleic acid molecules may optionally be treated to dephosphorylate 5′ ends or 3′ ends, for example, by using enzymes known in the art, such as phosphatases.

[0047] In some embodiments, appending the adaptor to the nucleic acid fragments generated by methods described herein can be achieved using a ligation reaction or a priming reaction. In some embodiments, appendage of an adaptor to the nucleic acid fragments comprises ligation. In some embodiments, ligation of the adaptor to the nucleic acid fragments can be following end repair of the nucleic acid fragments. In another embodiment, the ligation of the adaptor to the nucleic acid fragments can be following generation of the nucleic acid fragments without end repair of the nucleic acid fragments. The adaptor can be any type of adaptor known in the art including, but not limited to, conventional duplex or double stranded adaptors in which the adaptor comprises two complementary strands. In some embodiments, the adaptor can be a double stranded DNA adaptor. In some embodiments, the adaptor can be an oligonucleotide of known sequence and, thus, allow generation and/or use of sequence specific primers for amplification and/or sequencing of any polynucleotides to which the adaptor is appended or attached. In some embodiments, the adaptor can be a conventional duplex adaptor, wherein the adaptor comprises sequence well known in the art. In a some embodiments, the adaptor can be appended to the nucleic acid fragments generated by the methods described herein in multiple orientations. In some embodiment, the methods described herein can involve the use of a duplex adaptor comprising double stranded DNA of known sequence that is blunt ended and can bind to the double stranded nucleic acid fragments generated by the methods described herein in one of two orientations. In some embodiments, the adaptor can be ligated to each of the nucleic acid fragments such that each of the nucleic acid fragments comprises the same adaptor. In other words, each of the nucleic acid fragments comprises a common adaptor. In another embodiment, an adaptor can be appended or ligated to a library of nucleic acid fragments generated by the methods described herein such that each nucleic acid fragment in the library of nucleic acid

fragments comprises the adaptor ligated to one or both ends. In another embodiment, more than one adaptor can be appended or ligated to a library of nucleic acid fragments generated by the methods described herein. The multiple adaptors may occur adjacent to one another, spaced intermittently, or at opposite ends of the nucleic acid fragments. In some embodiments, the adaptor can be ligated or appended to the 5′ and/or 3′ ends of the nucleic acid fragments generated by the methods described herein. The adaptor can comprise two strands wherein each strand comprises a free 3′ hydroxyl group but neither strand comprises a free 5′ phosphate. In some embodiments, the free 3′ hydroxyl group on each strand of the adaptor can be ligated to a free 5′ phosphate present on either end of the nucleic acid fragments of the present invention. In this embodiment, the adaptor comprises a ligation strand and a non-ligation strand whereby the ligation strand can be ligated to the 5′ phosphate on either end of the nucleic acid fragment while a nick or gap can be present between the non-ligation strand of the adaptor and the 3′ hydroxyl on either end of the nucleic acid fragment. In some embodiments, the nick or gap can be filled in by performing a gap repair reaction. In some embodiments, the gap repair can be performed with a DNA dependent DNA polymerase with strand displacement activity. In some embodiments, the gap repair can be performed using a DNA-dependent DNA polymerase with weak or no strand displacement activity. In some embodiments, the ligation strand of the adaptor can serve as the template for the gap repair or fill-in reaction. The gap repair or fill-in reaction may comprise an extension reaction wherein the ligation strand of the adaptor serves as a template and leads to the generation of nucleic acid fragments with complementary termini or ends. In some embodiments, the gap repair can be performed using Taq DNA polymerase. In some embodiments, the ligation of the first adaptor to the nucleic acid fragments generated by the

methods described herein may not be followed by gap repair. The nucleic acid fragments may comprise the adaptor sequence ligated only at the 5′ end of each strand.

[0048] Ligation and, optionally gap repair, of the adaptor to the nucleic acid fragments generates an adaptor-nucleic acid fragment complex. In some embodiments, the adaptor-nucleic acid fragment complex can be denatured. Denaturation can be achieved using any of the methods known in the art including, but not limited to, physical, thermal, and/or chemical denaturation. In some embodiments, denaturation can be achieved using thermal or heat denaturation. In some embodiments, denaturation of the adaptor-nucleic acid fragment complex generates single stranded nucleic acid fragments comprising the adaptor sequence at only the 5′end of the nucleic acid fragments. In another embodiment, denaturation of the first adaptor-nucleic acid fragment complex generates single stranded nucleic acid fragments comprising adaptor sequence at both the 5′end and 3′end of the nucleic acid fragments.

Methods of Amplification

[0049] The methods, compositions and kits described herein can be useful to generate amplification-ready products directly from a nucleic acid source for downstream applications such as next generation sequencing, as well as generation of libraries with enriched population of sequence regions of interest. In some embodiments, the adapter-nucleic fragment ligated products, e.g., from the ligation of an adaptor to a 5' end of each nucleic acid fragment of a plurality of nucleic acid fragments from one or more samples, is amplified.

[0050] Methods of amplification are well known in the art. In some embodiments, the amplification is exponential, e.g. in the enzymatic amplification of specific double stranded sequences of DNA by a polymerase chain reaction (PCR). In other embodiments the amplification method is linear. In other embodiments the amplification method is isothermal. In

some embodiments, the amplification is exponential, e.g. in the enzymatic amplification of specific double stranded sequences of DNA by a polymerase chain reaction (PCR).

[0051] Suitable amplification reactions can be exponential or isothermal and can include any DNA amplification reaction, including but not limited to polymerase chain reaction (PCR), strand displacement amplification (SDA), linear amplification, multiple displacement amplification (MDA), rolling circle amplification (RCA), single primer isothermal amplification (SPIA, see e.g. U.S. Pat. No. 6,251,639), Ribo-SPIA, or a combination thereof. In some cases, the amplification methods for providing the template nucleic acid may be performed under limiting conditions such that only a few rounds of amplification (e.g., 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 etc.), such as for example as is commonly done for cDNA generation. The number of rounds of amplification can be about 1-30, 1-20, 1-15, 1-10, 5-30, 10-30, 15-30, 20-30, 10-30, 15-30, 20-30, or 25-30.

[0052] PCR is an in vitro amplification procedure based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by thermophilic template dependent polynucleotide polymerase, resulting in the exponential increase in copies of the desired sequence of the polynucleotide analyte flanked by the primers. The two different PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5′ ends of the oligonucleotide primers.

[0053] LCR uses a ligase enzyme to join pairs of preformed nucleic acid probes. The probes hybridize with each complementary strand of the nucleic acid analyte, if present, and ligase is

employed to bind each pair of probes together resulting in two templates that can serve in the next cycle to reiterate the particular nucleic acid sequence.

[0054] SDA (Westin et a 2000, Nature Biotechnology, 18, 199-202; Walker et al 1992, Nucleic Acids Research, 20, 7, 1691-1696), is an isothermal amplification technique based upon the ability of a restriction endonuclease such as HincII or BsoBI to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of an exonuclease deficient DNA polymerase such as Klenow exo minus polymerase, or Bst polymerase, to extend the 3′-end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as targets for an antisense reaction and vice versa.

[0055] Some aspects of the invention utilize linear amplification of nucleic acids or polynucleotides. Linear amplification generally refers to a method that involves the formation of one or more copies of the complement of only one strand of a nucleic acid or polynucleotide molecule, usually a nucleic acid or polynucleotide analyte. Thus, the primary difference between linear amplification and exponential amplification is that in the latter process, the product serves as substrate for the formation of more product, whereas in the former process the starting sequence is the substrate for the formation of product but the product of the reaction, i.e. the replication of the starting template, is not a substrate for generation of products. In linear amplification the amount of product formed increases as a linear function of time as opposed to exponential amplification where the amount of product formed is an exponential function of time.

Downstream Applications

[0056] One aspect of the present invention is that the methods and compositions disclosed herein can be efficiently and cost-effectively utilized for downstream analyses, such as in next generation sequencing or hybridization platforms, with minimal loss of biological material of interest. The methods of the present invention can also be used in the analysis of genetic information of selective genomic regions of interest (e.g., analysis of SNPs or other disease markers) as well as genomic regions which may interact with the selective region of interest. The methods of the present invention may further be used in the analysis of copy number variation as well as differential expression.

Sequencing

[0057] In some embodiments, a population of sequencing reads is generated from the amplified adapter-nucleic fragment ligated products. In some embodiments, a sequencing read comprises an index read, which comprises the sequence of an indexing site. In some embodiments, an index read comprises the sequence of an indexing site and the sequence of an identifier site. For example, the indexing site is sequenced with the identifier site. In some embodiments, the index read does not include the sequence of an identifier site. For example, the indexing site is not sequenced with the identifier site. In some embodiments, a sequencing read comprises the target sequence. In some embodiments, a sequencing read comprises a target sequence and an identifier sequence. For example, the target sequence is sequenced with the identifier site. In some embodiments, the target sequence is not sequenced with the identifier site.

[0058] The methods of the present invention are useful for sequencing by the method commercialized by Illumina, as described U.S. Pat Nos. 5,750,341; 6,306,597; and 5,969,119.

[0059] In general, double stranded fragment polynucleotides can be prepared by the methods of the present invention to produce amplified nucleic acid sequences tagged at one (e.g., (A)/(A′) or both ends (e.g., (A)/(A′) and (C)/(C′)). In some cases, single stranded nucleic acid tagged at one or both ends is amplified by the methods of the present invention (e.g., by SPIA or linear PCR).The resulting nucleic acid is then denatured and the single-stranded amplified polynucleotides are randomly attached to the inside surface of flow-cell channels. Unlabeled nucleotides are added to initiate solid-phase bridge amplification to produce dense clusters of double-stranded DNA. To initiate the first base sequencing cycle, four labeled reversible terminators, primers, and DNA polymerase are added. After laser excitation, fluorescence from each cluster on the flow cell is imaged. The identity of the first base for each cluster is then recorded. Cycles of sequencing are performed to determine the fragment sequence one base at a time.

[0060] In some embodiments, the methods of the invention are useful for preparing target polynucleotides for sequencing by the sequencing by ligation methods commercialized by Applied Biosystems (e.g., SOLiD sequencing). In other embodiments, the methods are useful for preparing target polynucleotides for sequencing by synthesis using the methods commercialized by 454/Roche Life Sciences, including but not limited to the methods and apparatus described in Margulies et al., *Nature (*2005) 437:376-380 (2005); and U.S. Pat. Nos. 7,244,559; 7,335,762; 7,211,390; 7,244,567; 7,264,929; and 7,323,305. In other embodiments, the methods are useful for preparing target polynucleotide(s) for sequencing by the methods commercialized by Helicos BioSciences Corporation (Cambridge, Mass.) as described in U.S. application Ser. No. 11/167,046, and U.S. Pat. Nos. 7,501,245; 7,491,498; 7,276,720; and in U.S. Patent Application Publication Nos. US20090061439; US20080087826; US20060286566; US20060024711;

US20060024678; US20080213770; and US20080103058. In other embodiments, the methods are useful for preparing target polynucleotide(s) for sequencing by the methods commercialized by Pacific Biosciences as described in U.S. Pat. Nos. 7,462,452; 7,476,504; 7,405,281; 7,170,050; 7,462,468; 7,476,503; 7,315,019; 7,302,146; 7,313,308; and US Application Publication Nos. US20090029385; US20090068655; US20090024331; and US20080206764.

[0061] An example of a sequencing technique that can be used in the methods of the provided invention is semiconductor sequencing provided by Ion Torrent (e.g., using the Ion Personal Genome Machine (PGM)). Ion Torrent technology can use a semiconductor chip with multiple layers, e.g., a layer with micro-machined wells, an ion-sensitive layer, and an ion sensor layer. Nucleic acids can be introduced into the wells, e.g., a clonal population of single nucleic can be attached to a single head, and the bead can be introduced into a well. To initiate sequencing of the nucleic acids on the beads, one type of deoxyribonucleotide (e.g., dATP, dCTP, dGTP, or dTTP) can be introduced into the wells. When one or more nucleotides are incorporated by DNA polymerase, protons (hydrogen ions) are released in the well, which can be detected by the ion sensor. The semiconductor chip can then be washed and the process can be repeated with a different deoxyribonucleotide. A plurality of nucleic acids can be sequenced in the wells of a semiconductor chip. The semiconductor chip can comprise chemical-sensitive field effect transistor (chemFET) arrays to sequence DNA (for example, as described in U.S. Patent Application Publication No. 20090026082). Incorporation of one or more triphosphates into a new nucleic acid strand at the 3' end of the sequencing primer can be detected by a change in current by a chemFET. An array can have multiple chemFET sensors.

[0062] Another example of a sequencing technique that can be used in the methods of the provided invention is nanopore sequencing (see e.g., Soni G V and Meller A. (2007) *Clin Chem*

53: 1996-2001). A nanopore can be a small hole of the order of 1 nanometer in diameter. Immersion of a nanopore in a conducting fluid and application of a potential across it can result in a slight electrical current due to conduction of ions through the nanopore. The amount of current that flows is sensitive to the size of the nanopore. As a DNA molecule passes through a nanopore, each nucleotide on the DNA molecule obstructs the nanopore to a different degree. Thus, the change in the current passing through the nanopore as the DNA molecule passes through the nanopore can represent a reading of the DNA sequence.

Data Analysis

[0063] In some embodiments, the sequence reads are used in the analysis of genetic information of selective genomic regions of interest as well as genomic regions which may interact with the selective region of interest. Amplification methods as disclosed herein can be used in the devices, kits, and methods known to the art for genetic analysis, such as, but not limited to those found in U.S. Pat. Nos. 6,449,562, 6,287,766, 7,361,468, 7,414,117, 6,225,109, and 6,110,709.

[0064] In some embodiments, the sequencing reads are used to detect duplicate sequencing reads. In some embodiments, a sequencing read is identified as a duplicate sequencing read when it contains an identifier site and target sequence that is the same as another sequencing read from the same population of sequencing reads.

[0065] In some embodiments, duplicate sequencing reads are differentiated from one another as being true duplicates versus apparent or perceived duplicates. Apparent or perceived duplicates may be identified from sequencing libraries and using conventional measures of duplicate reads (i.e., reads were mapped using bowtie), where all reads with the same start and end nucleic acid coordinates were counted as duplicates. True duplicates may be identified from sequencing

libraries having had an identifier site introduced through ligation to differentiate between fragments of DNA that randomly have the same start and end mapping coordinates.

[0066] In some embodiments, the sequence of the identifier sites from the index read of two nucleic acids generated from any dsDNA may have the same start site, as determined from the sequencing reads of the target sequence of the generated nucleic acid fragments. If the identifier site from the index read of the two nucleic acid fragments are not identical, then the target sequence reads were not generated from the same original dsDNA molecule and, therefore, are no true duplicate reads. The ligation of random sequences onto dsDNA molecules, accompanied by the methods of the invention, allow for the identification of true duplicate reads versus apparent or perceived duplicate reads.

[0067] In some embodiments, the sequence of the identifier sites from the index read of two nucleic acid fragments generated from a genomic DNA (gDNA) molecule that have the same start site, which can be determined from the sequencing reads of the target sequence of the nucleic acid fragments is determined. If the identifier site from the index read of the two nucleic acid fragments are not identical, then the target sequence reads were not generated from the same original gDNA molecule and, therefore, are not true duplicate reads. In another embodiment, an identifier site is inserted at the adapter insert junction. The sequence of the identifier site is carried through the library amplification step. The identifier site is the first sequence read during the forward read. As the identifier sequence is not logically present adjacent to naturally occurring sequence, this uniquely identifies the DNA fragment. Therefore, by ligating random sequences onto the original gDNA, the methods of the invention identify true duplicate reads.

[0068] In some embodiments, a duplicate sequencing read is detected and analyzed. Duplicate reads can be filtered using 'samtools rmdup', wherein reads with identical external coordinates

are removed, only one read with highest mapping quality is retained. After filtering, a filtered set of deduplicated reads can be used in any downstream analysis. Conversely, this filtering step can be skipped, and downstream analysis can be done using the unfiltered reads, including duplicates.

[0069] In some embodiments, sequencing reads are generated from a number of samples. In some embodiments, adaptors are ligated to a plurality of nucleic acid fragments from the samples, in which the nucleic acid fragments from each sample has the same indexing site. In some embodiments, a plurality of nucleic acid fragments is generated from a first sample and a second sample and adaptors are ligated to each nucleic acid fragment, in which adaptors ligated to each nucleic acid fragment from the first sample have the same first indexing site and adaptors ligated to nucleic acid fragments from the second sample has the same second indexing site. In some embodiments, the data associated with the nucleic acid fragments (e.g., sequencing reads) are separated based on the indexing site before sequencing reads of the target sequence and/or identifier site are analyzed. In some embodiments, the nucleic acid fragments or data associated with the nucleic acid fragments (e.g., sequencing reads) are separated based on the indexing site before duplicate sequencing reads are analyzed and/or removed.

[0070] In some embodiments, a method disclosed herein identifies or detects one or more true duplicate(s) with increased accuracy as compared to other methods. For example, in some embodiments, a method disclosed herein identify true duplicates (in contrast to identifying apparent or perceived duplicates) with increased accuracy as compared to other methods. The increased resolution and/or accuracy in identifying one or more true duplicate(s) can provide a considerable contribution to the state of the art in more accurately identifying true duplicates. In some embodiments, a method disclosed herein identifies or detects a true duplicate with

increased efficiency as compared to other methods, such as paired end sequencing. The increase in accuracy, resolution, and/or efficiency in detecting duplicate reads (e.g., true duplicate(s)) can increase confidence in sequencing results, such as for expression and CNV analysis.

Kits

[0071] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, the kit, in a suitable container, comprises: an adaptor or several adaptors, one or more of oligonucleotide primers and reagents for amplification.

[0072] The containers of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other containers, into which a component may be placed, and preferably, suitably aliquotted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a container.

[0073] When the components of the kit are provided in one or more liquid solutions, the liquid solution can be an aqueous solution. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent.

[0074] A kit may include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

[0075] In some embodiments, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. In some embodiments, a kit comprises a composition of the invention, in one or more containers. In some embodiments, the

invention provides kits comprising adapters, primers, and/or other oligonucleotides described herein. In some embodiments, the kit further comprises one or more of: (a) a DNA ligase, (b) a DNA-dependent DNA polymerase, (c) an RNA-dependent DNA polymerase, (d) a forward adapter (e) one or more oligonucleotides comprising reverse adaptor sequence and (f) one or more buffers suitable for one or more of the elements contained in said kit. The adapters, primers, other oligonucleotides, and reagents can be, without limitation, any of those described above. Elements of the kit can further be provided, without limitation, in any of the amounts and/or combinations (such as in the same kit or same container) described above. The kits may further comprise additional agents, such as those described above, for use according to the methods of the invention. For example, the kit can comprise a first forward adaptor that is a partial duplex adaptor as described herein, a second forward adapter, and a nucleic acid modifying enzyme specific for a restriction and/or cleavage site present in the first forward adaptor. The kit elements can be provided in any suitable container, including but not limited to test tubes, vials, flasks, bottles, ampules, syringes, or the like. The agents can be provided in a form that may be directly used in the methods of the invention, or in a form that requires preparation prior to use, such as in the reconstitution of lyophilized agents. Agents may be provided in aliquots for single-use or as stocks from which multiple uses, such as in a number of reaction, may be obtained.

[0076] In some embodiments, the kit comprises a plurality of adaptor oligonucleotides, wherein each of the adaptor oligonucleotides comprises at least one of a plurality of identifier site sequences, wherein each identifier site sequence of the plurality of identifier site sequences differs from every other identifier site sequence in said plurality of identifier site sequences at at least three nucleotide positions, and instructions for using the same. Adapters comprising

different identifier site sequences can be supplied individually or in combination with one or more additional adapters having a different identifier site sequence. In some embodiments, the kit can comprises a plurality of adapter oligonucleotides.

EXAMPLES

Example 1: Identification of Duplicate Sequencing Reads with NuGEN Ovation Target Enrichment Library System

[0077] Sample Description: l00ng of DNA from a human HapMap sample (NA19238) was fragmented to approximately 500 base pair in length by sonication with a Covaris system (Covaris, Inc., Woburn, MA). The resulting DNA was treated with end repair enzyme mix NuGEN R01280 and R01439 (NuGEN Technologies, Inc., San Carlos, CA) according to supplier's recommendation to produce blunt ended DNA fragments.

[0078] Library Generation, Enrichment, and Identifier Site Incorporation: An oligonucleotide with segments, from 5' to 3' of the top strand, 1) an Illumina indexing read priming site such as AGAGCACACGTCTGAACTCCAGTCAC (SEQ ID NO:2), 2) an indexing site, 3) an identifier site having a random 6 base sequence and, 4) a sequence compatible with an Illumina forward sequencing priming site such as TCTTTCCCTACACGACGCTCTTCCGATCT (SEQ ID NO:3) , was annealed to a second oligonucleotide to form a partially double stranded DNA adapter. Five uM of these adapters were ligated onto the end-repaired DNA using Ligase and Ligase reaction buffer from NuGEN's Ovation Ultralow Library System (NuGEN Technologies, Inc., San Carlos, CA) according to supplier's recommendations. Following 30 minutes of incubation at 25°C, the reaction mixture was diluted with water, 0.8X volume of Ampure XP magnetic beads (Agencourt Biosciences Corporation, A Beckman Coulter Company, Beverly, MA) was added and the solution thoroughly mixed. The beads were collected, washed and the ligated

DNA fragments eluted according to manufacturer's recommendations. A pool of targeting probes was annealed to the eluted DNA fragments by initially heating the solution to 95°C then slowly cooling the mixture from 80°C to 60°C by 0.6 degrees/minute. Targeting probes that were specifically annealed were extended with Taq DNA polymerase (New England Biolabs, Inc., Ipswich, MA) according to the manufacturer's protocols. Following extension, the DNA fragments were collected on Agencourt magnetic beads, washed and eluted according to manufacturer's recommendations. These libraries were enriched by 30 cycles of PCR using NuGEN library enrichment primers (Ovation Target Enrichment Library System, NuGEN Technologies, Inc., San Carlos, CA) that also contain the Illumina flow cell sequences (Illumina Inc., San Diego, CA) according to supplier's recommendation.

[0079] The resulting libraries were quantitated by qPCR using a kit provided by KAPA, diluted to 2nM and applied to an Illumina MiSeq DNA Sequencer (Illumina Inc., San Diego, CA). The following series was run: 36 base first read, 14 base second read, and 24 base third read.

[0080] *Data Analysis:* The sequencer output was processed in accordance with manufacturer's recommendation. In order to analyze the data, the indexing read was split into two files. The first file contained the first 8 bases of the indexing reads and is utilized as the library index file for standard library parsing. The other file contains only the random bases and is set aside for further sequence parsing.

[0081] Following our data analysis pipeline of sequence alignment with bowtie aligner (Langmead B. et al., Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 2009, 10:R2.), duplicate reads were identified by their genomic start positions. At this point, sequencing reads that started at the same genomic position were checked against the random bases file to see if they have the same or different set of random

bases that were ligated to them. Where two sequences with the same starting genomic coordinate had the same set of random bases, they were considered to have come from the same initial DNA ligation event, regardless of which Ovation Target Enrichment targeting probe was used to generate the sequence fragment in question. These sequences, therefore, did not provide unique information about the starting genomic DNA and are considered as one sequencing read for the purpose of variant analysis. Two sequence reads with the same starting genomic coordinate that have different random bases were derived from unique ligation events and were considered to both be valid sequencing reads for the purpose of variant identification. **Figure 5** provides the analysis results demonstrating the identification of the duplicate reads. The use of an identifier site allowed for the determination of the number of true duplications versus the number of apparent or perceived duplicates.

[0082] If the sequences of two libraries are identical, their duplication status is unknown since this could occur by chance in any library. If an identifier sequence is used in combination with the library reads, the status can be determined (duplicates if identical, distinct if different). With the SPET system, one end is common so the probability of two libraries having identical ends increases. These would appear to be duplicate sequences and their true status can be determined by looking at an identifier sequence.

[0083] Over the sampling of all randomly selected reads, the use of identifier sites provided an increased resolution on the presence of true duplicates. When evaluating two million random reads, the apparent duplicates were found to comprise 39% of all reads. However, the true duplicates, identified through the use of an identifier site, were found to comprise only 26% of all reads. The methods employing the use of an identifier site were found to considerably increase the resolution of the true number of duplicates within the pool of reads.

Example 2: Removal of Duplicate Sequencing Reads with 8 Base Identifier Site

[0084] In a standard RNA sequencing library, adaptors are ligated to the ends of double stranded cDNA. These adaptors contain universal sequences that allow for PCR amplification and sequencing on high throughput sequencing machines. The adaptors are synthesized with a large population of additional sequences, in which each additional sequence is an identifier site, at the ligation end. The identifier sites are present at the junction between the adaptor and the cDNA. The sequence read starts with the identifier site and follows with the cDNA sequence.

[0085] This pool of identifier sites is used for the detection of PCR duplicates, as PCR duplicates will contain the identical identifier site, whereas two different cDNA molecules will ligate to two different adaptors containing two different identifier sites. This identifier site is designed as eight random bases introduced onto the end of the adaptor. Sequence reads from libraries made with such adaptors contain the 8 bases of the identifier site followed by the cDNA sequence. Standard PCR duplicate removal software such as PICARD, Markduplicates, and/or SAMtools rndup is used to identify and remove the PCR duplicates, leaving behind for analysis any instances of multiple cDNA fragments that happen to have the same sequence.

Example 3: Removal of Duplicate Sequencing Reads with Mixture of Random 1-8 Base Identifier Sites

[0086] In a standard RNA sequencing library, adaptors are ligated to the ends of double stranded cDNA. These adaptors contain universal sequences that allow for PCR amplification and sequencing on high throughput sequencing machines. The adaptors are synthesized with a large population of additional sequences, in which each additional sequence is an identifier site, at the

ligation end. The identifier sites are present at the junction between the adaptor and the cDNA. The sequence read starts with the identifier site and follows with the cDNA sequence.

[0087] This pool of identifier sites is used for the detection of PCR duplicates, as PCR duplicates will contain the identical identifier site, whereas two different cDNA molecules will ligate to two different adaptors containing two different identifier sites.

[0088] One to eight random bases are introduced onto the end of the adaptor. Sequence reads from libraries made with such adaptors contain between 1 and 8 bases of the identifier site followed by the cDNA sequence. Standard PCR duplicate removal software such as PICARD, Markduplicates, and/or SAMtools rndup is used to identify and remove the PCR duplicates, leaving behind for analysis any instances of multiple cDNA fragments that happen to have the same sequence.

Example 4: Removal of Duplicate Sequencing Reads with Mixture of 96 Defined 6-Base Identifier Sites

[0089] In a standard RNA sequencing library, adaptors are ligated to the ends of double stranded cDNA. These adaptors contain universal sequences that allow for PCR amplification and sequencing on high throughput sequencing machines. The adaptors are synthesized with a large population of additional sequences, in which each additional sequence is an identifier site, at the ligation end. The identifier sites are present at the junction between the adaptor and the cDNA. The sequence read starts with the identifier site and follows with the cDNA sequence.

[0090] This pool of identifier sites is used for the detection of PCR duplicates, as PCR duplicates will contain the identical identifier site, whereas two different cDNA molecules will ligate to two different adaptors containing two different identifier sites.

[0091] A mixture of 96 defined six-bases sequences are introduced onto the end of the adaptors. Thus, each six-base sequence is an identifier site. Sequence reads from libraries made with such adaptors contain one of the 96 six-base identifier site followed by the cDNA sequence. Standard PCR duplicate removal software such as PICARD, Markduplicates, and/or SAMtools rndup is used to identify and remove the PCR duplicates, leaving behind for analysis any instances of multiple cDNA fragments that happen to have the same sequence.

Example 5: Identification of Duplicate Sequencing Reads in Determining mRNA Expression Levels

[0092] Sample Description: Total RNA is extracted from tumor and normal adjacent tissue for the purpose of finding differences in expression levels of transcripts between the two sample types. 100ng of each sample is converted into cDNA using the USP primers, reaction buffer, and Reverse Transcriptase provided in NuGEN's Encore Complete Library System (NuGEN Technologies, Inc., San Carlos, CA) according to the supplier's recommendations. This is followed by second strand synthesis, again using materials provided in the kit according to recommendations. Double stranded cDNA was prepared using the SuperScript® Double-Stranded cDNA Synthesis Kit from Life Technologies (Carlsbad, CA) according the manufacturer's instructions. DNA was sheared with a Covaris S-series device (Covaris, Inc., Woburn, MA) using the 200 bp sonication protocol provided with the instrument (10% duty cycle, 200 cycles/burst, 5 intensity, 180 seconds). DNA was treated with 1.5 uL l0X Blunting Buffer, 0.5 uL Blunting Enzyme (New England Biolabs, Inc., Ipswich, MA; p/n E1201) and 1.2 uL of 2.5mM of each dNTP mix in a total volume of 15 uL for 30 minutes at 25°C followed by 10 minutes at 70°C.

[0093] *Library Generation*: The DNA fragments were then subjected to end repair using end repair buffers and enzymes provided in NuGEN's Ovation Ultralow Library System (NuGEN Technologies, Inc., San Carlos, CA).

[0094] Forward adaptor 5'

[0095] AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTNNNNNNNN (SEQ ID NO:4),

[0096] Reverse adaptor 1) 5'

[0097] CAAGCAGAAGACGGCATACGAGATTCCCTTGTGACTGGAGTTCAGACGTGTG CTCTTCCGATCTNNNNNNNN (SEQ ID NO:5),

[0098] Reverse adaptor 2) 5'

[0099] CAAGCAGAAGACGGCATACGAGATTGAAGGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCNNNNNNNN (SEQ ID NO:6), and

[00100] Common partner 5' NNNNNNNNAGATCGGAAGAGC (SEQ ID NO:7) were all ordered from IDT (Integrated DNA Technologies, Coralville, IA). The reverse adaptors each contain a unique identifier (underlined) that enables libraries made with these adapters to be distinguished. In this case N represents an equimolar mixture of A, C, G, and T. A mixture of 5uM forward, 5uM reverse, and 10uM common in 10mM MgCl2, 50mM Tris pH 8 was heated to 95C for 5 minutes, then cooled to 20C. Adaptor ligation was performed by addition of 4.5 uL water, 3 uL Adaptor mix (prepared above), 6 uL 5X NEBNext Quick Ligation Reaction Buffer and 1.5 uL Quick T4 DNA Ligase (New England Biolabs, Inc., Ipswich, MA; p/n E6056), followed by incubation for 30 minutes at 25°C followed by 10 minutes at 70°C. Ligation products were purified by adding 70uL water and 80 uL of Ampure XP beads (Agencourt Genomics), washing twice with 70% ethanol and eluting with 20 uL of 10mM Tris pH 8.0.

Library products were amplified in a 50uL PCR containing 0.5uM each of primer (5' AATGATACGGCGACCACCGA (SEQ ID NO:8) , and 5' CAAGCAGAAGACGGCATACGA (SEQ ID NO:9), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgC12, 0.2mM each dNTP, and 1 unit Taq polymerase. The reaction was cycled 15 times under the conditions 95C for 15 seconds, 60C for 1 minute. PCR products were purified with 1 volume of Ampure XP beads (Agencourt Biosciences Corporation, A Beckman Coulter Company, Beverly, MA) as described above. The library was analyzed by HS DNA Bioanalyzer (Agilent Technologies, Santa Clara, CA) and quantitated with the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA; p/n KK4835) according to the supplied instructions. The resulting libraries are combined and are compatible with standard TruSeq single end or paired Illumina sequencing protocols for GAIIx, MiSeq, or Hi Seq sequencing instruments (Illumina Inc., San Diego, CA). The following series is run; 50 base first read, 6 base second read. A third read is not required for counting purposes or duplication analysis.

[00101] Data Analysis: The sequencer output was processed in accordance with manufacturer's recommendation. The 6 bases of the index read is used for standard library parsing, separating the data files from the two sample types. The first 50 bases of the target sequence read are compared to each other. Any read that has identical sequence to any other read is identified as a duplicate and removed from the population, thus, only a single copy is retained within the file. Once the duplicate reads have been removed, 8 bases are trimmed from each read. The trimmed reads are aligned to a reference genome. Differential expression is then determined by comparing FPKM (fragments per kilobase per million reads) values between libraries utilizing scripts such as cufflinks or cuffdiff (Trapnell et al. 2010, Nature Biotechnology, 28, 511-515; Trapnell et al. 2013, Nature Biotechnology, 31, 46-53).

Example 6: Sequencing of the Identifier Site With the Target Sequence in Paired End Reads

[00102] The Ovation Library System for Low Complexity Samples (NuGEN Technologies, Inc., San Carlos, CA) was used to generate four libraries, each from a single amplicon, following manufacturer's protocol. Purified libraries were mixed and sequenced as a multiplex on the Illumina MiSeq (Illumina Inc., San Diego, CA) to produce 125nt forward, 8nt index 1, 8nt index 2, and 25nt reverse reads. Because all amplicon reads start and end at the same sequence coordinates, the traditional method of marking library PCR duplicates (marking reads that start and end at the same genomic coordinates as duplicates) cannot be used. Instead, the 0-8nt of random sequence contained in the adaptors ligated to the amplicon were treated as an identifier sequence and used to mark duplicates. Any paired end reads that shared the same length and sequence of these random bases with any other paired end read was called a duplicate. The table below shows the results of this duplicate marking.

Table 1.

Library	Reads	Duplicate Reads	Reads from independent molecules
1	454249	376797	77452
2	439367	364001	75366
3	317760	253057	64703
4	476572	398380	78192

[00103] Table 1 demonstrates the accuracy of the method used to differentiate between duplicate reads and reads from truly independent molecules. The population of reads from independent molecules, depicted in the final column of Table 1, represent sequences from independent amplicon molecules used in generating the libraries.

Example 7: Sequencing of the Identifier Site With the Target Sequence in Reduced Representation Bisulfite (RRBS) Libraries

[00104] Reduced representation bisulfite (RRBS) libraries of the human genome are generated through the complete restriction enzyme digestion of 100ng input sample, followed by selection for short fragments. The resulting pool of fragments are ligated to adaptor sequences comprising an indexing site and an identifier site. The identifier sites comprise either 6 or 8 random nucleotides. The sequences are then sequenced to identify the identifier sites, thus revealing the true number of duplicates in the pool. In the absence of identifier sites, the number of apparent or perceived duplicates are greater than the number of true duplicates. The inclusion of an identifier site results in the identification of the number of true duplicates, as compared to the larger number of apparent or perceived duplicates.

[00105] Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patents, and patent publications cited are incorporated by reference herein in their entirety for all purposes.

[00106] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

[00107] All references, articles, publications, patents, patent publications, and patent applications cited herein are incorporated by reference in their entireties for all purposes. However, mention of any reference, article, publication, patent, patent publication, and

patent application cited herein is not, and should not be taken as an acknowledgment or any form of suggestion that they constitute valid prior art or form part of the common general knowledge in any country in the world.

[00108] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

ABSTRACT

The present invention provides methods, compositions and kits for detecting duplicate sequencing reads. In some embodiments, the duplicate sequencing reads are removed.

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	AMORESE, Douglas	Nonprovisional Application Number (if known):	
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.

2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.

3. The applicable box is checked below:

I. ☒ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. ☐ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Angie Mah/	Date November 13, 2014
Name (Print/Typed) Angie S. Mah	Practitioner Registration Number 64,902

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

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Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Filer:	Angie S.Y. Mah
Attorney Docket Number:	NUGN-001/01US

Filed as Large Entity

Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility application filing	1011	1	280	280
Utility Search Fee	1111	1	600	600
Utility Examination Fee	1311	1	720	720
Request for Prioritized Examination	1817	1	4000	4000
Pages:				
Claims:				
Claims in Excess of 20	1202	1	80	80
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Publ. Fee- Early, Voluntary, or Normal	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**5820**

CLAIMS

1. A method for detecting a duplicate sequencing read from a population of sample sequencing reads comprising:
    a) ligating an adaptor to a 5' end of each nucleic acid fragment of a plurality of nucleic acid fragments from one or more samples, wherein the adaptor comprises:
        (i) an indexing primer binding site;
        (ii) an indexing site;
        (iii) an identifier site; and
        (iv) a target sequence primer binding site;
    b) amplifying the adapter-nucleic acid fragment ligated products;
    c) generating a population of sequencing reads from amplified adapter-nucleic acid fragment ligated products; and
    d) detecting the population of sequencing reads comprising a sequencing read with a duplicate identifier site and target sequence.
2. The method of claim 1, wherein the method further comprises removing from the population of sequence reads the sequencing read with a duplicate identifier site and target sequence.
3. The method of claim 1, wherein the identifier site is sequenced with the indexing site.
4. The method of claim 1, wherein the identifier site is sequenced separately from the indexing site.
5. The method of claim 1, wherein the identifier site is sequenced with the target sequence.
6. The method of claim 1, wherein the identifier site is sequenced separately from the target sequence.
7. The method of claim 1, wherein the adaptor comprises from 5' to 3':

(i) the indexing primer binding site;

(ii) the indexing site;

(iii) the identifier site; and

(iv) the target sequence primer binding site.

8. The method of claim 1, wherein the adaptor comprises from 5' to 3':

(i) the indexing primer binding site;

(ii) the indexing site;

(iii) the target sequence primer binding site; and

(iv) the identifier site.

9. The method of claim 1, wherein the plurality of nucleic acid fragments is generated from more than one sample.

10. The method of claim 9, wherein the nucleic acid fragments from each sample has the same indexing site.

11. The method of claim 10, wherein the sequencing reads are separated based on the indexing site.

12. The method of claim 11, wherein the separation of sequencing reads is performed prior to step d).

13. The method of claim 1, wherein the nucleic acid fragments are DNA fragments, RNA fragments, or DNA/RNA fragments.

14. The method of claim 13, wherein the nucleic acid fragments are genomic DNA fragments or cDNA fragments.

15. The method of claim 1, wherein the indexing site is between 2 and 8 nucleotides in length.

16. The method of claim 1, wherein the indexing site is about 6 nucleotides in length.

17. The method of claim 1, wherein the identifier site is between 1 and 8 nucleotides in length.

18. The method of claim 1, wherein the identifier site is about 8 nucleotides in length.

19. The method of claim 1, wherein the indexing primer binding site is a universal indexing primer binding site.

20. The method of claim 1, wherein the target sequence primer binding site is a universal target sequence primer binding site.

21. A kit comprising a plurality of adaptors, wherein each adaptor comprises:

    (i) an indexing primer binding site;

    (ii) an indexing site;

    (iii) an identifier site; and

    (iv) a target sequencing primer binding site.

PTO/AIA/15 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	NUGN-001/01US
First Named Inventor	Doug AMORESE
Title	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUE
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [ ] **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. [ ] **Applicant asserts small entity status.** See 37 CFR 1.27
3. [ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. [✓] **Specification** [*Total Pages* 44] Both the claims and abstract must start on a new page. *(See MPEP § 608.01(a) for information on the preferred arrangement)*
5. [✓] **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 6]
6. **Inventor's Oath or Declaration** [*Total Pages* ____] *(including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))*
   a. [ ] Newly executed (original or copy)
   b. [ ] A copy from a prior application (37 CFR 1.63(d))
7. [✓] **Application Data Sheet** ** See note below.* See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program (*Appendix*)
   [ ] Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** (*if applicable, items a. – c. are required*)
   a. [✓] Computer Readable Form (CRF)
   b. [ ] Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**

ACCOMPANYING APPLICATION PAPERS

10. [ ] **Assignment Papers** (cover sheet & document(s)) Name of Assignee ____
11. [ ] **37 CFR 3.73(c) Statement** [ ] **Power of Attorney** (*when there is an assignee*)
12. [ ] **English Translation Document** (*if applicable*)
13. [ ] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
14. [ ] **Preliminary Amendment**
15. [ ] **Return Receipt Postcard** (*MPEP § 503*) (*Should be specifically itemized*)
16. [ ] **Certified Copy of Priority Document(s)** (*if foreign priority is claimed*)
17. [ ] **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. [✓] **Other:** Certification and Request for Prioritized Examination Under 37 CFR 1.102(e)

Note:* (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

19. CORRESPONDENCE ADDRESS

[✓] The address associated with Customer Number: 58249 **OR** [ ] Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Angie Mah/	Date	Nov. 13, 2014
Name (Print/Type)	Angie Mah	Registration No. (Attorney/Agent)	64902

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Application Number: 14540917 Document Date: 11/13/2014

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Form Revision Date: August 26, 2013

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 14540917 Document Date: 11/13/2014

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Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

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Form Revision Date: August 26, 2013

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 11/25/2014

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01 FC : 1051 140.00 DA

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Sequence Listing was accepted.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Anjum, Durreshwar (CGI Federal)
Timestamp: [year=2014; month=11; day=14; hr=9; min=57; sec=36; ms=55; ]
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Validated By CRFValidator v 1.0.4

Application No: 14540917 Version No: 1.1

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AMORESE, DOUGLAS
SCOLNICK, JONATHAN
SCHROEDER, BEN

<120> COMPOSITIONS AND METHODS FOR IDENTIFICATION
OF A DUPLICATE SEQUENCING READ

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PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	14540917
Filing Date	2014-11-13
First Named Inventor	Douglas AMORESE
Art Unit	1636
Examiner Name	Unassigned
Attorney Docket Number	NUGN-001/01US

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6251639	B1	2001-06-26	Nurith Kurn	
	2	5750341	A	1998-05-12	Stephen C. Macevicz	
	3	6306597	B1	2001-10-23	Stephen C. Macevicz	
	4	5969119	A	1999-10-19	Stephen C. Macevicz	
	5	7244559	B2	2007-07-17	Rothberg et al.	
	6	7335762	B2	2008-02-26	Rothberg et al.	
	7	7211390	B2	2007-05-01	Rothberg et al.	
	8	7244567	B2	2007-07-17	Chen et al.	

	9	7264929	B2	2007-09-04	Rothberg et al.	
	10	7323305	B2	2008-01-29	Leamon et al.	
	11	7501245	B2	2009-03-10	Quake et al.	
	12	7491498	B2	2009-02-17	Lapidus et al.	
	13	7276720	B2	2007-10-02	Kevin Ulmer	
	14	7462452	B2	2008-12-09	Williams et al.	
	15	7476504	B2	2009-01-13	Stephen Turner	
	16	7405281	B2	2008-07-29	Xu et al.	
	17	7170050	B2	2007-01-30	Turner et al.	
	18	7462468	B1	2008-12-09	Williams et al.	
	19	7476503	B2	2009-01-13	Turner et al.	

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	First Named Inventor	Douglas AMORESE
	Art Unit	1636
	Examiner Name	Unassigned
	Attorney Docket Number	NUGN-001/01US

	20	7315019	B2	2008-01-01	Turner et al.	
	21	7302146	B2	2007-11-27	Turner et al.	
	22	7313318	B2	2007-12-25	Turner et al.	
	23	6449562	B1	2002-10-10	Chandler et al.	
	24	6287766	B1	2001-09-11	Nolan et al.	
	25	7361468	B2	2008-04-22	Liu et al.	
	26	7414117	B2	2008-08-19	Saito et al.	
	27	6225109	B1	1999-05-01	Juncosa et al.	
	28	6110709	A	2000-08-29	Ausubel et al.	

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U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14540917
	Filing Date	2014-11-13
	First Named Inventor	Douglas AMORESE
	Art Unit	1636
	Examiner Name	Unassigned
	Attorney Docket Number	NUGN-001/01US

	1	20130231253	A1	2013-09-05	Amorese et al.	
	2	20060024711	A1	2006-02-02	Lapidus et al.	
	3	20090061439	B2	2010-08-03	Philip Richard Buzby	
	4	20080087826	A1	2012-11-01	Gore et al.	
	5	20060286566	A1	2006-12-21	Lapidus et al.	
	6	20060024711	A1	2006-02-02	Lapidus et al.	
	7	20060024678	A1	2006-02-02	Philip Buzby	
	8	20080213770	A1	2008-09-04	Williams et al.	
	9	20080103058	A1	2008-05-01	Suhaib Siddiqi	
	10	20090029385	A1	2009-01-29	Christians et al.	
	11	20090068655	A1	2009-03-12	John G.K. Williams	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14540917
	Filing Date	2014-11-13
	First Named Inventor	Douglas AMORESE
	Art Unit	1636
	Examiner Name	Unassigned
	Attorney Docket Number	NUGN-001/01US

	12	20090024331	A1	2009-01-22	Tomaney et al.	
	13	20080206764	A1	2008-18-28	Williams et al.	
	14	20090026082	A1	2009-01-29	Rothberg et al.	

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FOREIGN PATENT DOCUMENTS

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	1							☐

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	WESTIN et al., Anchored multiplex amplification on a microelectronic chip array. 2000. Nature Biotechnology. 18:199-204.	☐
	2	Walker et al., Strand displacement amplification--an isothermal, in vitro DNA amplification technique. 1992. Nucleic Acids Research. 20(7):1691-1696.	☐
	3	MARGULIES et al. Genome sequencing in open microfabricated high density picoliter reactors. 2005. Nature. 437 (7057):376-380.	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14540917
	Filing Date	2014-11-13
	First Named Inventor	Douglas AMORESE
	Art Unit	1636
	Examiner Name	Unassigned
	Attorney Docket Number	NUGN-001/01US

	4	SONI and MELLER. Progress toward ultrafast DNA sequencing using solid-state nanopores. 2007. Clinical Chemistry. 53(11):1996-2001.	☐
	5	LANGMEAD et al. Ultragast and memory-efficient alignment of short DNA sequences to the human genome. 2009. Genome Biology. 10:R25.	☐
	6	TRAPNELL et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. 2010. Nature Biotechnology. 28:511-515.	☐
	7	TRAPNELL et al. Differential analysis of gene regulation at transcript resolution with RNA-seq. 2013. Nature Biotechnology. 31:46-53.	☐

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14540917
	Filing Date	2014-11-13
	First Named Inventor	Douglas AMORESE
	Art Unit	1636
	Examiner Name	Unassigned
	Attorney Docket Number	NUGN-001/01US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Angie Mah/	Date (YYYY-MM-DD)	2014-12-04
Name/Print	Angie Mah	Registration Number	64902

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	20863062
Application Number:	14540917
International Application Number:	
Confirmation Number:	7076
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Customer Number:	58249
Filer:	Angie S.Y. Mah
Filer Authorized By:	
Attorney Docket Number:	NUGN-001/01US
Receipt Date:	04-DEC-2014
Filing Date:	13-NOV-2014
Time Stamp:	14:09:11
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	NUGN-001_01US_IDS_Dec_2014.pdf	75555 a4a75fba5f684ee5e9dafae6d2f5baecb9f9f389	no	8

Warnings:	
Information:	

This is not an USPTO supplied IDS fillable form					
2	Non Patent Literature	Langmead_2009.pdf	418882 617ee080bc27157a4689294d8e6de1f177062853	no	10
Warnings:					
Information:					
3	Non Patent Literature	Margulies_2005.pdf	746855 e43ecea5bb529f9e0058f2e961bce09f03575081	no	14
Warnings:					
Information:					
4	Non Patent Literature	Soni_and_Meller_2007.pdf	383298 93a337c85efb504915007effed5a4797944231d1	no	6
Warnings:					
Information:					
5	Non Patent Literature	Trapnell_2010.pdf	1463680 b7884f48141ee94f82108b312ac8a59d72d557e5	no	18
Warnings:					
Information:					
6	Non Patent Literature	Trapnell_2013.pdf	1564250 0bd974367512f5ee9e44aca4578d10e3d84aa884	no	19
Warnings:					
Information:					
7	Non Patent Literature	Walker_1992.pdf	11569484 f4b9a2c1656ed35008682dd74468ce2910230609	no	6
Warnings:					
Information:					
8	Non Patent Literature	Westin_2000.pdf	298035 941b7f18f1bc8946e11c60c988efb41422ed9f1b	no	6
Warnings:					
Information:					
			Total Files Size (in bytes):	16520039	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/540,917	11/13/2014	Douglas AMORESE	NUGN-001/01US

CONFIRMATION NO. 7076

58249
COOLEY LLP
ATTN: Patent Group
1299 Pennsylvania Avenue, NW
Suite 700
Washington, DC 20004

NOTICE

OC000000072136865

Date Mailed: 12/04/2014

INFORMATIONAL NOTICE TO APPLICANT

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

The item(s) indicated below are also required and should be submitted with any reply to this notice to avoid further processing delays.

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Douglas AMORESE
  Jonathan Scolnick
  Ben Schroeder

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/540,917

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	21 minus 20 =	* 1			OR	x 80 =	80
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1680

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/540,917	11/13/2014	1636	1820	NUGN-001/01US	21	2

CONFIRMATION NO. 7076

58249
COOLEY LLP
ATTN: Patent Group
1299 Pennsylvania Avenue, NW
Suite 700
Washington, DC 20004

FILING RECEIPT



Date Mailed: 12/04/2014

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Douglas AMORESE, Los Altos, CA;
Jonathan Scolnick, San Francisco, CA;
Ben Schroeder, San Mateo, CA;

Applicant(s)

Nugen Technologies, Inc., San Carlos, CA

Power of Attorney: None

Domestic Priority data as claimed by applicant

This appln claims benefit of 61/903,826 11/13/2013

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access - A proper **Authorization to Permit Access to Application by Participating Offices** (PTO/SB/39 or its equivalent) has been received by the USPTO.

If Required, Foreign Filing License Granted: 11/25/2014
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/540,917**
Projected Publication Date: 05/14/2015
Non-Publication Request: No
Early Publication Request: No

Title

COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

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COOLEY LLP
ATTN: PATENT GROUP
1299 PENNSYLVANIA AVENUE, NW
SUITE 700
WASHINGTON DC 20004

MAILED
DEC 10 2014
OFFICE OF PETITIONS

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 14/540,917

1. THE REQUEST FILED November 13, 2014 IS **GRANTED**.

The above-identified application has met the requirements for prioritized examination

A. ☒ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;
B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
C. filing a **request for continued examination**;
D. filing a notice of appeal;
E. filing a request for suspension of action;
F. mailing of a notice of allowance;
G. mailing of a final Office action;
H. completion of examination as defined in 37 CFR 41.102; or
I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to **Michelle R. Eason** at (571) 272-4231.

In his/her absence, calls may be directed to Brian W. Brown at (571) 272-5338.

/Michelle R. Eason/
(*Signature*)

Paralegal Specialist, Office of Petitions
(Title)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	AMORESE, Douglas, et al.	Confirmation No.:	7076
Serial No.:	14/540,917	Group Art Unit:	1636
Filed:	November 13, 2014	Examiner:	*To be assigned*

FOR: **COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

POWER BY ASSIGNEE AND STATEMENT UNDER 37 C.F.R. §3.73(c)

The Assignee of the entire right, title, and interest in the above-identified application hereby grants the registered practitioners of Cooley LLP included in the Customer Number provided below power to act, prosecute, and transact all business in the U.S. Patent and Trademark Office in connection with this application, any applications claiming priority to this application, and any patents issuing therefrom.

The Assignee certifies that to the best of its knowledge and belief it is the owner of the entire right, title, and interest in and to the above-identified application as evidenced by:

☒ An assignment document, a copy of which is enclosed herewith and is being recorded with the U.S. Patent and Trademark Office concurrently herewith;

☐ An assignment previously recorded in the U.S. Patent and Trademark Office at Reel ___, Frame ___.

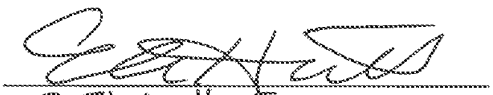
Please direct all telephone calls and correspondence to:

CUSTOMER NUMBER: **58249**

COOLEY LLP
ATTN: Patent Group
1299 Pennsylvania Avenue NW, Suite 700
Washington, DC 20004
Tel: (202) 842-7800
Fax: (202) 842-7899

The undersigned (whose title is supplied below) is empowered to sign this statement on behalf of the Assignee.

Date: 11 DEZ 14

Signature: 

Name: ELIZABETH A. HUNT
Title: CEO
Company: NuGen Technologies, Inc.

111916576 v2

ASSIGNMENT

AMORESE, Douglas, residing at Los Altos, California, and **SCHROEDER, Ben**, residing at San Mateo, California, and **SCOLNICK, Jonathan**, residing at San Francisco, California, (each referred to as "Assignor") have made an invention(s) (the "Invention(s)") set forth in an application for patent of the United States, entitled **COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ**, and which is a:

(1) ☐ provisional application
- (a) ☐ to be filed herewith; or
- (b) ☐ bearing Application No. , and filed on ;

(2) ☒ non-provisional application
- (a) ☐ to be filed herewith; or
- (b) ☒ bearing Application No. 14/540,917, and filed on November 13, 2014; and/or

(3) ☐ PCT application
- (a) ☐ bearing Application No. , and filed on .

WHEREAS, NuGen Technologies, Inc., a corporation duly organized under and pursuant to the laws of California, and having its principal place of business at 201 Industrial Road, Suite 310, San Carlos, California 94070 (the "Assignee"), is desirous of acquiring the entire right, title, and interest in: the Invention(s); the application(s) for patent identified in paragraph (1), (2) and/or (3); the right to file applications for patent of the United States or other countries on the Invention(s); any application(s) for patent of the United States or other countries claiming priority to, and/or the benefit of, these applications; any provisional or other right to recover damages, including royalties, for prior infringements of these application(s); and any patent(s) of the United States or other countries that may be granted therefor or thereon.

NOW, THEREFORE, for good and sufficient consideration, the receipt of which is hereby acknowledged, and to the extent that the Assignor has not done so already via a prior agreement with the Assignee, or if the Assignor has already done so via a prior agreement with the Assignee then in confirmation of any obligation to do so in said prior agreement, the Assignor has sold, assigned, transferred, and set over, and by these presents does sell, assign, transfer, and set over, unto the Assignee, its successors, legal representatives, and assigns, the Assignor's entire right, title, and interest in:

(a) the Invention(s);

(b) the application(s) for patent identified in paragraph (1), (2) and/or (3);

(c) the right to file applications for patent of the United States or other countries on the Invention(s), including all rights under the Hague Convention, the Paris Convention for the Protection of Industrial Property, and under the Patent Cooperation Treaty, and all rights of claiming priority in any country of the world;

(d) any application(s) for patent of the United States or other countries claiming the Invention(s);

(e) any application(s) for patent of the United States or other countries claiming priority to, and/or the benefit of, at least one of the application(s) for patent identified in paragraph (1), (2) and/or (3) or any application(s) for patent claiming the Invention(s), including any substitute application(s), division(s), continuation(s), and continuation(s)-in-part;

(f) any provisional or other right to recover damages, including royalties, for prior infringements of any application for patent identified in the preceding paragraphs (b)-(e); and

(g) any patent(s) of the United States or other countries that may be granted for or on any application for patent identified in the preceding paragraphs (b)-(e), including any reissue(s), reexamination(s), revival(s), renewal(s) and extension(s) of said patent(s).

The above-granted rights, titles, and interests are to be held and enjoyed by the Assignee, for its own use and behalf and the use and behalf of its successors, legal representatives, and assigns, as fully and entirely as the same would have been held and enjoyed by the Assignor had this sale and assignment not been made.

The Assignor hereby represents to the Assignee, its successors, legal representatives, and assigns, that, at the time of execution and delivery of these presents, or if applicable, at such time said prior agreement was executed, the Assignor is a lawful owner of an undivided interest in the entire right, title, and interest in and to the Invention(s), that the Invention(s) are unencumbered, except, if applicable, by obligation to assign in accordance with said prior agreement, and that the Assignor has good and full right and lawful authority to sell and convey the same in the manner set forth herein, and that Assignor will not make or enter into any assignment, sale, agreement or encumbrance which would conflict with these presents.

The Assignor hereby covenants and agrees to and with the Assignee, its successors, legal representatives, and assigns, that the Assignor will sign all papers and documents, take all lawful oaths, and do all acts necessary or required to be done in connection with any and all proceedings for the procurement, maintenance, enforcement and defense of the Invention(s), said application(s), and said patent(s), including interference and derivation proceedings, and any post-grant proceedings (e.g., opposition

proceedings, post-grant reviews, *Inter partes* reviews, supplemental examinations, etc.) without charge to the Assignee, its successors, legal representatives, and assigns, but at the cost and expense of the Assignee, its successors, legal representatives, and assigns.

The Assignor hereby authorizes and requests the attorneys of COOLEY LLP to insert in the spaces provided above the filing date, the application number, and the attorney docket number of the application(s) identified in paragraph (1), (2) and/or (3) when known.

The Assignor hereby requests the Commissioner of Patents to issue said patents of the United States to the Assignee for the sole use and behalf of the Assignee, its successors, legal representatives, and assigns.

Date: Dec 11, 2014 By: [signature]
Douglas Amorese

State of CALIFORNIA)
) ss.
County of SANTA CLARA)

On 12/11/14, before me, KYLE DEVIN EISENBERG, Notary Public, personally appeared DOUGLAS ALAN AMORESE, who proved to me on the basis of satisfactory evidence, to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

REQUIRED SENTENCE IF NOTARIZED IN CALIFORNIA: I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

KYLE DEVIN EISENBERG
Commission # 2057666
Notary Public - California
San Francisco County
My Comm. Expires May 10, 2018

WITNESS my hand and official seal.

[signature]
Signature of Notary Public

Place Notary Seal Above

My Commission Expires: 5-10-2018

111916878 v2

Date: 11 Dec 2014 By: Ben Schroeder

State of CALIFORNIA)
) ss.
County of SANTA CLARA)

On 12/11/14, before me, KYLE DEVIN EISENBERG, Notary Public, personally appeared BENJAMIN GEORGE SCHROEDER, who proved to me on the basis of satisfactory evidence, to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

REQUIRED SENTENCE IF NOTARIZED IN CALIFORNIA: I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

KYLE DEVIN EISENBERG
Commission # 2067568
Notary Public - California
San Francisco County
My Comm. Expires May 10, 2018

Signature of Notary Public

Place Notary Seal Above

My Commission Expires: 5-10-18

111916878 v2

Date: 11 December 14 By: _____
Jonathan Scolnick

State of CALIFORNIA)
) ss.
County of SANTA CLARA)

On 12/11/14, before me, KYLE DEVIN EISENBERG, Notary Public, personally appeared JONATHAN ADAM SCOLNICK, who proved to me on the basis of satisfactory evidence, to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

REQUIRED SENTENCE IF NOTARIZED IN CALIFORNIA: I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

KYLE DEVIN EISENBERG
Commission # 2067666
Notary Public - California
San Francisco County
My Comm. Expires May 10, 2018

WITNESS my hand and official seal.

Signature of Notary Public

Place Notary Seal Above

My Commission Expires: 5-10-18

111916878 v2

Date: 11 DEC 14 By: [signature]
Name: ELIZABETH A. HUTT
Title: CEO
Company: NuGen Technologies, Inc.

State of CALIFORNIA)
) ss.
County of SANTA CLARA)

On 12/11/14, before me, KYLE DEVIN EISENBERG, Notary Public, personally appeared ELIZABETH ANN HUTT POLLARD, who proved to me on the basis of satisfactory evidence, to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

REQUIRED SENTENCE IF NOTARIZED IN CALIFORNIA: I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

KYLE DEVIN EISENBERG
Commission # 2067666
Notary Public - California
San Francisco County
My Comm. Expires May 10, 2018

WITNESS my hand and official seal.

[signature]
Signature of Notary Public

Place Notary Seal Above

My Commission Expires: 5-10-2018

111916878 v2

Electronic Acknowledgement Receipt

EFS ID:	21077651
Application Number:	14540917
International Application Number:	
Confirmation Number:	7076
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Customer Number:	58249
Filer:	Angie S.Y. Mah
Filer Authorized By:	
Attorney Docket Number:	NUGN-001/01US
Receipt Date:	31-DEC-2014
Filing Date:	13-NOV-2014
Time Stamp:	08:15:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Oath or Declaration filed	NUGN_001_01US_Decs.pdf	132009 c8cd06b8f7ad2226ccdfcdda6b1aac392f3d8ea9	no	3

Warnings:

The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
2	Power of Attorney	NUGN_001_01US_POA.pdf	254191 1223823626f57d91c8794ef732aaf0ce9c219ff4	no	9
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
			Total Files Size (in bytes):	386200	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 14/540,917

filed on 11/13/2014.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Ben Schroeder Date (Optional) : _____

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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LEGAL NAME OF INVENTOR

Inventor: Douglas Amorese Date (Optional) : .....

Signature: .....

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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☐ The attached application, or

☒ United States application or PCT international application number 14/540,917

filed on 11/13/2014.

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LEGAL NAME OF INVENTOR

Inventor: Jonathan Scolnick Date (Optional) : _____

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/540,917	11/13/2014	Douglas AMORESE	NUGN-001/01US	7076

58249 7590 01/07/2015

COOLEY LLP
ATTN: Patent Group
1299 Pennsylvania Avenue, NW
Suite 700
Washington, DC 20004

EXAMINER
STRZELECKA, TERESA E

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
01/07/2015	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

zpatdcdocketing@cooley.com

Office Action Summary	Application No. 14/540,917	Applicant(s) AMORESE ET AL.	
	Examiner TERESA E. STRZELECKA	Art Unit 1637	AIA (First Inventor to File) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *2* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-21* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☐ Claim(s) _____ is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☒ Claim(s) *1-21* are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Election/Restrictions

2. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-20, drawn to a method for detecting a duplicate sequencing read from a population of sample sequencing reads comprising:

a) ligating an adaptor to a 5' end of each nucleic acid fragment of a plurality of nucleic acid fragments from one or more samples, wherein the adaptor comprises:

(i) an indexing primer binding site;

(ii) an indexing site;

(iii) an identifier site; and

(iv) a target sequence primer binding site;

b) amplifying the adapter-nucleic acid fragment ligated products;

c) generating a population of sequencing reads from amplified adapter-nucleic acid fragment ligated products; and

d) detecting the population of sequencing reads comprising a sequencing read with a duplicate identifier site and target sequence, classified in C12Q 1/6806, for example.

II. Claim 21, drawn to a kit comprising a plurality of adaptors, wherein each adaptor comprises:

(i) an indexing primer binding site;

(ii) an indexing site;

(iii) an identifier site; and

(iv) a target sequencing primer binding site, classified in C12Q 6855, for example.

The inventions are distinct, each from the other because of the following reasons:

3. Inventions I and II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case the kit of Group II can be used in an entirely different method, such as library construction from different tissues, rather than in the method of Group I.

Searching the inventions of Groups I and II together would impose serious search burden. The inventions of Groups I and II have a separate status in the art as shown by their different classifications. Moreover, in the instant case, the search for the adaptor oligonucleotide and the method of sequencing using adaptor oligonucleotides are not coextensive. Group II encompasses all possible oligonucleotides, since the terms "primer binding site", "indexing site", identifier site" and "target sequence binding site" are not claimed in terms of their specific structure, i.e., a nucleic acid sequence. In contrast, the search for group I would require a text search for the method of using adaptors in sequencing. Prior art which teaches an adaptor of Group II would not necessarily be applicable to the method of using adaptors in sequencing. Moreover, even if the adaptor product were known, the method of using the product may be novel and unobvious in view of the preamble or active steps.

4. Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a

serious search and/or examination burden if restriction were not required because one or more of the following reasons apply:

Because these inventions are distinct for the reasons given above, have acquired a separate status in the art as shown by their different classification, and the search required for each group is not required for the other groups because each group requires a different non-patent literature search due to each group comprising different products and/or method steps, restriction for examination purposes as indicated is proper.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of an invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence

or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other invention.

5. This application contains claims directed to the following patentably distinct species:

Group I

Species of sequencing the identifier site

A) identifier site is sequenced with the indexing site (claim 3),

B) identifier site is sequenced separately from the indexing site (claim 4),

C) identifier site is sequenced with the target sequence (claim 5),

D) identifier site is sequenced separately from the target sequence (claim 6).

Species of adaptor

E) adaptor comprises from 5' to 3': (i) the indexing primer binding site; (ii) the indexing site; (iii) the identifier site; and (iv) the target sequence primer binding site (claim 7),

F) adaptor comprises from 5' to 3': (i) the indexing primer binding site; (ii) the indexing site; (iii) the target sequence primer binding site; and (iv) the identifier site (claim 8).

Species of nucleic acid fragments

G) genomic DNA fragments (claim 13, 14),

H) cDNA fragments (claim 13, 14),

I) RNA fragments (claim 13),

J) DNA/RNA fragments (claim 13).

6. The species are independent or distinct because claims to the different species recite the mutually exclusive characteristics of such species. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species from each of the sets of species if Group I is elected, or a single grouping of patentably indistinct species, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claim 1 is generic.

There is a search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply:

The species require a different field of search (e.g., searching different classes/subclasses or electronic resources, or employing different search queries); and/or the prior art applicable to one species would not likely be applicable to another species; and/or the species are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species or grouping of patentably indistinct species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

The election may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under

37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species or grouping of patentably indistinct species.

Should applicant traverse on the ground that the species, or groupings of patentably indistinct species from which election is required, are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing them to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other species.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

7. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

8. The examiner has required restriction between product or apparatus claims and process claims. Where applicant elects claims directed to the product/apparatus, and all product/apparatus claims are subsequently found allowable, withdrawn process claims that include all the limitations of the allowable product/apparatus claims should be considered for

rejoinder. All claims directed to a nonelected process invention must include all the limitations of an allowable product/apparatus claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product/apparatus claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product/apparatus are found allowable, an otherwise proper restriction requirement between product/apparatus claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product/apparatus claim will not be rejoined. See MPEP § 821.04. Additionally, in order for rejoinder to occur, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product/apparatus claims. **Failure to do so may result in no rejoinder.** Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to TERESA E. STRZELECKA whose telephone number is (571)272-0789. The examiner can normally be reached on M-F (8:30-5:30).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

TERESA E STRZELECKA
Primary Examiner
Art Unit 1637

/TERESA E STRZELECKA/
Primary Examiner, Art Unit 1637
January 2, 2015

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/540,917	11/13/2014	Douglas AMORESE	NUGN-001/01US

CONFIRMATION NO. 7076

POA ACCEPTANCE LETTER

58249
COOLEY LLP
ATTN: Patent Group
1299 Pennsylvania Avenue, NW
Suite 700
Washington, DC 20004

OC000000072703872

Date Mailed: 01/08/2015

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 12/31/2014.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/hteffera/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

REMARKS

Restriction

The Office has issued a restriction requirement stating that the application claims two distinct inventions that are related as product and process of use. Specifically, the Office identifies the two inventions as being:

> Group I - claims 1-20, drawn to a method for detecting a duplicate sequencing read from a population of sample sequencing reads; and
>
> Group II - claim 21, drawn to a kit comprising a plurality of adaptors, wherein each adaptor comprises an indexing primer binding site, an indexing site, an identifier site, and a target sequencing primer binding site.

Applicant hereby elects, with traverse, Group I, reading on claims 1-20. The Office alleges there would be a serious search and/or examination burden if restriction were not required because each group alleged requires distinct and non-coextensive search queries. Applicant respectfully disagrees. The claim in Group II is directed to a kit comprising the adaptor recited in Group I. Accordingly, it would not be an undue burden for the Office to search and examine the claim in Group II along with Group I.

In the event the Office maintains the restriction requirement, Applicant reserves the right to pursue claims drawn to the non-elected subject matter of Group II in one or more divisional application(s).

Election of Species

The Office has indicated that the claims are directed to patentably distinct species and has requested an election of species. Applicant must elect a single species from each of the three sets of species:

Species of sequencing the identifier site

A) identifier site is sequenced with the indexing site (claim 3),

B) identifier site is sequenced separately from the indexing site (claim 4),

C) identifier site is sequenced with the target sequence (claim 5),

D) identifier site is sequenced separately from the target sequence (claim 6).

Species of adaptor

E) adaptor comprises from 5' to 3': (i) the indexing primer binding site; (ii) the indexing site; (iii) the identifier site; and (iv) the target sequence primer binding site (claim 7),

F) adaptor comprises from 5' to 3': (i) the indexing primer binding site; (ii) the indexing site; (iii) the target sequence primer binding site; and (iv) the identifier site (claim 8).

Species of nucleic acid fragments

G) genomic DNA fragments (claims 13 and 14),

H) cDNA fragments (claims 13 and 14),

I) RNA fragments (claim 13),

J) DNA/RNA fragments (claim 13).

Applicant hereby elects species A), E) and G). Claims 1-3, 7, and 9-20 encompass the elected species.

Applicant reserves the right to request examination of additional species upon allowance of the elected species in this application, and to pursue claims to the non-elected species in this application in the event that a generic claim is found to be allowable.

Applicant also reserves the right to request rejoinder of claims to non-elected subject matter, upon the allowance of a claim directed to the elected invention. Applicant further reserves the right to file one or more continuation or divisional applications directed to non-elected subject matter.

CONCLUSION

In view of the foregoing, Applicant respectfully submits that submit that this paper fully addresses the Restriction Requirement dated January 7, 2015. However, the Examiner is requested to call the undersigned if any questions or comments arise.

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283.

Dated: March 6, 2015

Respectfully submitted,
COOLEY LLP

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By: /Angie Mah/
Angie S. Mah
Reg. No. 64,902

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	AMORESE, Douglas, et al.	Confirmation No.:	7076
Application No.:	14/540,917	Group Art Unit:	1637
Filed:	November 13, 2014	Examiner:	Teresa E. Strzelecka

FOR: **COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ**

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO RESTRICTION REQUIREMENT

This paper is in response to the Restriction Requirement dated January 7, 2015. This response is timely filed before the two-month statutory period for reply ending on March 7, 2015.

Remarks begin on page 2 of this paper.

Reconsideration of the application in view of the following remarks is respectfully requested.

Electronic Acknowledgement Receipt

EFS ID:	21693478
Application Number:	14540917
International Application Number:	
Confirmation Number:	7076
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Customer Number:	58249
Filer:	Angie S.Y. Mah
Filer Authorized By:	
Attorney Docket Number:	NUGN-001/01US
Receipt Date:	06-MAR-2015
Filing Date:	13-NOV-2014
Time Stamp:	11:42:55
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Response to Election / Restriction Filed	NUGN-001_01US_OARtoRestriction_SpeciesRequirement_.pdf	99625 3c4da50339e19780f7024d63e0523843a68a4428	no	4

Warnings:	
Information:	

Total Files Size (in bytes):	99625

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	21917420
Application Number:	14540917
International Application Number:	
Confirmation Number:	7076
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Customer Number:	58249
Filer:	Angie S.Y. Mah
Filer Authorized By:	
Attorney Docket Number:	NUGN-001/01US
Receipt Date:	31-MAR-2015
Filing Date:	13-NOV-2014
Time Stamp:	12:03:41
Application Type:	Utility under 35 USC 111(a)

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Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	WO2008093098A2.pdf	2267793 70f0a59bf895afa2f6ec4ac3df5441b07590a177	no	111
Warnings:					
Information:					

2	Foreign Reference	WO2012061832A1.pdf	758620 a73d4bd7edf16a3658dd0a074d9f50b6200f2dba	no	103
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Information:					
3	Foreign Reference	WO2014144092A1.pdf	558648 b075a2ede6e478166f251eef3887421fb38f887f	no	68
Warnings:					
Information:					
4	Non Patent Literature	MYERS_2013.pdf	844636 324d3f99777ec6fe2e74fc11c9833fab1512f61b	no	15
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Information:					
5	Non Patent Literature	PCTUS2014065530__ISR_and_WrittenOpinion.pdf	1037971 b58cb700b1f5390602b5f7353d3e45bdf0d56f40	no	14
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Information:					
6	Transmittal Letter	NUGN-001_01US-IDSTransmittal.pdf	78149 3d269f11858d8841787f783608d134fbab552e9a	no	2
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Information:					
7	Information Disclosure Statement (IDS) Form (SB08)	NUGN-001_01US-SB08.pdf	164578 8d2de668fea53e4b5a38fbf77c1f7d7d1325a1de	no	1
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Information:					
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New Applications Under 35 U.S.C. 111

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New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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(71) Applicant: **NUGEN TECHNOLOGIES, INC.** [US/US]; 201 Industrial Road, Suite 310, San Carlos, CA 94070 (US).

(72) Inventors: **AMORESE, Doug**; 357 South El Monte Avenue, Los Altos, CA 94022 (US). **SCHROEDER, Benjamin, G.**; 1964 Shoreview Avenue, San Mateo, CA 94401 (US). **SCOLNICK, Jonathan**; 451 Kansas Street, #518, San Francisco, CA 94107 (US).

(74) Agents: **HSU, Jennifer** et al.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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[Continued on next page]

(54) **Title**: SEQUENTIAL SEQUENCING

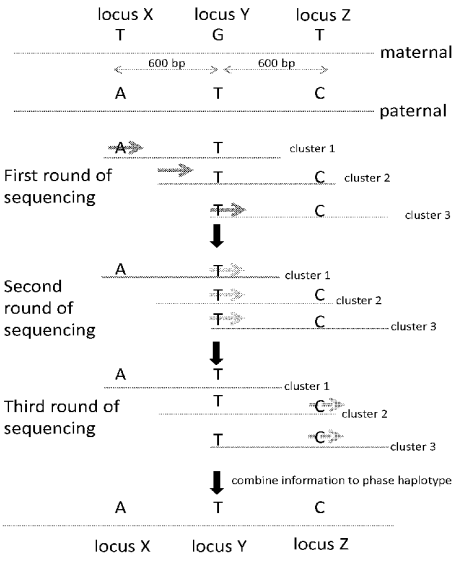


FIG. 3

(57) **Abstract**: Described herein are improved methods, compositions and kits for next generation sequencing (NGS). The methods, compositions and kits described herein enable phasing of two or more nucleic acid sequences in a sample, i.e. determining whether the nucleic acid sequences (which can comprise regions of sequence variation) are located on the same chromosome and/or the same chromosomal fragment. Phasing information can be obtained by performing multiple, successive sequencing reactions from the same immobilized nucleic acid template. The methods, compositions and kits provided herein can be useful, for example, for haplotyping, SNP phasing, or for determining downstream exons in RNA-seq.

WO 2014/144092 A1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

SEQUENTIAL SEQUENCING

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/801,600, filed March 15, 2013, which application is incorporated herein by reference.

BACKGROUND

[0002] Next generation sequencing (NGS) can be utilized for a wide variety of life science applications, such as, e.g., diagnostics, epidemiology, and forensics. Widely used NGS technologies commonly involve sequencing of short reads (e.g., 100 nt reads) which can then be mapped to a reference genome. While such technologies can be useful for the detection of sequence variants (e.g., mutations), they are also associated with certain limitations. For example, NGS can be used to sequence genomes or genomic regions of diploid organisms. A genome of a diploid organism can comprise sets of chromosome pairs, wherein each chromosome pair comprises a maternal chromosome and a paternal chromosome. For example, a diploid organism can inherent a maternal allele and a paternal allele at a single locus. Thus, alleles detected at a plurality of loci which map to a particular chromosome might exist within a single chromosome (maternal or paternal) of a chromosome pair, or across both chromosomes of a chromosome pair. While many NGS platforms are useful for detecting alleles at a plurality of loci, they currently do not provide phasing and/or haplotype information e.g., to distinguish whether alleles detected at a plurality of loci that map to a particular chromosome are co-located on the same chromosome or are located on separate chromosomes in a chromosome pair. Determining whether a plurality of alleles are co-located on the same (maternal or paternal) chromosome or are located on different chromosomes can be useful for a variety of reasons, as discussed further below.

[0003] The pattern of alleles within each individual chromosome can be referred to as haplotype. Haplotyping can have many diagnostic and clinical applications. For example, two inactivating mutations across different loci within a single gene might be of little or no consequence if present on the same individual chromosome (i.e. chromosome of either maternal or paternal origin), because the other copy of the gene product will remain functional. On the other hand, if one of the inactivating mutations is present in the maternal chromosome and the other in the paternal chromosome, there can be no functional copy of the gene product, which can result in a negative phenotype (non-viability, increased risk for disease and others). Haplotyping can also be used to predict risk or susceptibility to specific

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genetic diseases, as many genetic associations are tied to haplotypes. For example, the various haplotypes of the human leukocyte antigen (HLA) system can be associated with genetic diseases ranging from autoimmune disease to cancers.

[0004] Another instance in which phasing information can be useful is distinguishing between functional genes and their non-functional pseudogene counterparts within the genome. One well known functional gene/pseudogene pair is the genes SMN1 and SMN2, which differ in sequence by only five nucleotides over many Kb of sequence, yet one of the nucleotide differences renders the SMN2 gene almost completely non-functional. Using short read sequencing, a mutation may be found in one of the two genes, but unless the mutation happens to occur within the sequencing read that also covers one of the known nucleotide differences between SMN1 and SMN2, it will be difficult to know which of the genes (the functional gene, or the nonfunctional pseudogene) is mutated.

[0005] The present NGS methods can employ short read sequencing to query regions of variable DNA sequence (polymorphisms etc.) interspersed within regions of conserved DNA sequence. As significant blocks of conserved sequence can be interspersed between the variable regions, short read sequencing does not lend itself to phasing analysis. Although methods have been developed to obtain phasing information, these methods (for example, Sanger sequencing and subcloning), can be labor intensive and/or costly.

[0006] There is a need for improved NGS methods that provide phasing information. Such methods can provide a highly parallel platform for performing multiple sequencing reactions from the same immobilized templates. Methods described herein fulfill this need.

SUMMARY OF THE INVENTION

[0007] Provided herein are novel methods, compositions and kits for phasing two or more nucleic acid sequences in a sample. Specifically, in one aspect methods and compositions are provided that can allow for determining whether two or more nucleic acid sequences (typically comprising regions of sequence variation) are located on the same nucleic acid template, chromosome, or chromosomal fragment. The methods and compositions disclosed herein can also be used to distinguish and differentiate between two closely related nucleic acid sequences by compiling and aligning data from sequential sequencing reads.

[0008] The methods, kits, and compositions described herein can employ sequential paired sequencing reads from the same immobilized nucleic acid template. The reads can be generated by successive rounds of priming, sequencing, denaturing and repriming, and the

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results from multiple reads originating from the same template can be compiled to obtain phasing information.

[0009] Additionally, the methods, kits, and compositions described herein can employ pools of oligonucleotides which may be used to prime sequencing reactions. Such sequencing reactions can target specific regions of specific nucleic acids for sequencing. These oligonucleotide pools can be used onboard a sequencer to extend the sequencing of nucleic acids that have already undergone a first round of sequencing.

[0010] In one aspect, provided herein is a method for relating multiple nucleic acid sequences (e.g., comprising regions of sequence variation) to the same nucleic acid template. In some embodiments, the method comprises: a) creating a nucleic acid library; b) sequencing the library with an oligonucleotide primer; c) denaturing the extension strand; d) performing a second round of sequencing by introducing a new oligonucleotide primer containing sequence complementary to conserved regions present in some of the nucleic acid templates within the nucleic acid library; e) repeating steps c) and d) as needed; and f) compiling sequencing data from the successive sequencing reads to differentiate between closely related nucleic acid sequences. In some embodiments, the nucleic acid library is a directional nucleic acid library. In some embodiments, the nucleic acid library is not a directional nucleic acid library.

[0011] In some embodiments, the nucleic acid library comprises closely related nucleic acid sequences as inserts. In some embodiments, the conserved regions within the nucleic acid inserts are located adjacent to variable regions. In some embodiments, alignment of multiple variable regions enables differentiating between and/or typing of related transcripts. In some embodiments, alignment of multiple variable regions enables differentiating between and/or typing of related micro-organisms. In some embodiments, the nucleic acid library is a directional nucleic acid library. In some embodiments, the nucleic acid library is not a directional nucleic acid library.

[0012] In another aspect, provided herein is a method for differentiating between closely related nucleic acid sequences (such as genes and pseudogenes) by using specific sets of oligonucleotide primers containing sequence complementary to a common region shared by the closely related sequences. In some embodiments, the method comprises: a) creating a sequencing library with related nucleic acid sequences as inserts; b) sequencing the library with an oligonucleotide primer; c) denaturing the extension strand; d) performing a second round of sequencing by introducing a new oligonucleotide primer containing sequence complementary to conserved regions present in some of the nucleic acid templates within the

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nucleic acid library; e) repeating steps c) and d) as needed; and f) compiling sequencing data from the successive sequencing reads to differentiate between closely related nucleic acid sequences.

[0013] Kits for performing any of the methods described herein are provided. Such kits can include reagents, enzymes and platforms for amplification and sequencing of nucleic acids. In one embodiment, a kit is provided comprising: a) an adaptor or several adaptors, b) one or more of oligonucleotide primers, and c) reagents for amplification. In another embodiment, the kit further comprises reagents for sequencing. A kit can include instructions for employing the kit components as well as the use of any other reagent not included in the kit.

[0014] Also provided herein is a method for relating at least two nucleic acid sequences or regions of sequence variation to the same nucleic acid template, the method comprising: (a) creating a nucleic acid library wherein a template is immobilized on a solid support; (b) generating a first sequencing read from the immobilized template with a first oligonucleotide primer, wherein a 3' terminal nucleotide of the first oligonucleotide primer binds to a first binding site in the template; (c) denaturing the first sequencing read from the immobilized template; (d) annealing a second oligonucleotide primer that is complementary to a region or regions within the immobilized template, wherein a 3' terminal nucleotide of the second oligonucleotide primer binds to a second binding site in the template that is more than 5 nt away from the first binding site in the template; (e) generating a second sequencing read with the second oligonucleotide primer; and (f) compiling data from the first sequencing read and the second sequencing read to map reads originating from the template. In some embodiments, the nucleic acid libraries are generated from amplicons. In some embodiments, the amplicons comprise conserved regions which flank a variable region. In some embodiments, alignment of multiple variable regions enables differentiation and/or typing of related transcripts. In some embodiments, alignment of multiple variable regions enables differentiation and/or typing of related micro-organisms. In some embodiments, the libraries are reduced complexity. In some embodiments, the reduced complexity is achieved by target enrichment. In some embodiments, the nucleic acid library comprises closely related nucleic acid sequences as inserts. In some embodiments, the nucleic acid library is a directional nucleic acid library. In some embodiments, the nucleic acid library is not a directional nucleic acid library.

[0015] Also provided herein is a method comprising: (a) coupling at least one nucleic acid template to a sequencing platform; (b) subjecting the at least one nucleic acid template to a first round of sequencing using a first sequencing primer, wherein the first sequencing primer

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comprises a 3' terminal nucleotide which anneals to a first binding site in the at least one nucleic acid template, wherein the first round of sequencing produces a first sequencing read of said at least one nucleic acid template; (c) removing said first annealed sequencing primer and said first sequencing read from said at least one nucleic acid template; and (d) subjecting said at least one nucleic acid template to a second round of sequencing using a second sequencing primer, wherein the second sequencing primer comprises a 3' terminal nucleotide which anneals to a second binding site in the at least one nucleic acid template which is more than 5 nt away from the first binding site, wherein the second round of sequencing produces a second sequencing read of the at least one nucleic acid template.

[0016] In some embodiments of any of the foregoing methods, the first and/or the second round of sequencing comprises sequencing by synthesis. In some embodiments, the first and/or the second round of sequencing comprises sequencing by ligation. In some embodiments, the coupling comprises conjugating the at least one nucleic acid template to a solid support of the sequencing platform. In some embodiments, the solid support comprises a bead, a well, an array, or a flow cell. In some embodiments, the at least one nucleic acid template comprises an adaptor oligonucleotide ligated to one or both ends of the at least one nucleic acid template. In some embodiments, the first sequencing primer is selectively hybridizable to a primer binding site of the adaptor oligonucleotide. In some embodiments, the second sequencing primer is not selectively hybridizable to the primer binding site of the adaptor oligonucleotide. In some embodiments, the second sequencing read comprises an informative locus. In some embodiments, the informative locus is a polymorphic locus. In some embodiments, the first sequencing read comprises a first informative locus and the second sequencing read comprises a second informative locus. In some embodiments, the method comprises determining that a first allele detected at the first informative locus and a second allele detected at the second informative locus are in phase. In some embodiments, the second sequencing primer is designed to hybridize to a location that is within x nt upstream of the informative locus, wherein x is a number that is less than an average sequencing read length produced by the first or second round of sequencing. In some embodiments, the removing comprises a denaturing step. In some embodiments, the removing further comprises a washing step. In some embodiments, the method further comprises subjecting the at least one nucleic acid template to one or more additional rounds of sequencing, wherein the one or more additional rounds of sequencing comprise the steps of : (i) removing a previously annealed sequencing primer and previously generated sequencing read from the at least one nucleic acid template; (ii) annealing an additional

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sequencing primer to the at least one nucleic acid template; and (iii) subjecting the at least one nucleic acid template to an additional round of sequencing using the additional sequencing primer, thereby producing an additional sequencing read of the at least one nucleic acid template. In some embodiments, the additional sequencing primer comprises a 3' terminal nucleotide which hybridizes to a binding site that is more than 5 nt away from a binding site of the 3' terminal nucleotide of the previously annealed sequencing primer. In some embodiments, a first allele detected by the first sequencing read, a second allele detected by the second sequencing read, and an additional allele detected by the additional sequencing read are determined to be in phase. In some embodiments, the additional sequencing primer and the previously annealed sequencing primer are designed to hybridize to genomic locations that map to a single chromosome. In some embodiments, the genomic locations that map to a single chromosome span a distance that is less than a length of the at least one nucleic acid template. In some embodiments, the genomic locations that map to a single chromosome span a distance that is less than three-quarters of the length of the at least one nucleic acid template. In some embodiments, the genomic locations that map to a single chromosome span a distance that is less than two-thirds of the length of the at least one nucleic acid template. In some embodiments, the genomic locations that map to a single chromosome span a distance that is less than one half of the length of the at least one nucleic acid template. In some embodiments, the genomic locations that map to a single chromosome span a distance that is about a sequencing read length of the sequencing platform. In some embodiments, the at least one nucleic acid template is a member of a sequencing library. In some embodiments, the sequencing library is a whole-genome sequencing library. In some embodiments, the sequencing library is a target-enriched sequencing library. In some embodiments, the sequencing library is a directional sequencing library. In some embodiments, the sequencing library is not a directional sequencing library. In some embodiments, the sequencing library comprises nucleic acid fragments of an average length. In some embodiments, the average length is about 50 bases to about 10 kb. In some embodiments, the first, second, and optionally additional sequencing reads comprise sequence information from a plurality of loci. In some embodiments, phasing of alleles detected at the plurality of loci is determined based upon the first, second, and optionally additional sequencing reads. In some embodiments, the at least one nucleic acid template comprises a plurality of nucleic acid templates. In some embodiments, the at least one nucleic acid template comprises at least 10,000 nucleic acid templates. In some embodiments, the at least one nucleic acid template comprises 3 billion nucleic acid

templates. In some embodiments, the at least one nucleic acid template is coupled to a unique addressable location of the sequencing platform. In some embodiments, the method comprises compiling sequencing reads from the unique addressable location into one data file. In some embodiments, the method comprises determining phasing of a plurality of alleles detected across two or more unique addressable locations. In some embodiments, the two or more unique addressable locations each comprise two or more detected alleles, wherein the two or more detected alleles of each of the two or more unique addressable locations are determined to be in phase if at least one common detected allele is shared by the two or more unique addressable locations. In some embodiments, step (d) of a method disclosed herein comprises subjecting the at least one nucleic acid template to a second round of sequencing using a mixture of second sequencing primers. In some embodiments, the mixture of second sequencing primers comprises a pair of primers, wherein each primer in the primer pair is designed to sequence a forward or reverse complement strand containing a locus. In some embodiments, primers within the mixture are designed and/or selected to have minimal potential to hybridize to the same nucleic acid template.

[0017] In some embodiments of any of the aforementioned methods, the template comprises DNA. In some embodiments, the template comprises RNA. In some embodiments, one or more methods disclosed herein is used to provide phasing information for two or more alleles separated by a distance of over 150 bases, over 300 bases, over 500 bases, over 600 bases, over 700 bases, over 800 bases, over 900 bases, over 1000 bases (1kb), over 5 kb, over 10 kb, over 50 kb, over 100 kb, over 500 kb, over 1000 kb (1 Mb), over 5 Mb, over 10 Mb, over 50 Mb, over 100 Mb, over 150 Mb, over 200 Mb, over 250 Mb, or over 300 Mb.

[0018] Also provided herein is a kit, comprising: (a) one or more oligonucleotide primers for performing a sequencing reaction; and (b) instructions for practicing a method of any of the preceding claims using the one or more oligonucleotide primers.

[0019] Also provided herein is a computer readable medium comprising computer executable code that, upon execution by a computer processor, implements a method comprising: (a) accessing a data file in computer memory that comprises a plurality of sequencing reads corresponding to a first addressable location of a sequencing platform, wherein the sequencing reads are generating by subjecting a first nucleic acid template coupled to the addressable location to a first and at least a second round of sequencing, wherein the first and at least second rounds of sequencing are performed using a first and second sequencing primer, respectively; (b) using the data file, detecting a first detected allele

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at a first locus of the nucleic acid template and detecting a second detected allele at a second locus of the nucleic acid template; and (c) determining the first and second detected allele to be in phase based upon said detecting. In some embodiments, the method further comprises: (d) accessing a second data file in computer memory that comprises a plurality of sequencing reads corresponding to a second addressable location of a sequencing platform, wherein the sequencing reads are generated by subjecting a second nucleic acid template coupled to the addressable location to the first and the at least second round of sequencing; (e) using the second data file, detecting a first detected allele at a first locus of the second nucleic acid template and detecting a second detected allele at a second locus of the second nucleic acid template; and (f) determining the first and second detected alleles of the second nucleic acid template to be in phase with the first and second detected alleles of the first nucleic acid template if the first or second detected allele of the second nucleic acid template is shared in common with the first nucleic acid template.

INCORPORATION BY REFERENCE

[0020] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0022] **FIGS. 1A** and **1B** illustrate the current limitations of NGS sequencing in obtaining phasing information for a plurality of alleles.

[0023] **FIG. 2** depicts an exemplary workflow of a method described herein.

[0024] **FIG. 3** depicts an exemplary embodiment of a method described herein.

[0025] **FIG. 4** depicts another exemplary embodiment of a method described herein.

[0026] **FIG. 5** depicts an exemplary computer system disclosed herein.

[0027] **FIG. 6** depicts sequential sequencing method as applied to 16S microbial rRNA characterization, as described in Example 1.

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[0028] **FIG. 7** depicts the use of specific oligonucleotide pools and the generation of mated pairs of sequencing reads to differentiate between two closely related nucleotide sequences, such as a gene/pseudogene pair.

DETAILED DESCRIPTION OF THE INVENTION

[0029] Throughout this application, various embodiments are presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0030] Unless otherwise specified, terms and symbols of genetics, molecular biology, biochemistry and nucleic acid used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, *DNA Replication,* Second Edition (W.H. Freeman, New York, 1992); Lehninger, *Biochemistry,* Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human *Molecular Genetics,* Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, *Oligonucleotides and Analogs: A Practical Approach* (Oxford University Press, New York, 1991); Gait, editor, *Oligonucleotide Synthesis: A Practical Approach* (IRL Press, Oxford, 1984); Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Fourth Edition (2012); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins, eds., 1984); *A Practical Guide to Molecular Cloning* (B. Perbal, 1984); and a series, *Methods in Enzymology* (Academic Press, Inc.) and the like.

Definitions

[0031] As used in the specification and claims, the singular forms "a", "an" and "the" can include plural references unless the context clearly dictates otherwise. For example, the term "a primer" can include a plurality of primers, including mixtures thereof.

[0032] The terms "determining", "measuring", "evaluating", "assessing," "assaying," and "analyzing" can be used interchangeably herein to refer to any form of measurement, and include determining if an element is present or not. These terms can include both quantitative and/or qualitative determinations. Assessing may be relative or absolute. "Assessing the

presence of" can include determining the amount of something present, as well as determining whether it is present or absent.

[0033] As used herein, the term "phasing" can refer to the process of determining whether two or more nucleic acid sequences are located on the same nucleic acid template, the same fragment of a chromosome, or the same chromosome.

[0034] As used herein, the term "haplotype" can refer to a haploid genotype, e.g., a combination or set of nucleic acid sequences detected at a plurality of loci which are typically inherited as a unit and are linked, for example during a translocation event. A haplotype can provide a distinctive genetic pattern of an individual. A haplotype can be determined for one locus, several loci, or an entire chromosome depending on the number of recombination events that occur between a given set of loci. The nucleic acid sequences are not limited to any specific type and can include, by way of example only, conserved genetic sequences (e.g., non-variant) or variant genetic sequences (e.g., polymorphisms). For example, single nucleotide polymorphisms (SNPs), short tandem repeats (STRs), including , for example, di-, tri-, and tetra-nucleotide repeats, copy number variations (CNVs), insertions, deletions, inversions, methylations, etc. can be considered variant genetic sequences. Such markers can be found in various publications and databases including, for example, the ATCC short tandem repeat (STR) database, the Human SNP Database, the NCBI dbSNP database, Celera Human SNP database, SNP Database of the Genome Analysis Group, and the like.

[0035] The term "phased alleles" can refer to the distribution of the particular alleles on a nucleic acid template, a single chromosomal region, and/or a single chromosome. Accordingly, the "phase" of two alleles can refer to a characterization or determination of whether the alleles are located on a single chromosome or two separate chromosomes (e.g., a maternally or paternally inherited chromosomes). Unless otherwise stated, "haplotype" and "phased alleles" can be considered synonymous.

[0036] "Nucleotides" and "nt" can be used interchangeably herein to generally refer to biological molecules that can form nucleic acids. Nucleotides can have moieties that contain not only the known purine and pyrimidine bases, but also other bases that have been modified. Such modifications include acylated purines or pyrimidines, methylated purines or pyrimidines, alkylated riboses, or other heterocycles.

[0037] As used herein, the term "oligonucleotide" can refer to a polynucleotide chain, typically less than 200 residues long, most typically between 15 and 100 nucleotides long, but also intended to encompass longer polynucleotide chains. Oligonucleotides can be single-or double-stranded. The terms "oligonucleotide probe" or "probe", as used herein, can refer to

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an oligonucleotide capable of hybridizing to a complementary nucleotide sequence. As used herein, the term "oligonucleotide" can be used interchangeably with the terms "primer", "adaptor" and "probe".

[0038] The term "polynucleotide", "nucleic acid", or grammatical equivalents, can refer to two or more nucleotides covalently linked together. A nucleic acid described herein can contain phosphodiester bonds, although in some cases, as outlined below (for example in the construction of primers and probes such as label probes), nucleic acid analogs are included that can have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Pat. No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid (also referred to herein as "PNA") backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids (also referred to herein as "LNA"), Koshkin et al., J. Am. Chem. Soc. 120.13252 3 (1998); positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Pat. Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y. S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Pat. Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y. S. Sanghui and P. Dan Cook, all of which are incorporated by reference. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp 169-176, hereby incorporated by reference). Several nucleic acid analogs are described in Rawls, C & E News

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Jun. 2, 1997 page 35, hereby incorporated by reference. "Locked nucleic acids" are also included within the definition of nucleic acid analogs. LNAs can be a class of nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2'-O atom with the 4'-C atom. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone can be done to increase the stability and half-life of such molecules in physiological environments. For example, PNA:DNA and LNA-DNA hybrids can exhibit higher stability and thus can be used in some embodiments. The target nucleic acids can be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. Depending on the application, the nucleic acids can be DNA (including, e.g., genomic DNA, mitochondrial DNA, and cDNA), RNA (including, e.g., mRNA and rRNA) or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc.

[0039] As used herein, the terms “hybridization”, “hybridizing” and “annealing” can be used interchangeably and can refer to the pairing of complementary nucleic acids.

[0040] The term “primer”, as used herein, can refer to an oligonucleotide, generally with a free 3’ hydroxyl group, that is capable of hybridizing with a template (such as a target polynucleotide, target DNA, target RNA or a primer extension product) and is also capable of promoting polymerization of a polynucleotide complementary to the template. A primer can contain a non-hybridizing sequence that constitutes a tail of the primer. A primer can still hybridize to a target even though its sequences are not fully complementary to the target.

[0041] “Complementary”, as used herein, can refer to complementarity to all or only to a portion of a sequence. The number of nucleotides in the hybridizable sequence of a specific oligonucleotide primer can be such that stringency conditions used to hybridize the oligonucleotide primer can prevent excessive random non-specific hybridization. The number of nucleotides in the hybridizing portion of the oligonucleotide primer can be at least as great as the defined sequence on the target polynucleotide that the oligonucleotide primer hybridizes to, namely, at least 5, 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 about 20, or from about 6 to about 10 or 6 to about 12, or 12 to about 200 nucleotides, or about 10 to about 50 nucleotides. In general, the target polynucleotide can be larger than the oligonucleotide primer or primers as described previously.

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[0042] The term "genomic sequence", as used herein, can refer to a sequence that occurs in a genome. RNAs can be transcribed from a genome; this term can encompass sequence that exist in the nuclear genome of an organism, as well as sequences that are present in a cDNA copy of an RNA (e.g., an mRNA) transcribed from such a genome.

[0043] The term "target polynucleotide,", "target region", or "target", as used herein, can refer to a polynucleotide of interest under study. A target polynucleotide can contain one or more sequences or loci that are of interest and under study. A target polynucleotide can comprise, for example, a genomic region. The genomic region can comprise all or a portion of a chromosome. The genomic region can comprise a plurality of loci. The genomic region can comprise a plurality of loci for which phasing and/or haplotype information is desired.

[0044] The terms "template", "template strand", "template DNA" and "template nucleic acid" can be used interchangeably herein to refer to a strand of nucleic acid (e.g., DNA) that is copied by an amplification cycle.

[0045] The term "mutation", as used herein, can refer to a change of the nucleotide sequence of a genome. Mutations can involve large regions of a genome (e.g., copy number variation). Mutations can involve small regions of a genome. Examples of mutations involving small sections of a genome include, e.g., point mutations or single nucleotide polymorphisms, multiple nucleotide polymorphisms, insertions (e.g., insertion of one or more nucleotides at a locus), multiple nucleotide changes, deletions (e.g., deletion of one or more nucleotides at a locus), and inversions (e.g., reversal of a sequence of one or more nucleotides).

[0046] The term "locus", as used herein, can refer to a location of a gene, a nucleotide, or a sequence on a chromosome and/or in a genome. A locus can be considered "polymorphic" or to have a "polymorphism" if there exists in a population two or more forms of a nucleotide or sequence at the locus (e.g., if the locus comprises variant genetic sequences). An "allele" of a locus, as used herein, can refer to an alternative form of a nucleotide or sequence at the locus. A "wild-type allele" can refer to an allele that has the highest frequency in a population of subjects. A "mutant allele" can refer to an allele that has a lower frequency that a "wild-type allele". An "informative" locus, as used herein, can refer to a locus which can be used to determine phasing information of two or more alleles. The informative locus can be a polymorphic locus.

[0047] The term "single nucleotide polymorphism", or "SNP", as used herein, can refer to a type of genomic sequence variation resulting from a single nucleotide substitution within a

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sequence. "SNP alleles" or "alleles of a SNP" can refer to alternative forms of the SNP at a given locus.

[0048] The term "genotyping", as used herein, can refer to a process of determining differences in the genetic make-up (genotype) of an individual, e.g., by examining the individual's DNA sequence using a biological assay. The biological assay can comprise a sequencing reaction.

[0049] As used herein, a "sample" or "nucleic acid sample" can refer to any substance containing or presumed to contain nucleic acid. The sample can be a biological sample obtained from a subject.

[0050] An "addressable location", as used herein, can refes to a location that can be recorded and/or tracked throughout any of the procedures carried out during one or more methods described herein.

OVERVIEW

[0051] Provided herein are methods, compositions, and kits useful for sequence analysis of nucleic acids. The subject methods, compositions, and kits can be useful for obtaining accurate phasing and/or haplotyping information using NGS technologies. For example, the methods, compositions, and kits provided herein can be used to determine phasing of a plurality of alleles that are separated by a distance that is greater than a distance of an NGS sequence read or paired read. The methods and kits provided herein can be used to obtain phasing information for detected alleles that map to two or more loci. For example, the methods and kits provided herein can be used for determining whether detected alleles that map to two or more loci are located on the same nucleic acid template, on the same chromosome or chromosome region, or on different chromosomes or chromosomal regions in a chromosome pair. The methods and kits can be used to determine a plurality of polymorphic markers at a plurality of loci within a nucleic acid template. The methods and kits described herein can also be used to obtain phasing information for a plurality of alleles that are detected on different nucleic acid templates. One or more phasing methods disclosed herein can be used to provide phasing information for two or more alleles. The two or more alleles may be separated by a distance of over 150 bases, over 300 bases, over 500 bases, over 600 bases, over 700 bases, over 800 bases, over 900 bases, over 1000 bases (1kb), over 5 kb, over 10 kb, over 50 kb, over 100 kb, over 500 kb, over 1000 kb (1 Mb), over 5 Mb, over 10 Mb, over 50 Mb, over 100 Mb, over 150 Mb, over 200 Mb, over 250 Mb, or over 300 Mb.

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[0052] The methods and kits described herein can also be used to differentiate between closely related nucleic acid sequences, e.g., to distinguish a gene from a closely related pseudogene. Such methods can be useful, for example, for haplotyping, SNP phasing, determining downstream exons in RNA-seq, and in genetic diagnostics applications. The methods, kits and compositions described herein can utilize sequential sequencing of a nucleic acid template. Altogether, the phasing methods disclosed herein provide an improvement over the existing methods by offering a highly parallel, efficient method for obtaining phasing information.

[0053] **FIGS. 1A** and **1B** illustrate current limitations of NGS sequencing in obtaining phasing information for a plurality of alleles. **FIG. 1A** depicts a chromosome pair comprising a maternal and paternal chromosome obtained from genomic DNA from a subject. The chromosomes comprise a plurality of loci: locus X, locus Y, which is located, e.g., 600 bp downstream of locus X, and locus Z, which is located, e.g., 600 bp downstream of locus Y. The maternal chromosome comprises the following alleles: allele T at locus X (X_T, allele G at locus Y (Y_G), and allele T at locus Z (Z_T). By contrast, the paternal chromosome comprises the following alleles, allele A at locus X (X_A), allele T at locus Y (Y_T), and allele C at locus Z (Z_C). FIG. 1B depicts a current embodiment NGS sequencing method which is unable to determine phasing of alleles at loci X, Y, and Z. Genomic DNA from the subject is fragmented into ~1000 bp DNA fragments. The fragments are spatially separated and clonally amplified into clusters. By way of example only, clusters 1-3 tile overlapping regions which span loci X, Y, and Z. The clusters then undergo next generation sequencing, e.g., on an Illumina platform. Next generation sequencing typically produces sequencing reads from one or both ends of the DNA fragments, due to the sequencing adaptors attached to one or both ends of the fragments, wherein the sequencing adaptors comprise sequencing primer binding sites (not shown in the figure). The sequencing reads can have read lengths of, e.g., 100 nt, so the next generation sequencing in this example does not provide sequencing information across the entire template molecule. In this particular example, paired end sequencing is employed, producing sequencing reads from both ends of the template DNA fragments, each read having a read length of 100 nt (as depicted by thick gray arrows in **FIG. 1B**). Accordingly, the DNA template fragment of cluster 1 comprises X_A and Y_T, however, only X_A is detected by the sequencing reads. The DNA template fragment of cluster 2 comprises Y_T and Z_C, however, only Z_C is detected by the sequencing reads. The DNA template fragment of cluster 3 comprises Y_T and Z_C, however, only Y_T is detected by the sequencing reads. Because sequencing reads from clusters 1-3 only detect a

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single allele at a single locus for each cluster, phasing information is not generated for the detected alleles X_A, Y_T, and Z_C, e.g., whether they are located on the same template molecule, the same chromosomal region, or the same chromosome.

[0054] **FIG. 2** depicts an exemplary workflow of a phasing method described herein. The method can comprise a first step 210 of coupling a nucleic acid template to a sequencing platform. The coupling can comprise attaching the nucleic acid template to a solid support of the sequencing platform. Coupling can in some cases include formation of a covalent bond (e.g., formed by ligation), or a noncovalent bond (e.g., formed by hybridization) between the template and the solid support of the sequencing platform. In some cases, coupling does not involve formation of a covalent bond or noncovalent bond between the template and the solid support of the sequencing platform. In some cases, coupling can include associating two entities, e.g., placing an entity, such as a nucleic acid template, in a well. In some cases, the nucleic acid template is coupled to a unique addressable location of the sequencing platform. For example, a plurality of nucleic acid templates can be coupled to a sequencing platform, wherein each of the plurality of templates is coupled to a unique addressable location of the sequencing platform. Coupling of nucleic acid templates to unique addressable locations of a sequencing platform can comprise attaching the templates to the unique addressable locations and/or can comprise confining the templates within the unique addressable locations (e.g., confining to a well). For example, a template can be attached to a solid support (e.g., a bead) and the solid support confined within the unique addressable location, such as, e.g., depositing the bead in a well. The method can further comprise a second step 220 of subjecting the nucleic acid template to a first sequencing reaction using a first sequencing primer. The first sequencing primer can initiate sequencing at a first location on the nucleic acid template. The first sequencing reaction can generate a first sequencing read. The first sequencing read can provide the sequence of a first region of the nucleic acid template. The method can further comprise a second step 230 of subjecting the nucleic acid template to a second sequencing reaction using a second sequencing primer. The second sequencing primer can initiate sequencing at a second location on the nucleic acid template. The second location can be distinct from the first location. In some cases, a 3' terminal nucleotide of the second primer can hybridize to a location that is more than 5 nt away from a binding site of a 3' terminal nucleotide of the first primer. The second sequencing reaction can generate a second sequencing read. The second sequencing read can provide the sequence of a second region of the nucleic acid template which is distinct from the first region of the nucleic acid template. In some embodiments, the nucleic acid template is optionally subjected to one or

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more additional rounds of sequencing (depicted as step 240) using additional sequencing primers.

[0055] Nucleic acid templates can be subjected to any number of rounds of sequencing as described herein. For example, nucleic acid templates can be subjected to 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 rounds of sequencing according to one or more phasing methods described herein. Nucleic acid templates can be subjected to about 2 to about 8 rounds of sequencing, about 6 to about 20 rounds of sequencing, about 10 to about 100 rounds of sequencing, about 50 to about 500 rounds of sequencing, about 100 to about 1000 rounds of sequencing, about 500 to about 2000 rounds of sequencing, about 1000 to about 10000 rounds of sequencing, or more than about 10000 rounds of sequencing according to one or more phasing methods described herein.

[0056] The rounds of sequencing can produce any number of sequencing reads for the nucleic acid template. For example, about 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 sequencing reads can be generated for any given nucleic acid template. About 1 to about 8 sequencing reads, about 6 to about 20 sequencing reads, about 10 to about 100 sequencing reads, about 50 to about 500 sequencing reads, about 100 to about 1000 sequencing reads, about 500 to about 2000 sequencing reads, about 1000 to about 10000 sequencing reads, or more than about 10000 sequencing reads can be generated for any given nucleic acid template,

[0057] In some embodiments, the first, second, and/or any additional rounds of sequencing are conducted using a plurality of primers. For example, any round of sequencing designed to sequence a particular locus can comprise use of a pair of primers, wherein each primer in the primer pair is designed to sequence a forward or reverse complement strand containing

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the locus. For example, any round of sequencing can comprise sequencing using a mixture of primers. Primers within a mixture can be designed and/or selected to have minimal potential to hybridize to the same nucleic acid template. For example, primers within a mixture can be designed to hybridize to locations that are at least 1 kb, 2 kb, 3kb, 4 kb, 5 kb, 10 kb, 20 kb, 30 kb, 40 kb, 50 kb, 60 kb, 70 kb, 80 kb, 90 kb, 100 kb, 200 kb, 300 kb, 400 kb, 500 kb, 600 kb, 700 kb, 800 kb, 900 kb, 1000 kb (1Mb), 2 Mb, 3 Mb, 4 Mb, 5 Mb, 6 Mb, 7 Mb, 8 Mb, 9 Mb, 10 Mb, , 20 Mb, 30 Mb, 40 Mb, 50 Mb, 60 Mb, 70 Mb, 80 Mb, 90 Mb, 100 Mb, or 200 Mb apart. Primers within a mixture can be designed to hybridize to different chromosomes. A mixture of primers used for any round of sequencing can comprise any number of primers, for example, can comprise 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 primers. A mixture of priemrs used for any round of sequencing can comprise about 1 to about 8 primers, about 6 to about 20 primers, about 10 to about 100 primers, about 50 to about 500 primers, about 100 to about 1000 primers, about 500 to about 2000 primers, about 1000 to about 10000 primers, or more than about 10000 primers.

[0058] In some cases, the first, second, and optionally additional sequencing reads can be compiled to produce sequence information for the nucleic acid template. For example, first, second, and optionally additional sequencing reads from a unique addressable location can be compiled to produce sequence information for the nucleic acid template coupled to that unique addressable location.

[0059] **FIG. 3** depicts an exemplary embodiment of a phasing method described herein, and highlights advantages of the subject method for obtaining phasing information for a plurality of alleles. **FIG. 3** depicts the same chromosome pair comprising a maternal and paternal chromosome obtained from genomic DNA from a subject as depicted in **FIG. 1**. As in **FIG. 1**, the chromosomes comprise a plurality of loci: locus X, locus Y, which is located, e.g., 600 bp downstream of locus X, and locus Z, which is located, e.g., 600 bp downstream of locus Y. As in **FIG. 1**, the maternal chromosome comprises the following alleles: allele T at locus X ($X_{T)}$, allele G at locus Y (Y_G), and allele T at locus Z (Z_T). As in **FIG. 1**, the paternal chromosome comprises the following alleles, allele A at locus X (X_A), allele T at

locus Y (Y_T), and allele C at locus Z (Z_C). As in **FIG. 1**, genomic DNA from the subject is fragmented into ~1000 bp DNA fragments. As in **FIG. 1**, the fragments are spatially separated and clonally amplified into clusters. As in **FIG. 1**, clusters 1-3 tile overlapping regions which span loci X, Y, and Z. The clusters are then subjected to sequential rounds of sequencing according to a phasing method described herein. A first round of sequencing is performed using a first sequencing primer. The first sequencing primer can be designed to selectively hybridize to a sequencing primer binding site of a sequencing adaptor attached to a first end of the cluster templates. The first round of sequencing using the first sequencing primer provides a first set of sequencing reads (dark gray arrows) of a first end of the cluster templates. Because locus X happens to be located near the end of the first end of cluster 1, Allele A_A is detected by the first read of cluster 1. The extension strands resulting from extension of the first sequencing primer are then denatured and removed from the clusters. In a next step, a second round of sequencing is performed using a second sequencing primer. The second sequencing primer can be designed to hybridize to a genomic location upstream of locus Y and to sequence locus Y. Accordingly, the second round of sequencing produces a second set of sequencing reads (medium gray arrows). Because clusters 1, 2, and 3 comprise locus Y, the second set of sequencing reads comprise reads from clusters 1, 2, and 3. The second set of sequencing reads detect allele T at locus Y (Y_T) in clusters 1, 2, and 3. The extension strands resulting from extension of the second sequencing primer are then denatured and removed from the clusters. In a next step, a third round of sequencing is performed using a third sequencing primer. The third sequencing primer can be designed to hybridize to a genomic location upstream of locus Z and to sequence locus Z. Accordingly, the third round of sequencing produces a third set of sequencing reads (stippled medium gray arrows). Because clusters 2 and 3, but not cluster 1, comprise locus Z, the third set of sequencing reads comprise reads from clusters 2 and 3 but not from cluster 1. The third set of sequencing reads detect allele C at locus Z (Z_C) in clusters 2 and 3. Sequence data from the first, second, and third sets of sequencing reads is compiled and used to determine phasing of the detected alleles at loci X, Y, and Z. Because the first and second set of sequencing reads reveal X_A and Y_T in cluster 1, it can be determined that X_A and Y_T are in phase (e.g., on the same nucleic acid fragment). Because the second and third sets of sequencing reads reveal Y_T and Z_C in clusters 2 and 3, it can be determined that Y_T and Z_C are in phase (e.g., on the same nucleic acid fragment). Moreover, because the first, second, and third clusters share a common allele (Y_T), it can be determined that X_A, Y_T, and Z_C are all in

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phase. Because X_A, Y_T, and Z_C are all in phase, a haplotype of the subject can be determined to be X_A Y_T Z_C.

[0060] **FIG. 4** depicts another exemplary embodiment of a phasing method described herein. **FIG. 4** depicts the same chromosome pair comprising a maternal and paternal chromosome obtained from genomic DNA from a subject as depicted in **FIG. 1**. As in **FIG. 1**, the chromosomes comprise a plurality of loci: locus X, locus Y, which is located, e.g., 600 bp downstream of locus X, and locus Z, which is located, e.g., 600 bp downstream of locus Y. As in **FIG. 1**, the maternal chromosome comprises the following alleles: allele T at locus X (X_T), allele G at locus Y (Y_G), and allele T at locus Z (Z_T). As in **FIG. 1**, the paternal chromosome comprises the following alleles, allele A at locus X (X_A), allele T at locus Y (Y_T), and allele C at locus Z (Z_C). As in **FIG. 1**, genomic DNA from the subject is fragmented into ~1000 bp DNA fragments. As in **FIG. 1**, the fragments are spatially separated and clonally amplified into clusters. As in **FIG. 1**, clusters 1-3 tile overlapping regions which span loci X, Y, and Z. The clusters are then subjected to sequential rounds of sequencing according to a phasing method described herein. A first round of sequencing is performed using a first sequencing primer. The first sequencing primer can be designed to selectively hybridize to a genomic location upstream of locus X and to sequence locus X. The first round of sequencing produces a first set of sequencing reads. Because cluster 1, but not clusters 2 or 3, comprise locus X, the first set of sequencing reads comprises a read from cluster 1 but not from clusters 2 or 3. The first set of sequencing reads detects allele A at locus X(X_A). The extension strands resulting from extension of the first sequencing primer are then denatured and removed from the clusters. In a next step, a second round of sequencing is performed using a second sequencing primer. The second sequencing primer can be designed to hybridize to a genomic location upstream of locus Y and to sequence locus Y. Accordingly, the second round of sequencing produces a second set of sequencing reads (medium gray arrows). Because clusters 1, 2, and 3 all comprise locus Y, the second set of sequencing reads comprise reads from clusters 1, 2, and 3. The second set of sequencing reads detect allele T at locus Y ((Y_T) in clusters 1, 2, and 3. The extension strands resulting from extension of the second sequencing primer are then denatured and removed from the clusters. In a next step, a third round of sequencing is performed using a third sequencing primer. The third sequencing primer can be designed to hybridize to a genomic location upstream of locus Z and to sequence locus Z. Accordingly, the third round of sequencing produces a third set of sequencing reads (stippled medium gray arrows). Because clusters 2 and 3, but not cluster 1, comprise locus Z, the third set of sequencing reads

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comprise reads from clusters 2 and 3 but not from cluster 1. The third set of sequencing reads detect allele C at locus Z (Z_C) in clusters 2 and 3. Sequence data from the first, second, and third sets of sequencing reads is compiled and used to determine phasing of the detected alleles at loci X, Y, and Z. Because the first and second set of sequencing reads reveal X_A and Y_T in cluster 1, it can be determined that X_A and Y_T are in phase. Because the second and third sets of sequencing reads reveal Y_T and Z_C in clusters 2 and 3, it can be determined that Y_T and Z_C are in phase. Moreover, because the first, second, and third clusters share a common allele (Y_T), it can be determined that X_A, Y_T, and Z_C are all in phase. Because X_A, Y_T, and Z_C are all in phase, a haplotype of the subject can be determined to be X_A Y_T Z_C.

[0061] Reference will now be made in detail to exemplary embodiments. While the disclosed methods and compositions will be described in conjunction with the exemplary embodiments, it will be understood that these exemplary embodiments are not intended to limit the methods and compositions described herein, but can encompass alternatives, modifications and equivalents, which may be included in the spirit and scope described herein

Exemplary nucleic acid templates

[0062] Nucleic acid templates to be sequenced by a method provided herein can comprise DNA. The DNA can be complex DNA, for example genomic DNA. The DNA can be from a linear or circular genome. The DNA can be plasmid DNA, cosmid DNA, bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC). The DNA can be from more than one individual or organism. The DNA can be double stranded or single stranded. The input DNA can be part of chromatin. The DNA can be associated with histones. The DNA can comprise one or more chromosomes. For example, if the DNA is from a human, the DNA can comprise one or more of chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, or Y. The DNA can be mitochondrial DNA. The DNA can be cell-free DNA. The DNA can be cDNA. The cDNA can be generated from RNA, e.g., mRNA. Nucleic acid tempaltes to be sequenced by a method provided herein can comprise RNA.

[0063] Nucleic acid templates to be sequenced by a method provided herein can be obtained from and/or can be in a nucleic acid sample. The nucleic acid sample can be obtained from a subject. A subject can be any biological entity of interest from which phasing and/or haplotype information is desired. The subject can be an animal, plant, fungus, insect, bacteria, algae, virus, cell, tissue, and the like. The animal can be human, non-human primate, dog, cat, cow, pig, sheep, guinea pig, hamster, bird, frog, fish, rat, mouse, other

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rodent or other animal. A nucleic acid sample can also comprise a mixture of genomes of different species such as host-pathogen, bacterial populations, viral populations, and the like. For example, a DNA sample can comprise DNA (e.g., cDNA) made from a mixture of genomes of different species. Alternatively, the nucleic acid sample can be from a synthetic source.

[0064] The nucleic acid sample can be obtained from a biological sample from the subject. The biological sample can be, e.g., a liquid sample, a solid sample, or a combination thereof. Exemplary liquid samples include, but are not limited to ascites, buccal sample, cavity rinse, cerebrospinal fluid, whole blood, plasma, serum, sweat, tears, sputum, saliva, urine, or organ rinse. The sample can be a cell-free sample. Exemplary cell-free samples include, but are not limited to plasma, serum, plasma, urine, sweat, saliva, sputum, and tears. Exemplary solid biological samples include, e.g., tissue biopsy, feces, and the like. A biological sample can also comprise cultured cells or constituents of culture cells (including, e.g., conditioned medium resulting from the growth of cells in cell culture medium, recombinant cells and cell components). The sample can be a single cell. Nucleic acid samples can be obtained from biological samples by any means known to those of skill in the art. For example, nucleic acid can be extracted from a biological sample using liquid extraction (e.g, Trizol, DNAzol) techniques. Nucleic acid can also be extracted using commercially available kits. Nucleic acids can be concentrated by, e.g., centrifugation and/or precipitation. Nucleic acids can be purified by binding to a selective membrane and then by elution. In some cases, gentle handling steps may be taken to avoid shearing of chromosomal or intact DNA during the extraction process. Exemplary gentle handling strategies can include, e.g., pipetting with a wide-bore pipette tip and avoidance of vigorous pipetting and/or vortexing. In some cases, gentle handling steps are unnecessary for preparation of nucleic acid samples.

[0065] The nucleic acid sample can be fragmented by any means known in the art or otherwise described herein. The nucleic acids can be fragmented to result in a population of nucleic acid fragments of a desired length. The nucleic acid fragments can be double stranded or single stranded fragments. The length of the nucleic acid fragments can be any length. A nucleic acid fragment to be sequenced by a method described herein can have a length that is less than 300 Mb, less than 200 Mb, less than 100 Mb, less than 50 Mb, less than 40 Mb, less than 30 Mb, less than 20 Mb, less than 10 Mb, less than 5 Mb, less than 3 Mb, less than 2 Mb, less than 1 Mb (1000 kb), less than 900 kb, less than 800 kb, less than 700 kb, less than 600 kb, less than 500 kb, less than 400 kb, less than 300 kb, less than 200 kb, less than 100 kb (100,000 bases), less than 100,000 bases, less than 90,000 bases, less

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than 80,000 bases, less than 70,000 bases, less than 60,000 bases, less than 50,000 bases, less than 40000 bases, less than 30,000 bases, less than 20,000 bases, less than 10,000 bases, less than 9000 bases, less than 8000 bases, less than 7000 bases, less than 6000 bases, less than 5000 bases, less than 4000 bases, less than 3000 bases, less than 2000 bases, less than 1500 bases, less than 1400 bases, less than 1300 bases, less than 1200 bases, less than 1100 bases, less than 1000 bases, less than 900 bases, less than 800 bases, less than 700 bases, less than 600 bases, less than 500 bases, less than 400 bases, less than 300 bases, less than 250 bases, less than 200 bases, less than 150 bases, less than 100 bases, less than 50 bases, less than 40 bases, less than 30 bases, less than 20 bases, or less than 10 bases. In some cases, a nucleic acid fragment to be sequenced by a method described herein has a length that is about 10 bases, about 20 bases, about 30 bases, about 40 bases, about 50 bases, about 100 bases, about 150 bases, about 200 bases, about 250 bases, about 300 bases, about 400 bases, about 500 bases, about 600 bases, about 700 bases, about 800 bases, about 900 bases, about 1000 bases, about 1100 bases, about 1200 bases, about 1300 bases, about 1400 bases, about 1500 bases, about 1600 bases, about 1700 bases, about 1800 bases, about 1900 bases, about 2000 bases, about 3000 bases, about 4000 bases, about 5000 bases, about 6000 bases, about 7000 bases, about 8000 bases, about 9000 bases, about 10000 bases, about 20000 bases, about 30000 bases, about 40000 bases, about 50000 bases, about 60000 bases, about 70000 bases, about 80000 bases, about 90000 bases, about 100000 bases (100 kb), about 200 kb, about 300 kb, about 400 kb, about 500 kb, about 600 kb, about 700 kb, about 800 kb, about 900 kb, about 1000 kb (1Mb), about 2 Mb, about 3 Mb, about 5 Mb, about 10 Mb, about 10 Mb, about 30 Mb, about 40 Mb, about 50 Mb, about 100 Mb, about 200 Mb, or about 300 Mb. In some cases, a nucleic acid fragment to be sequenced by a method described herein has a length that is more than about 10 bases, more than about 20 bases, moer than about 30 bases, more than about 40 bases, more than about 50 bases, more than about 100 bases, more than about 150 bases, more than about 200 bases, more than about 250 bases, more than about 300 bases, more than about 400 bases, more than about 500 bases, more than about 600 bases, more than about 700 bases, more than about 800 bases, more than about 900 bases, more than about 1000 bases, more than about 1100 bases, more than about 1200 bases, more than about 1300 bases, more than about 1400 bases, more than about 1500 bases, more than about 1600 bases, more than about 1700 bases, more than about 1800 bases, more than about 1900 bases, more than about 2000 bases, more than about 3000 bases, more than about 4000 bases, more than about 5000 bases, more than about 6000 bases, more than about 7000 bases, more than about 8000 bases, more than about 9000 bases, more than about 10000 bases, more than about

20000 bases, more than about 30000 bases, more than about 40000 bases, more than about 50000 bases, more than about 60000 bases, more than about 70000 bases, more than about 80000 bases, more than about 90000 bases, more than about 100000 bases (100 kb), more than about 200 kb, more than about 300 kb, more than about 400 kb, more than about 500 kb, more than about 600 kb, more than about 700 kb, more than about 800 kb, more than about 900 kb, more than about 1000 kb (1Mb), more than about 2 Mb, more than about 3 Mb, more than about 5 Mb, more than about 10 Mb, more than about 10 Mb, more than about 30 Mb, more than about 40 Mb, more than about 50 Mb, more than about 100 Mb, more than about 200 Mb, or more than about 300 Mb. A nucleic acid fragment to be sequenced by a method described herein can have a length that is between about 10 to about 100 bases, about 50 to about 200 bases, about 100 to about 250 bases, about 150 to about 500 bases, about 300 to about 700 bases, about 400 to about 800 bases, about 600 to about 900 bases, about 800 to about 1000 bases, about 200 to about 1000 bases, about 500 bases to about 10000 bases, or about 2000 bases to about 100000 bases. In some embodiments, the nucleic acid fragment has a length that is over 9000 bases. For example, the nucleic acid fragments can be about 0.9 to about 2 kb, about 1.5 to about 4 kb, about 2 to about 6 kb, about 5 to about 8 kb, or about 7 to about 10 kb, about 10 to about 100 kb, about 50 kb to about 1 Mb, about 1 Mb to about 50 Mb, about 10 Mb to about 300 Mb. One of skill in the art will readily understand that the length of the nucleic acid templates to be sequenced by a method disclosed herein can be any length. Length of nucleic acid templates to be sequenced by a method disclosed herein can vary depending on the sequencing platform used in practicing a method disclosed herein. In some embodiments, the nucleic acid sample is not fragmented.

[0066] The nucleic acid sample can be enriched for target polynucleotides of interest. Target polynucleotides can comprise polynucleotides that map to genomic regions of interest. A genomic region of interest can be any genomic region from which phasing and/or haplotype information is desired. The genomic region of interest can comprise a region of a chromosome. The genomic region of interest can comprise a whole chromosome. The chromosome can be a diploid chromosome. In a human genome, for example, the diploid chromosome can be any of chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23. In some cases, the chromosome can be an X or Y chromosome. In some cases, the genomic region of interest comprises a portion of a chromosome. A genomic region of interest can be of any length. The genomic region of interest can have a length that is between, e.g., about 1 to about 10 bases, about 5 to about 50 bases, about 10 to about 100 bases, about 70 to about 300 bases, about 200 bases to about 1000 bases (1 kb), about 700

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bases to about 2000 bases, about 1 kb to about 10 kb, about 5 kb to about 50 kb, about 20 kb to about 100 kb, about 50 kb to about 500 kb, about 100 kb to about 2000 kb (2 Mb), about 1 Mb to about 50 Mb, about 10 Mb to about 100 Mb, about 50 Mb to about 300 Mb. For example, a genomic region of interest can be over 1 base, over 10 bases, over 20 bases, over 50 bases, over 100 bases, over 200 bases, over 400 bases, over 600 bases, over 800 bases, over 1000 bases (1kb), over 1.5 kb, over 2 kb, over 3 kb, over 4 kb, over 5 kb, over 10 kb, over 20 kb, over 30 kb, over 40 kb, over 50 kb, over 60 kb, over 70 kb, over 80 kb, over 90 kb, over 100 kb, over 200 kb, over 300 kb, over 400 kb, over 500 kb, over 600 kb, over 700 kb, over 800 kb, over 900 kb, over 1000 kb (1Mb), over 2 Mb, over 3 Mb, over 4 Mb, over 5 Mb, over 6 Mb, over 7 Mb, over 8 Mb, over 9 Mb, over 10 Mb, , over 20 Mb, over 30 Mb, over 40 Mb, over 50 Mb, over 60 Mb, over 70 Mb, over 80 Mb, over 90 Mb, over 100 Mb, or over 200 Mb.

[0067] A genomic region of interest can comprise one or more informative loci. An informative locus can be a polymorphic locus, e.g., comprising two or more alleles. In some cases, the two or more alleles comprise a minor allele. In particular embodiments, genomic regions of interest comprise one or more HLA loci.

[0068] Enrichment of target polynucleotides can be by any means known in the art or otherwise described herein. For example, nucleic acids can be enriched using target-specific primers, or by hybridization to target-selective probes. Target-selective probes can comprise a nucleic acid sequence that is selectively hybridizable to a target polynucleotide of interest. The target-selective probes can comprise a capture moiety which enables capture by a capture reagent. Exemplary capture moiety/reagents include, e.g., biotin/avidin, streptavidin, neutravidin, digoxigenin/wheat germ agglutinin, histidine/cobalt, iron. Nucleic acids can be enriched by a target-selective sequencing library preparation method. Target-selective sequencing library preparation kits and technology are commercially available from, e.g., NuGEN, Agilent, Illumina, or Nimblegen. Target-selective sequencing library preparation methods from Agilient are described in, e.g., US Patent No. 7,867,703 and US Patent Application Publication No. 20120289426, which are hereby incorporated by reference. Target-selective sequencing library preparation methods from Illumina are described in, e.g., US Patent Application Pub No. 20070141604, hereby incorporated by reference. Target-selective sequencing library preparation methods from Nimblegen are described in, e.g., US Patent Application Pub. No. 20090105081, hereby incorporated by reference. Target-selective sequencing library preparation methods from NuGEN are described in, e.g., US Patent Application Pub. No. 20130231253, hereby incorporated by reference. Other

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exemplary target-selective sequencing library preparation methods are described in US Patent Application Publication No. 20120003657, hereby incorporated by reference. Enrichment of target genomic regions can, in some cases, comprise isolation of intact chromosomes or chromosomal regions from a biological sample. In some embodiments, the nucleic acid sample is not enriched for target polynucleotides, e.g., represents a whole genome.

[0069] The nucleic acid template to be sequenced can be a member of a sequencing library. Sequencing libraries can comprise any number of library members. Sequencing libraries can comprise 2, 10, 50, 100, 500, 1000, 5000, 10000, 50000, 100000, 500000, 1000000, 5000000, 10000000, 50000000, 100000000, 500000000, 1000000000, 2000000000, 3000000000, 4000000000, 5000000000, 6000000000, 7000000000, 8000000000, 9000000000, 10000000000, or more than 10000000000 members. In some embodiments, nucleic acid libraries are sequenced according to one or more methods described herein. The sequencing library can be prepared from a nucleic acid sample by any means known to those of skill in the art or otherwise described herein. In some embodiments, library preparation comprises attachment of one or more adaptor oligonucleotides to one or more ends of nucleic acid fragments. In some cases, adaptor oligonucleotides are attached to both ends of nucleic acid fragments. The term "adaptor", as used herein, can refer to an oligonucleotide. In some cases, the attachment of an adaptor to a nucleic acid template can enable the generation of amplification-ready products of the nucleic acid template. An adaptor can have various designs. In some embodiments, an adaptor comprises a primer binding site for a platform-specific sequencing primer. In some embodiments, an adaptor comprises a barcode sequence. The barcode sequence can be a unique sequence of nucleotides that can encode information about an assay. A barcode sequence can, for example, encode information relating to the identity of an interrogated allele, identity of a target region of interest, identity of a sample, identity of a subject, or any combination thereof.

[0070] Attachment of adaptor oligonucleotides to nucleic acid fragments can be by any means known in the art, including, e.g., hybridization, amplification, ligation, and the like. Various ligation processes and reagents are known in the art and can be useful for attachment of adaptor oligonucleotides to nucleic acid fragments. For example, blunt ligation can be employed. Similarly, a single dA nucleotide can be added to the 3'-end of the double-stranded DNA product, by a polymerase lacking 3'-exonuclease activity and can anneal to an adaptor comprising a dT overhang (or the reverse). This design allows the hybridized components to be subsequently ligated (e.g., by T4 DNA ligase). Other ligation strategies and the corresponding reagents and known in the art and kits and reagents for carrying out

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efficient ligation reactions are commercially available (e.g, from New England Biolabs, Roche).

[0071] In some cases, it is useful to preserve information about the direction of single-stranded nucleic acid molecules while generating double-stranded polynucleotides. This may be accomplished by creation of a strand specific, e.g., directional sequencing library. The term "strand specific" or "directional", as used herein, can refer to the ability to differentiate in a double-stranded polynucleotide between the original template strand and the strand that is complementary to the original template strand. In some cases, one of the strands of the double-stranded polynucleotide is synthesized so that it has at least one modified nucleotide incorporated into it along the entire length of the strand. In some embodiments, the incorporation of the modified nucleotide marks the strand for degradation or removal. In some embodiments, the methods provided herein contemplate construction of directional nucleic acid libraries as described in pending US Application, Serial No. 13/643,056, titled COMPOSITIONS AND METHODS FOR DIRECTIONAL NUCLEIC ACID AMPLIFICATION AND SEQUENCING, Serial No. 13/643,056, hereby incorporated by reference in its entirety. In some cases, information about the direction of single-stranded nucleic acid molecules is not preserved while generating double-stranded polynucleotides. In some cases, the sequencing library is not a directional sequencing library.

[0072] Nucleic acid library members can optionally be amplified prior to sequencing. Methods of amplification are well known in the art. In some embodiments, the amplification is exponential, e.g. in the enzymatic amplification of specific double stranded sequences of DNA by a polymerase chain reaction (PCR). In other embodiments the amplification method is linear. In other embodiments the amplification method is isothermal. In some embodiments, the amplification comprises digital PCR. The digital PCR can be droplet digital PCR. In some embodiments, the amplification comprises single primer isothermal amplification (SPIA). In some embodiments, the amplification comprises reverse transcription PCR. In some embodiments, the amplification comprises real-time PCR. In some embodiments, the amplification comprises quantitative PCR (Q-PCR).

[0073] Nucleic acid library members can be clonally amplified prior to sequencing. Clonal amplification can involve generation of identical copies of individual nucleic acid templates in a nucleic acid library. Nucleic acid templates can be clonally amplified by any means known to those of skill in the art or otherwise described herein. In some cases, clonal amplification can comprise spatial separating individual nucleic acid template molecules and performing amplification of the separated nucleic acid template molecules. In certain

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instances, clonal amplification can comprise emulsion PCR (454 Life Sciences, Life Technologies). Emulsion PCR can comprise attachment of an individual nucleic acid template to a bead or particle. Beads and/or particles can be isolated in a water-in-oil emulsion, wherein droplets of the water-in-oil emulsion comprise a bead or particle with a template molecule attached thereon, and reaction components for carrying out an amplification reaction. Amplification of templates within the emulsion can result in clonal amplification of the templates. Clonal amplification can comprise bridge amplification (Illumina, Inc.). Bridge amplification is described, e.g, in US Patent No. 7,985,565, which is hereby incorporated by reference. Clonal amplification can comprise template walking (Life Technologies), described in US Patent Application Publication No. 20120156728, hereby incorporated by reference.

Primers

[0074] Primers can be oligonucleotides that are employed in an extension reaction by a polymerase along a polynucleotide template, such as in PCR or cDNA synthesis, for example. An oligonucleotide primer can be a synthetic polynucleotide that is single stranded, containing a sequence at its 3'-end that is capable of hybridizing with a sequence of the target polynucleotide. In some cases, the 3' region of the primer that hybridizes with the target nucleic acid has at least 80%, 90%, 95%, or 100% complementarity to a sequence or primer binding site.

[0075] Primers can be sufficiently long to prime synthesis of an extension strand along a template. Primers used in one or more methods described herein can have a length. The length can be any length, e.g., about 5 to about 15 nt, about 10 to about 20 nt, about 15 to about 30 nt, about 20 to about 40 nt, about 30 to about 60 nt, about 50 to about 100 nt, or more than about 100 nt.

[0076] Primers used in one or more methods described herein can include primers designed to hybridize to a portion or all of a sequencing adaptor. In some cases, primers described herein are designed to hybridize to a primer-binding sequence of a sequencing adaptor. Exemplary adaptors are described herein. In some cases, a first sequencing primer to be used in a sequential sequencing method (e.g., primer or primers to be used in a first round of sequencing) is designed to hybridize to a portion or all of a sequencing adaptor.

[0077] Primers used in one or more methods can include primers designed to sequence one or more loci from which phasing information is desired. The one or more loci can comprise one or more informative loci. For example, in some cases, the first sequencing primer, second sequencing primer (e.g., primer or primers to be used in a first or second round of

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sequencing, respectively), or any subsequent sequencing primer used in a sequential sequencing method is designed to sequence an informative locus.

[0078] A primer designed to sequence an informative locus can be designed to hybridize to a genomic location that is a distance upstream of the informative locus to be sequenced. The distance can be of any length. A skilled artisan will readily appreciate that the distance can vary depending on the read length of the sequencing technology utilized in the sequential sequencing method. In some cases, the distance is shorter than a read length of the sequencing technology utilized in the sequential sequencing method. The primer designed to sequence an informative locus can be designed to hybridize to a genomic location that is about, e.g., 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 nt upstream of the informative locus. The primer designed to sequence an informative locus can be designed to hybridize to a genomic location that is about 1 to about 10, about 5 to about 50, about 20 to about 100, about 40 to about 200, about 80 to about 500, about 100 to about 1000, about 500 to about 10000, about 5000 to about 100000, or more than 100000 nt upstream of the informative locus.

[0079] In some cases, the primer designed to sequence an informative locus can be designed to hybridize to a conserved region of the genome. The conserved region can be a region that does not comprise a polymorphic locus, e.g., is substantially not polymorphic. In some embodiments, the conserved region exhibits a polymorphism (e.g., sequence variant) at a population frequency that is less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.9%, less than 0.8%, less than 0.7%, less than 0.6%, less that 0.5%, less than 0.4%, less than 0.3%, less than 0.2%, less than 0.1%, less than 0.05%, less than 0.01%, less than 0.05%, less than 0.001%, less than 0.005%, less than 0.0001%, %, less than 0.0005%, less than 0.00001%,%, less than 0.00005%, less than 0.000001%, less than 0.000005%, less than 0.0000001%, less than 0.0000005%, or less than 0.00000001%. In some cases, the primer designed to sequence an informative locus can be designed to hybridize to an unconserved region of the genome.

[0080] Primers used in a first, second, or additional round of sequencing can hybridize to distinct locations on the nucleic acid template. For example, a first primer used in a first

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round of sequencing may comprise a 3' terminal nucleotide that hybridizes to a first location of a nucleic acid template. A second primer used in a second round of sequencing may comprise a 3' terminal nucleotide that hybridizes to a second location that is more than 5 nt away from the first location of the nucleic acid template (e.g., hybridizes to a location that is at least 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 nt away from the first location of the nucleic acid template. In some cases, a third primer used in a third round of sequencing may comprise a 3' terminal nucleotide that hybridizes to a third location that is more than 5 nt away from the second location of the nucleic acid template (e.g., hybridizes to a nucleotide that is at least 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 nt away from the second location of the nucleic acid template. Any primer used in a subsequent round of sequencing can have a 3' terminal nt that hybridizes to a location of the nucleic acid template that is at least 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 nt away from a binding site of the 3' terminal nt of the previous sequencing primer used in the previous round of sequencing.

[0081] Primers used in a first, second, or additional round of sequencing can initiate sequencing at distinct locations on the nucleic acid template. For example, a first primer used in a first round of sequencing may initiate sequencing at a first location of a nucleic acid template. A second primer used in a second round of sequencing may initiate sequencing at a

second location that is more than 5 nt away from the first location of the nucleic acid template (e.g., initiates sequencing at a location that is at least 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 nt away from the first location of the nucleic acid template. In some cases, a third primer used in a third round of sequencing may initiate sequencing at a third location that is more than 5 nt away from the second location of the nucleic acid template (e.g., initiates sequencing at a location that is at least 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 nt away from the second location of the nucleic acid template. Any primer used in a subsequent round of sequencing can initiate sequencing at a location of the nucleic acid template that is more than 5 nt away from the location of initiated sequencing for the previous round of sequencing, e.g., at least 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, 51, 52, 53, 54, 55 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 100000, or more than 100000 nt away from the location of initiated sequencing for the previous round of sequencing.

[0082] In some embodiments, standard or universal sequencing primers are used. For example, primers designed to hybridize to a primer binding site of a sequencing adaptor can be used. A primer can be a polyA or polyT oligonucleotide. In some embodiments, sequence-specific primers that hybridize to a conserved region or conserved regions within the nucleic acid inserts in the sequencing library are used. In some embodiments, the sequence-specific primers are designed to hybridize to conserved regions adjacent to regions of variable sequence within the nucleic acid inserts, thereby enabling differentiating between

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closely related sequences. In some embodiments, a set of oligonucleotide primers that hybridize to sequences shared in closely related sequences, such as gene/pseudogene pairs, are used.

[0083] Primers can be prepared by any suitable method. Methods for preparing oligonucleotides of specific sequence are known in the art, and include, for example, cloning and restriction of appropriate sequences or direct chemical synthesis. Chemical synthesis methods may include, for example, the phosphodiester method disclosed by Brown et al., 1979, Methods in Enzymology 68:109, the phosphotriester method described by Narang et al., 1979, Methods in Enzymology 68:90, the diethylphosphoramidate method disclosed in Beaucage et al., 1981, Tetrahedron Letters 22:1859, and the solid support method disclosed in U.S. Pat. No. 4,458,066, all of which publications are hereby incorporated by reference. Primers and/or reporter probes can also be obtained from commercial sources such as Amersham Pharmacia Biotech, Operon Technologies, Sigma, IDT Technologies, and Life Technologies. Computer programs can also be used to design primers, including but not limited to Array Designer Software (Arrayit Inc.), NetPrimer, Oligonucleotide Probe Sequence Design Software for Genetic Analysis (Olympus Optical Co.), Primer3, and DNAsis from Hitachi Software Engineering.

Methods of Sequencing

[0084] The methods provided herein contemplate sequential sequencing of nucleic acid templates. Sequencing methods are also well known in the art. Sequencing can be performed by a sequencing platform. The sequencing platform can be a next-generation sequencing (NGS) platform. In some cases, the NGS platform can sequence clonally amplified DNA templates or single DNA molecules in a massively parallel fashion (e.g. as described in Volkerding et al. Clin Chem 55:641-658 [2009]; Metzker M Nature Rev 11:31-46 [2010]), hereby incorporated by reference. Sequential sequencing methods provided herein can also employ Sanger sequencing.

[0085] The next-generation sequencing platform can be a commercially available platform. Commercially available platforms useful for performing a method described herein include, e.g., platforms for sequencing-by-synthesis, ion semiconductor sequencing (ChemFET sequencing), reversible dye terminator sequencing, pyrosequencing, sequencing by ligation, single-molecule sequencing, and sequencing by hybridization.

[0086] Platforms for sequencing by synthesis are available from, e.g., 454 Life Sciences, Illumina, Helicos Biosciences, and Qiagen. Illumina platforms can include, e.g., Illumina's Solexa platform, Illumina's Genome Analyzer, Illumina's MiSeq, Illumina's HiSeq,

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Illumina's NextSeq, and are described in Gudmundsson et al (Nat. Genet. 2009 41:1122-6), Out et al (Hum. Mutat. 2009 30:1703-12) and Turner (Nat. Methods 2009 6:315-6), U.S. Patent Application Publication Nos. 20080160580 and 20080286795, U.S. Patent Nos. 6,306,597, 7,115,400, and 7,232,656, which are hereby incorporated by reference. 454 Life Science platforms include, e.g., the GS Flex and GS Junior, and are described in U.S. Patent No. 7,323,305, hereby incorporated by reference. Platforms for ion semiconductor sequencing include, e.g., the Ion Torrent Personal Genome Machine (PGM) and are described in U.S. Patent No. 7,948,015, hereby incorporated by reference. Platforms for pryosequencing include the GS Flex 454 system and are described in U.S. Patent Nos. 7,211,390; 7,244,559; 7,264,929, hereby incorporated by reference. Platforms for sequencing by ligation include, e.g., the SOLiD sequencing platform and are described in U.S. Patent No. 5,750,341, hereby incorporated by reference. Platforms for single-molecule sequencing include the SMRT system from Pacific Bioscience and the Helicos True Single Molecule Sequencing platform.

[0087] For example, a sequencing technique that can be used in one or more phasing methods described herein is the method commercialized by Illumina, as described, e.g., in U.S. Pat. Nos. 5,750,341; 6,306,597; and 5,969,119. Such method can employ cluster amplification of library members onto a flow cell and a sequencing-by-synthesis approach. In a round of sequencing, cluster-amplified library members can be subjected to repeated cycles of polymerase-directed single base extension. Single-base extension can involve incorporation of reversible-terminator dNTPs, each dNTP labeled with a different removable fluorophore. The reversible-terminator dNTPs can be 3' modified to prevent further extension by the polymerase. After incorporation of the dNTP, the incorporated nucleotide can be identified by fluorescence imaging. Following fluorescence imaging, the fluorophore can be removed and the 3' modification can be removed resulting in a 3' hydroxyl group, thereby allowing another cycle of single base extension.

[0088] In one embodiment of a phasing method employing Illumina sequencing methodology, a sequencing library is prepared from a nucleic acid sample obtained from a subject. In some cases, the sequencing library is a directional sequencing library. In some cases, the sequencing library is not a directional sequencing library. Single-stranded nucleic acids from the library can optionally be amplified, for example, by PCR, prior to coupling to the Illumina sequencing platform. To couple template nucleic acids to the Illumina platform, the nucleic acids can be denatured into single-stranded templates. The single-stranded templates can be randomly attached to the inside surface of flow-cell channels. In some

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embodiments the single-stranded templates are randomly attached to unique addressable locations of the flow-cell. Unlabeled nucleotides can be added to initiate solid-phase bridge amplification to produce dense clusters of clonally amplified templates. In some embodiments, each cluster is coupled to a unique addressable location of the flow-cell. To initiate a first round of sequencing, labeled reversible terminator dNTPs, a first sequencing primer, and DNA polymerase can be added to the Illumina flow cell. The clusters can be subjected to repeated cycles of polymerase-directed single base extension and label detection as described above, thereby producing a first sequencing read from one or more clusters. Upon completion of the first round of sequencing, the extended strands and sequencing primers can be denatured from the cluster templates and removed, e.g., by washing. To initiate a second round of sequencing, labeled reversible terminator dNTPS, a second sequencing primer, and DNA polymerase can be added to the Illumina flow cell. The clusters can then be subjected to repeated cycles of polymerase-directed single base extension and label detection as described above, thereby producing a second sequencing read from one or more clusters. Subsequent rounds of sequencing can optionally be performed by removing the extended strands and primers from the preceding round of sequencing, using subsequent primers that are designed to hybridize, for example, downstream or upstream of the preceding sequencing primer. Sequence reads for the first, second, and optionally additional rounds of sequencing can be compiled for each cluster. The compiling can be used to generate sequence information for the clonally amplified templates. Sequence information for the clonally amplified templates can be used to determine phasing of one or more alleles detected by the sequencing.

[0089] In some embodiments, one or more phasing methods described herein employ sequencing by ligation methods commercialized by Applied Biosystems (e.g., SOLiD sequencing). Library preparation for SOLiD sequencing can comprise ligation of adaptors attached to the 5' and 3' ends of the fragments to generate a fragment library. Alternatively, internal adaptors can be introduced by ligating adaptors to the 5' and 3' ends of the fragments, circularizing the fragments, digesting the circularized fragment to generate an internal adaptor, and attaching adaptors to the 5' and 3 ' ends of the resulting fragments to generate a mate-paired library. Next, nucleic acid templates can be clonally amplified on beads via PCR. Following PCR, the templates can be denatured. Beads can be enriched for beads with extended templates. The clonally amplified templates on the beads can be subjected to a 3' modification that permits bonding to a glass slide. The beads can be deposited on a glass slide for sequencing by ligation. In some embodiments, each bead is deposited onto a unique

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addressable location of the glass slide. A round of SOLiD sequencing can comprise the following steps: (1) hybridization of a first sequencing primer to the clonally amplified templates. The first sequencing primer can be designed to hybridize to a sequencing primer binding site on one or more adaptors attached to the template nucleic acids. (2) Following hybridization, a set of four fluorescently labeled di-base probes can compete for ligation to the sequencing primer. Specificity of the di-base probe can be achieved by interrogating every 1st and 2nd base in each ligation reaction. (3) Cycles of ligation, detection and cleavage can be performed with the number of cycles determining the eventual read length. (4) Following a series of ligation cycles, the extension product can be removed and the template can be reset with a primer complementary to the n-1 position for a second round of ligation cycles. For SOLiD sequencing, one round of sequencing can comprise five rounds of primer reset and ligation cycles as described above, wherein each subsequent primer hybridizes to an n-1 position of the adaptor sequencing.

[0090] In one embodiment of a phasing method described herein employing SOLiD sequencing methodology, a sequencing library is prepared from a nucleic acid sample obtained from a subject using a library preparation method for SOLiD sequencing as described herein. In some cases, the sequencing library is a directional sequencing library. In some cases, the sequencing library is not a directional sequencing library. The sequencing library members can be deposited onto unique addressable locations of the SOLiD platform as described herein. A first round of SOLiD sequencing can be performed as described herein. Following the first round of sequencing, the extension product can be removed and a second set of sequencing primers can be used for a second round of SOLiD sequencing. The second round of SOLiD sequencing can comprise multiple cycles of primer hybridization and ligation as described above. The second set of sequencing primers can comprise a primer "primer A" that is designed to hybridize to a genomic location residing 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 than 50 nucleotides upstream of an informative locus "A" as well as 2, 3, 4, 5, or more additional sequencing primers that are designed to hybridize to an n-1 genomic location, n-2 genomic location, n-3 genomic location, n-4 genomic location, n-5 genomic location, etc., of "Primer A" Following a second round of SOLiD sequencing, the extension product can be removed and a third set of sequencing primers can be used for a third round of SOLiD sequencing. The third set of sequencing primers can comprise a primer "primer B" that is designed to hybridize to a genomic location residing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21,

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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 than 50 nucleotides upstream of an informative locus "B"., as well as 2, 3, 4, 5, or more additional sequencing primers that are designed to hybridize to an n-1 location, n-2 location, n-3 location, n-4 location, n-5 location, etc., of "Primer B". The informative locus "B" can be located downstream or upstream of informative locus "A". The third round of SOLiD sequencing can comprise multiple cycles of primer hybridization and ligation as described above. Additional rounds of SOLiD sequencing can be performed to sequence further downstream informative loci, as described above. Sequence reads for the first, second, and optionally additional rounds of sequencing can be compiled for each addressable location. The compiling can be used to generate sequence information for the clonally amplified templates. Sequence information for the clonally amplified templates can be used to determine phasing of one or more alleles detected by the sequencing.

[0091] In other embodiments, one or more phasing methods disclosed herein employ sequencing by synthesis using the methods commercialized by 454/Roche Life Sciences, including but not limited to the methods and apparatus described, e.g., in Margulies et al., *Nature(2005)* 437:376-380 (2005); and U.S. Pat. Nos. 7,244,559; 7,335,762; 7,211,390; 7,244,567; 7,264,929; and 7,323,305, hereby incorporated by reference. 454 sequencing library preparation can comprise ligation of adaptors to the ends of nucleic acid fragments. The adaptors can serve as primers for amplification and can provide a sequencing primer binding site for sequencing-by-synthesis. One of the adaptors can comprise a capture reagent, e.g., a biotin. The nucleic acid fragments can be attached to capture beads, e.g., streptavidin-coated beads. The fragments attached to the beads can be PCR amplified within droplets of an oil-water emulsion, resulting in multiple copies of clonally amplified nucleic acid fragments on each bead. Following 454 library preparation, library members can be coupled to a 454 sequencing platform by capture of beads in wells of the 454 platform, which can be pico-liter sized. In some cases, a well captures one bead. Each well can accordingly be an addressable location of the 454 platform. Pyrosequencing can be performed on each DNA fragment in parallel. Pyrosequencing can comprise hybridization of a sequencing primer to a sequencing primer binding site on an adaptor, followed by extension of the primer in a pyrosequencing reaction. During the pyrosequencing reaction, dTNPs are added such that only one out of four of the possible A/T/C/G nucleotides are added and available at a time. Pyrosequencing can detect release of pyrophosphate (PPi) upon nucleotide incorporation. PPi can be converted to ATP by ATP sulfurylase in the presence of adenosine 5' phosphosulfate. Luciferase can use ATP to convert luciferin to oxyluciferin, thereby

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generating a light signal that is detected. A detected light signal can be used to identify the incorporated nucleotide. The intensity of the light signal can be used to determine how many bases are incorporated. Following detection of the light signal, unincorporated dNTPs can be removed and the next one out of four possible A/T/C/G nucleotides can be added.

[0092] In one embodiment of a phasing method employing pyrosequencing methodology (e.g., 454 sequencing), a sequencing library is prepared from a nucleic acid sample obtained from a subject. In some cases, the sequencing library is a directional sequencing library. In some cases, the sequencing library is not a directional sequencing library. Sequencing library members can be coupled to addressable locations of a pyrosequencing platform (e.g., a 454 sequencing platform) as described above. A first round of pyrosequencing can be performed as described above, generating a first sequencing read from the library members. Following the first round of pyrosequencing, the extension strands and primers are removed from the library members, e.g., by denaturation and washing. A second round of sequencing can then be performed by hybridization of a second sequencing primer to one or more library members. The second sequencing primer can be designed to hybridize to a location upstream of an informative locus "A" and to sequence informative locus "A". A second pyrosequencing reaction can then be performed as described above. Subsequent removal of the second sequencing primer and additional rounds of pyrosequencing can be performed to sequence additional informative loci. The additional informative loci can be located downstream or upstream of locus "A". Sequence reads for the first, second, and optionally additional rounds of sequencing can be compiled for each addressable location. The compiling can be used to generate sequence information for the clonally amplified templates. Sequence information for the clonally amplified templates can be used to determine phasing of two or more alleles detected by the sequencing.

[0093] In other embodiments, one or more phasing methods disclosed herein may employ the sequencing methods commercialized by Helicos BioSciences Corporation (Cambridge, Mass.) as described, e.g., in U.S. Patent Application Ser. No. 11/167,046, and U.S. Pat. Nos. 7,501,245; 7,491,498; 7,276,720; and in U.S. Patent Application Publication Nos. US20090061439; US20080087826; US20060286566; US20060024711; US20060024678; US20080213770; and US20080103058, hereby incorporated by reference. For example, a method can employ the true Single-Molecule Sequencing technology by Helicos Biosciences Corporation. tSMS library preparation can comprise ligation of a polyA adaptor to 3 ' end of DNA fragments. The adapted fragments can then be coupled to a Helicos tSMS sequencer by the following steps: (1) fragments are hybridized to poly-T oligonucleotides immobilized on

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the tSMS flow cell; (2) the flow cell is loaded into an instrument, e.g., HeliScope™ sequencer. Each fragment can be hybridized to a unique addressable location on the tSMS flow cell. A laser can illuminate the surface of the flow cell, revealing the position of each template. A CCD camera can map the position of the templates on the flow cell surface. tSMS sequencing can then proceed by subjecting library members to repeated cycles of polymerase-directed single base extension. A round of tSMS sequencing reaction can commence by introducing a DNA polymerase and a fluorescently labeled nucleotide. The polymerase can incorporate the labeled nucleotides to the primer in a template directed manner. The polymerase and unincorporated nucleotides can be removed. The templates that have directed incorporation of the fluorescently labeled nucleotide can be discerned by imaging the flow cell surface. After imaging, a cleavage step can remove the fluorescent label, and the process can be repeated with other fluorescently labeled nucleotides until a desired read length is achieved. Sequence information can be collected with each nucleotide addition step.

[0094] In one embodiment of a phasing method employing the Helicos tSMS methodology, a tSMS sequencing library is prepared as described above from a nucleic acid sample obtained from a subject. In some cases, the sequencing library is a directional sequencing library. In some cases, the sequencing library is not a directional sequencing library. The sequencing library members can be coupled to unique addressable locations on the tSMS platform as described herein, e.g., by hyridization to polyT oligonucleotides immobilized on the flow cell. Reverse complement complement strands of the hybridized library members can be synthesized, thus creating immobilized templates for sequencing. The original library members hybridized to the flowcell can be denatured and/or removed. A first round of tSMS sequencing can be performed on immobilized templates as described above, generating a first sequencing read from the immobilized templates. Following the first round of tSMS sequencing, the first sequencing reads can be denatured and/or removed. The immobilized templates can be retained on the flow cell. A second round of tSMS sequencing can then be performed by hybridization of a sequencing primer to the immobilized templates. The second sequencing primer can be designed to hybridize to a location upstream of an informative locus "A" and to sequence informative locus "A". A second tSMS sequencing reaction can then be performed as described above. Subsequent denaturation and/or removal of the sequencing primer and sequence reads, followed by additional rounds of tSMS sequencing can be performed on the immobilized templates to sequence additional informative loci. The additional informative loci can be located

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downstream or upstream of locus "A". Sequence reads for the first, second, and optionally additional rounds of sequencing can be compiled for each addressable location. The compiling can be used to generate sequence information for the templates. Sequence information for the templates can be used to determine phasing of one or more alleles detected by the sequencing.

[0095] Another example of a sequencing technique that can be used in the methods described herein is semiconductor sequencing provided by Ion Torrent (e.g., using the Ion Personal Genome Machine (PGM)). Ion Torrent technology can use a semiconductor chip with multiple layers, e.g., a layer with micro-machined wells, an ion-sensitive layer, and an ion sensor layer. Sequencing library preparation for the Ion Torrent platform can involves ligation of two distinct adaptors at both ends of a DNA fragment. Individual library members can be attached to beads, as described herein. The library members can then be clonally amplified by, e.g., emulsion PCR. The clonally amplified library members can be coupled to an Ion Torrent sequencing platform, for example, by introducing individual beads comprising the clonally amplified library members into wells of the Ion Torrent platform. Each well can be considered a unique addressable location of the Ion Torrent platform. A round of sequencing can comprise annealing a first sequencing primer to the library members. The first sequencing primer can be designed to hybridize to a sequencing primer binding region of an Ion Torrent library adaptor. To initiate sequencing of the nucleic acids on the beads, one type of deoxyribonucleotide (e.g., dATP, dCTP, dGTP, or dTTP) can be introduced into the wells. When one or more nucleotides are incorporated by DNA polymerase, protons (hydrogen ions) can be released in the well, which can be detected by the ion sensor. The semiconductor chip can then be washed and the process can be repeated with a different deoxyribonucleotide. A plurality of nucleic acids can be sequenced in the wells of a semiconductor chip. The semiconductor chip can comprise chemical-sensitive field effect transistor (chemFET) arrays to sequence DNA (for example, as described in U.S. Patent Application Publication No. 20090026082). Incorporation of one or more triphosphates into a new nucleic acid strand at the 3' end of the sequencing primer can be detected by a change in current by a chemFET. An array can have multiple chemFET sensors.

[0096] In one embodiment of a phasing method described herein employing Ion Torrent methodology, an Ion Torrent sequencing library is prepared as described above from a nucleic acid sample obtained from a subject. In some cases, the sequencing library is a directional sequencing library. In some cases, the sequencing library is not a directional sequencing library. Sequencing library members can be coupled to unique addressable

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locations of the Ion Torrent platform as described herein. A first round of Ion Torrent sequencing can be performed on library members as described above, generating a first sequencing read from the library members. Upon completion of the first round of sequencing, the extended strands and sequencing primers can be denatured from the templates and removed, e.g., by washing. A second round of sequencing can be performed using a second sequencing primer. The second sequencing primer can be designed to hybridize to a location upstream of an informative locus "A" and to sequence informative locus "A". A second sequencing reaction can then be performed as described above. Subsequent removal of the second sequencing primer and additional rounds of sequencing can be performed to sequence additional informative loci. The additional informative loci can be located downstream or upstream of locus "A". Sequence reads for the first, second, and optionally additional rounds of sequencing can be compiled for each addressable location. The compiling can be used to generate sequence information for the clonally amplified templates. Sequence information for the clonally amplified templates can be used to determine phasing of one or more alleles detected by the sequencing.

[0097] Sequential sequencing by any of the means described herein can produce one or more sequencing reads for nucleic acid templates. The sequencing reads can have a length which may vary depending on the particular sequencing technology utilized. NGS platforms can provide sequence reads that vary in size from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, tens to hundreds, or thousands of base pairs. In some embodiments of the method described herein, the sequence reads are about 1 base long, about 2 bases long, about 3 bases long, about 4 bases long, about 5 bases long, about 6 bases long, about 7 bases long, about 8 bases long, about 9 bases long, about 10 bases long, about 12 bases long, about 14 bases long, about 16 bases long, about 18 bases long, about 20 bases long, about 25 bases long, about 30 bases long, about 35 bases long, about 40 bases long, about 45 bases long, about 50 bases long, about 55 bases long, about 60 bases long, about 65 bases long, about 70 bases long, about 75 bases long, about 80 bases long, about 85 bases long, about 90 bases long, about 95 bases long, about 100 bases long, about 110 bases long, about 120 bases long, about 130, about 140 bases long, about 150 bases long, about 200 bases long, about 250 bases long, about 300 bases long, about 350 bases long, about 400 bases long, about 450 bases long, about 500 bases long, about 600 bases long, about 700 bases long, about 800 bases long, about 900 bases long, about 1000 bases, about 2000 bases, about 3000 bases, about 4000 bases, about 5000 bases, about 6000 bases, about 7000 bases, about 8000 bases, about 9000 bases, about 10,000 bases long, about 20,000 bases long, about 30,000 bases long, about 40,000 bases long, about

50,000 bases long, about 60,000 bases long, about 70,000 bases long, about 80,000 bases long, about 90,000 bases long, about 100,000 bases, about 150,000 bases long, about 200,000 bases long, about 250,000 bases long, or more than 250,000 bases long.

[0098] In some cases, a sequencing read is at least 1 base long, at least 2 bases long, at least 3 bases long, at least 4 bases long, at least 5 bases long, at least 6 bases long, at least 7 bases long, at least 8 bases long, at least 9 bases long, at least 10 bases long, at least 12 bases long, at least 14 bases long, at least 16 bases long, at least 18 bases long, at least 20 bases long, at least 25 bases long, at least 30 bases long, at least 35 bases long, at least 40 bases long, at least 45 bases long, at least 50 bases long, at least 55 bases long, at least 60 bases long, at least 65 bases long, at least 70 bases long, at least 75 bases long, at least 80 bases long, at least 85 bases long, at least 90 bases long, at least 95 bases long, at least 100 bases long, at least 110 bases long, at least 120 bases long, at least 130 bases long, at least 140 bases long, at least 150 bases long, at least 200 bases long, at least 250 bases long, at least 300 bases long, at least 350 bases long, at least 400 bases long, at least 450 bases long, at least 500 bases long, at least 600 bases long, at least 700 bases long, at least 800 bases long, at least 900 bases long, at least 1000 bases long, at least 10,000 bases long, at least 20,000 bases long, at least 30,000 bases long, at least 40,000 bases long, at least 50,000 bases long, at least 60,000 bases long, at least 70,000 bases long, at least 80,000 bases long, at least 90,000 bases long, at least 100,000 bases, at least 150,000 bases long, at least 200,000 bases long, or more than 200,000 bases long.

[0099] A sequencing read can be between about 1 to about 10 bases long, between about 5 to about 50 bases long, between about 20 to about 100 bases long, between about 50 to about 200 bases long, between about 100 to about 500 bases long, between about 200 to about 1000 bases long, between about 500 to about 2000 bases long, between about 1000 to about 5000 bases long, between about 2000 to about 10000 bases long, between about 5000 to about 50000 bases long, abetween about 10000 to about 100000 bases long, or more than 100000 bases long.

Sequence analysis

[00100] Sequencing reads (e.g., sequence tags) can be aligned to assemble a partial or complete map of the subject's genome. Sequence tags can be aligned (e.g., mapped), for example, to a reference genome. The reference genome can be a human reference genome. The human reference genome can be the NCBI36/hg18 sequence, available on the world wide web at genome.ucsc.edu/cgi-bin/hgGateway?org=Human&db=hgl 8&hgsid=166260105). Sequencing reads can be mapped using an alternative source of

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public sequence information. Other sources of public sequence information include GenBank, dbEST, dbSTS, EMBL (the European Molecular Biology Laboratory), and the DDBJ (the DNA Databank of Japan). In some cases, the reference genome can be an artificial target sequence genome comprising polymorphic target sequences.

[00101] Mapping of the sequence tags can be achieved, for example, by aligning the sequence of the tag to a sequence of the reference genome. Such methods can be used to map a sequencing read to a genomic region, a chromosomal region, or a chromosome. A number of computer algorithms can be used for aligning sequences. Such algorithms include, without limitation, BLAST (Altschul et al., 1990), BLITZ (MPsrch) (Sturrock & Collins, 1993), BOWTIE (Langmead et al, Genome Biology 10:R25.1-R25.10 [2009]), FASTA (Person & Lipman, 1988), SAMtools (SAMtools, Bioinformatics, 2009, 25(16):2078-9), and ELAND (Illumina, Inc., San Diego, CA, USA). In one embodiment, sequence reads produced by Illumina sequencing are processed by bioinformatic alignment analysis for the Illumina Genome Analyzer, which uses the Efficient Large-Scale Alignment of Nucleotide Databases (ELAND) software. Additional software includes the Burroughs-Wheeler block sorting compression procedure which involves block sorting or preprocessing to make compression more efficient.

[00102] Reads from the sequential rounds of sequencing can be compiled for each sequenced template to assemble complete or partial sequence information of the template. Reads from the sequential rounds of sequencing can be compiled according to unique addressable locations of the sequencing platform. For example, all reads generated from a unique addressable location of a sequencing platform can be compiled into a data file. Reads can be compiled with aid of a computer processor. Compiled reads from a unique addressable location can be used to determine partial or complete sequence information for a nucleic acid template (either single template or clonally amplified template). The partial or complete sequence information can comprise information over a longer distance than an average read length of the sequencing platform. Because the sequence information is over a longer distance than an average read length of the sequencing platform, the probability that the sequence information comprises information over two or more informative loci can be increased.

[00103] Compiled reads from a unique addressable location may comprise sequence information for two or more detected alleles. Such compiled reads can be used to determine that the two or more detected alleles are in phase, since the detected alleles are located on the sample template or clonally amplified template. Alleles detected across two or more

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addressable locations can also be determined to be in phase, if the two or more addressable locations each share at least one detected allele in common. Alleles detected across a set of addressable locations comprising any number of addressable locations can be determined to be in phase, if the addressable locations in the set share at least one detected allele in common with at least one other addressable location in the set.

[00104] Compiling of reads, determination of phasing information, and/or haplotype analysis can be performed with the aid of a computer system. In some embodiments, the computer system executes instructions contained in a computer-readable medium. The computer system can comprise a processor. The processor can be associated with one or more controllers, calculation units, and/or other units of a computer system, or implanted in firmware. In some embodiments, one or more steps of the method are implemented in hardware. In some embodiments, one or more steps of the method are implemented in software. Software routines may be stored in any computer readable memory unit such as flash memory, RAM, ROM, magnetic disk, laser disk, or other storage medium as described herein or known in the art. Software may be communicated to a computing device by any known communication method including, for example, over a communication channel such as a telephone line, the internet, a wireless connection, or by a transportable medium, such as a computer readable disk, flash drive, etc. The one or more steps of the methods described herein may be implemented as various operations, tools, blocks, modules and techniques which, in turn, may be implemented in firmware, hardware, software, or any combination of firmware, hardware, and software. When implemented in hardware, some or all of the blocks, operations, techniques, etc. may be implemented in, for example, an application specific integrated circuit (ASIC), custom integrated circuit (IC), field programmable logic array (FPGA), or programmable logic array (PLA).

[00105] **FIG. 5** depicts an exemplary computer system adapted to implement one or more methods provided herein. The system **500** includes a central computer server **501** that is programmed to implement exemplary methods described herein. The server **501** includes a central processing unit (CPU, also "processor") **505** which can be a single core processor, a multi core processor, or plurality of processors for parallel processing. The server **501** also includes memory **510** (e.g. random access memory, read-only memory, flash memory); electronic storage unit **515** (e.g. hard disk); communications interface **520** (e.g. network adaptor) for communicating with one or more other systems; and peripheral devices **525** which may include cache, other memory, data storage, and/or electronic display adaptors. The memory **510**, storage unit **515**, interface **520**, and peripheral devices **525** are in

communication with the processor **505** through a communications bus (solid lines), such as a motherboard. The storage unit **515** can be a data storage unit for storing data. The server **501** is operatively coupled to a computer network ("network") **530** with the aid of the communications interface **520**. The network **530** can be the Internet, an intranet and/or an extranet, an intranet and/or extranet that is in communication with the Internet, a telecommunication or data network. The network **530** in some cases, with the aid of the server **501**, can implement a peer-to-peer network, which may enable devices coupled to the server **501** to behave as a client or a server.

[00106] The storage unit **515** can store files, such as subject reports, and/or communications with the caregiver, sequencing data, data about individuals, or any aspect of data generated by one or more phasing methods disclosed herein.

[00107] The server can communicate with one or more remote computer systems through the network **530**. The one or more remote computer systems may be, for example, personal computers, laptops, tablets, telephones, Smart phones, or personal digital assistants.

[00108] In some situations the system **500** includes a single server **501**. In other situations, the system includes multiple servers in communication with one another through an intranet, extranet and/or the Internet.

[00109] The server **501** can be adapted to store sequencing information, and/or information on a subject, such as, for example, polymorphisms, mutations, patient history of the subject, demographic data and/or other information of potential relevance. Such information can be stored on the storage unit **515** or the server **501** and such data can be transmitted through a network.

[00110] Methods as described herein can be implemented by way of machine (or computer processor) executable code (or software) stored on an electronic storage location of the server **501**, such as, for example, on the memory **510**, or electronic storage unit **515**. During use, the code can be executed by the processor **505**. In some cases, the code can be retrieved from the storage unit **515** and stored on the memory **510** for ready access by the processor **505**. In some situations, the electronic storage unit **515** can be precluded, and machine-executable instructions are stored on memory **510**. Alternatively, the code can be executed on a second computer system **540**.

[00111] Aspects of the systems and methods provided herein, such as the server **501**, can be embodied in programming. Various aspects of the technology may be thought of as "products" or "articles of manufacture" typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine

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readable medium. Machine-executable code can be stored on an electronic storage unit, such memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical, and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless likes, optical links, or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" can refer to any medium that participates in providing instructions to a processor for execution.

[00112] Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, tangible storage medium, a carrier wave medium, or physical transmission medium. Non-volatile storage media can include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such may be used to implement the system. Tangible transmission media can include: coaxial cables, copper wires, and fiber optics (including the wires that comprise a bus within a computer system). Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include, for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD, DVD-ROM, any other optical medium, punch cards, paper tame, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables, or links transporting such carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

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[00113] The computer readable medium can comprise computer executable code. The computer executable code can implement a method comprising: (a) accessing a data file in computer memory that comprises a plurality of sequencing reads corresponding to a first addressable location of a sequencing platform, wherein the sequencing reads are generating by subjecting a first nucleic acid template coupled to the addressable location to a first and at least a second round of sequencing, wherein the first and at least second rounds of sequencing are performed using a first and second sequencing primer, respectively; (b) using said data file, detecting a first detected allele at a first locus of the nucleic acid template and detecting a second detected allele at a second locus of the nucleic acid template; and (c) determining the first and second detected allele to be in phase based upon said detecting. The method executed by the computer executable code can further comprise the steps of (d) accessing a second data file in computer memory that comprises a plurality of sequencing reads corresponding to a second addressable location of a sequencing platform, wherein the sequencing reads are generating by subjecting a second nucleic acid template coupled to the addressable location to the first and the at least second round of sequencing; (e) using the second data file, detecting a first detected allele at a first locus of the second nucleic acid template and detecting a second detected allele at a second locus of the second nucleic acid template; and (f) determining the first and second detected alleles of the second nucleic acid template to be in phase with the first and second detected alleles of the first nucleic acid template if the first or second detected allele of the second nucleic acid template is shared in common with the first nucleic acid template.

Kits

[00114] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, the kit, in a suitable container, comprises: an adaptor or several adaptors, one or more of oligonucleotide primers and reagents for ligation, primer extension and amplification. The kit may also comprise means for purification, such as a bead suspension, and nucleic acid modifying enzymes. Kits can further comprise instructions for practicing any of the phasing methods described herein.

Products

[00115] Products based on the methods provided herein may be commercialized by the Applicants under the Ovation® family. Ovation is a registered trademark of NuGEN Technologies, Inc.

[00116] The information gained from the phasing of alleles in a genome as provided by practicing the methods described herein can be of great utility in research and discovery as

well as for clinical, diagnostic, forensic, archeological, geneological, and/or epidemiological applications. For example, haplotype information can be used to aid in the diagnosis, prognosis, and/or theranosis of a disease. For other example, haplotype information of a potential tissue or organ donor and organ recipient can aid in the assessment of HLA compatibilities of the donor and recipient for decreasing transplant rejection. In some cases, information gained from phasing of alleles in a genome of a subject can be used to tailor a healthcare or therapeutic regimen for the subject. In some cases, one or more methods described herein can be used to construct a database of haplotypes associated with one or more disease or biological trait. A haplotype obtained from a subject using a method as described herein can be compared against a haplotype database, thereby allowing for diagnosis and/or prognosis of a disease or condition. In some cases, haplotype information can be used to aid in the treatment of an infectious disease. For example, bacteria or virus infections can be associated with a plurality of drug resistant markers. Use of one or more phasing methods described herein can help determine if the plurality of drug resistant markers are due to co-infection with single-drug resistant strains or due to infection with a single strain that is multi-drug resistant. One or more phasing methods described herein can also be used to characterize T-cell receptor and/or immunoglobulin repertoire in a subject. For example, one or more phasing methods described herein can be used to assess V(D)J recombination in a subject. One or more phasing methods described herein can be used for diagnosing, predicting, determining or assessing the genetic characteristics of a fetus or embryo. A phasing method described herein can be used to assess genetic phasing within the genome of a developing fetus. In some cases, one or more phasing methods can be used to combat bioterrorism and/or to assess an epidemic or pandemic (e.g., by characterizing or identifying an infectious agent such as a virus or bacterium). In some cases, one or more phasing methods can be used for paternity testing, to establish a family tree, or to determine ancestry. In some cases, one or more phasing methods can be used to determine alternative splicing of RNA transcripts.

EXAMPLES

Example 1 — Characterization of the human oral microbiome by sequential sequencing of bacterial 16S ribosomal operon

[00117] This example describes the characterization of the human oral microbiome by sequencing of the 16S rRNA gene sequences of a number of related bacterial organisms. 16S rRNA gene sequences contain species-specific hypervariable regions that can provide means

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for bacterial identification. An exemplary embodiment of 16S rRNA sequential sequencing, described below, is depicted in **FIG. 6**.

Sample nucleic acid

[00118] Microbial genomic DNA is isolated from human saliva using the OMNIgene-DISCOVER sample collection kit (DNA Genotek) according to the manufacturer's instructions. The different line shadings and hash marks indicate different microbial genomes (microbial DNA). The relative numbers (e.g., 4 hatched lines vs 1 gray line) reflect the different ratios of the organisms in the saliva sample. Extracted DNA is then fragmented via sonication to an average length of 1000 bp and purified using Agencourt AMPure XP beads (Beckman Coulter Genomics). In some cases, extracted DNA is not subjected to fragmentation. For example, the microbial genetic DNA can be optionally enriched for target polynucleotides comprising the 16S genomic regions using primers to conserved 16S regions of the genome, thereby generating 16S amplicons.

Generation 16 S libraries with ligated adapters

[00119] The NuGEN Ovation Ultralow Library System (NuGEN Technologies) is used to attach library adaptors to the DNA fragments, thereby generating next generation sequencing libraries from 100 ng of the purified sample according to manufacturer's instructions. Ligation products with a minimum length of 200 bp in length are purified by selective binding to Agencourt AMPure XP beads.

Sequential primer sequencing

[00120] Library members are clonally amplified by bridge amplification on an Illumina flowcell, thereby generating spatially separated clusters, wherein the clusters comprise identical copies of an original sequencing library member. In a first round of sequencing, all clusters, including those containing 16 S ribosomal DNA fragments, are sequenced by Illumina sequencing system using an Illumina standard forward primer. The standard forward primer can be designed to hybridize to a sequencing primer binding site of a library adaptor. Alternatively, a custom primer may be used. The custom primer can be designed to hybridize to a location upstream of and to sequence an informative locus. In such cases, clusters comprising a primer binding site for the first custom primer can be sequenced. The first round of sequencing can generate a first sequencing read ("Read 1"). Following the first sequencing read, the DNA is denatured to wash away the newly synthesized strand comprising Read 1. In cases where the first sequencing primer is a standard forward primer, Read 1 can contain sequence information for the forward end of the cluster templates. In cases where the first sequencing primer is a custom primer, Read 1 can comprise sequence

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information for the informative locus. A second round of sequencing can then be performed using a second sequencing primer. The second sequencing primer can be a custom primer that is designed to hybridize to a location upstream of and to sequence an informative locus. In cases where the first round of sequencing is performed using a custom primer that sequences an informative locus, the second sequencing primer is a designed to hybridize to a location upstream of and to sequence informative locus which is downstream of the first informative locus. The second round of sequencing can produce a second sequencing read "Read 2" in clusters comprising a primer binding site for the second sequencing primer. Read 2 can comprise sequence information for the informative locus sequenced by the second sequencing primer. Successive rounds of denaturation, re-priming and sequencing are optionally performed with primers that are designed to sequence additional informative loci. Sequence reads from successive priming and sequencing are compiled for each cluster and aligned to map reads originating from the same nucleic acid fragments.

Example 2 — Genomic DNA sequencing — distinguishing between a gene and its pseudogene using sequential sequencing

[00121] FIG. 7 depicts an exemplary application of a method described herein to distinguish a gene from a pseudogene in a gene/pseudogene pair. Genomic DNA sequencing libraries are prepared using the NuGEN's Encore system. These libraries are sequenced on a DNA sequencing system such as those made by Illumina or Ion Torrent. Clusters include, but are not limited to cluster 1, from a different genomic region from a gene/pseudogene pair, cluster 2, which comprises a portion of the gene of a gene/psuedogene pair, and cluster 3, which comprises a portion of the pseudogene of the gene/pseudogene pair. A first round of sequencing is performed using a first sequencing primer, thereby generating a first sequencing read. In some cases, the first sequencing primer is a standard forward primer as described in Example 1. The first sequencing read reveals that clusters 2 and 3 exhibit different alleles at a certain locus (a T in cluster 2 and a C in cluster 3). However, it is impossible to determine from read 1 which cluster represents the gene or the pseudogene. Following a first sequencing read, the DNA is denatured to wash away the extension strand. An oligonucleotide designed to anneal to a common sequence shared by the gene and pseudogene is injected into the sequencer to act as the second sequencing primer. The second sequencing primer can be designed to hybridize to a conserved sequence shared by the gene and pseudogene, wherein extension of the primer produces a second sequencing read that extends across a locus that can distinguish the gene and pseudogene. An exemplary gene/pseudogene pair which can be distinguished using a method described herein is the

SMN1 gene/SMN2 pseudogene pair. In some embodiments, the second sequencing primer comprises a sequence which is designed to hybridize to a conserved squence shared by SMN1 and SMN2, wherein extension of the primer produces a second sequencing read that extends across a locus that can distinguish SMN1 and SMN2. In one embodiment, the second sequencing primer comprises the sequence CTTCCTTTATTTTCCTTACAGGGT (SEQ. ID. NO: 1). The second sequencing read reveals a downstream C base call at the locus in cluster 3. The C base call reveals that cluster 3 maps to the pseudogene but that cluster 2 maps to the gene. An oligonucleotide set may include primers that will sequence through one of the nucleotide differences between the gene and pseudogene as well as primers that will generate sequence to read nucleotide differences, and therefore determine whether a sequencing read is from a gene or its pseudogene. A combination of such primers will allow multiple gene/pseudogene pairs across the genome to be analyzed simultaneously for genetic mutations.

Example 3 — Targeted DNA sequencing library

[00122] A targeted DNA sequencing library is made using a target-selective library preparation kit from NuGEN, Agilent, Illumina, or Nimblegen, described herein. These libraries are sequenced on a DNA sequencing system such as those made by Illumina or Ion Torrent. Following a first sequencing read, the DNA is denatured to wash away the extension strand. A pool of primers that hybridize to common sequences in gene/pseudogene pairs are injected into the sequencer to act as a priming site for a second sequencing read. A primer set may include primers that will sequence through one of the nucleotide differences between a gene and its pseudogene (e.g., SMN1 and SMN2) as well as primers that will generate sequence to read nucleotide differences, and therefore determine whether a sequencing read is, for example, from a gene or pseudogene. A combination of such primers will allow multiple gene/pseudogene pairs across the genome to be analyzed simultaneously for genetic mutations. This type of technology is useful for genetic diagnostics.

Example 4 — RNA-sequencing library

[00123] An RNA sequencing library is made from NuGEN's Encore Complete RNA-Seq Library System. The library is sequenced on an Illumina DNA sequencer. Following the first sequencing read, a pool of primers that will hybridize to specific exons of interest is injected into the sequencing machine. These primers are used to generate a second sequencing read in a downstream exon. The second, targeted sequencing read provides information about which exons have been spliced together to generate a particular RNA transcript.

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[00124] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments provided herein described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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CLAIMS

WHAT IS CLAIMED IS:

1. A method for relating at least two nucleic acid sequences or regions of sequence variation to the same nucleic acid template, the method comprising:

a) creating a nucleic acid library wherein a template is immobilized on a solid support;

b) generating a first sequencing read from the immobilized template with a first oligonucleotide primer, wherein a 3' terminal nucleotide of the first oligonucleotide primer binds to a first binding site in the template;

c) denaturing the first sequencing read from the immobilized template;

d) annealing a second oligonucleotide primer that is complementary to a region or regions within the immobilized template, wherein a 3' terminal nucleotide of the second oligonucleotide primer binds to a second binding site in the template that is more than 5 nt away from the first binding site in the template;

e) generating a second sequencing read with the second oligonucleotide primer; and

f) compiling data from the first sequencing read and the second sequencing read to map reads originating from the template.

2. The method of claim 1, wherein the nucleic acid libraries are generated from amplicons.

3. The method of claim 2, wherein the amplicons comprise conserved regions which flank a variable region.

4. The method of claim 3, wherein alignment of multiple variable regions enables differentiation and/or typing of related transcripts.

5. The method of claim 3, wherein alignment of multiple variable regions enables differentiation and/or typing of related micro-organisms.

6. The method of claim 1, wherein libraries are of reduced complexity.

7. The method of claim 6, wherein reduced complexity is achieved by target enrichment.

8. The method of any of the preceding claims, wherein the nucleic acid library comprises closely related nucleic acid sequences as inserts.

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9. A method, comprising:

a) coupling at least one nucleic acid template to a sequencing platform;

b) subjecting the at least one nucleic acid template to a first round of sequencing using a first sequencing primer, wherein the first sequencing primer comprises a 3' terminal nucleotide which anneals to a first binding site in the at least one nucleic acid template, wherein the first round of sequencing produces a first sequencing read of the at least one nucleic acid template;

c) removing the first annealed sequencing primer and the first sequencing read from the at least one nucleic acid template; and

d) subjecting the at least one nucleic acid template to a second round of sequencing using a second sequencing primer, wherein the second sequencing primer comprises a 3' terminal nucleotide which anneals to a second binding site in the at least one nucleic acid template which is more than 5 nt away from the first binding site, wherein the second round of sequencing produces a second sequencing read of the at least one nucleic acid template.

10. The method of claim 9, wherein the first and/or the second round of sequencing comprises sequencing by synthesis.

11. The method of claim 9, wherein the first and/or the second round of sequencing comprises sequencing by ligation.

12. The method of claim 9, wherein the coupling comprises conjugating the at least one nucleic acid template to a solid support of the sequencing platform.

13. The method of claim 12, wherein the solid support comprises a bead, a well, an array, or a flow cell.

14. The method of claim 9, wherein the at least one nucleic acid template comprises an adaptor oligonucleotide ligated to one or both ends of the at least one nucleic acid template.

15. The method of claim 14, wherein the first sequencing primer is selectively hybridizable to a primer binding site of the adaptor oligonucleotide.

16. The method of claim 14, wherein the second sequencing primer is not selectively hybridizable to the primer binding site of the adaptor oligonucleotide.

17. The method of claim 9, wherein the second sequencing read comprises an informative locus.

18. The method of claim 17, wherein the informative locus is a polymorphic locus.

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19. The method of claim 17, wherein the first sequencing read comprises a first informative locus and wherein the second sequencing read comprises a second informative locus.

20. The method of claim 19, comprising determining that a first allele detected at the first informative locus and a second allele detected at the second informative locus are in phase.

21. The method of claim 17, wherein the second sequencing primer is designed to hybridize to a location that is within *x* nt upstream of the informative locus, wherein *x* is a number that is less than an average sequencing read length produced by the first or second round of sequencing.

22. The method of claim 9, wherein the removing comprises a denaturing step.

23. The method of claim 22, wherein the removing further comprises a washing step.

24. The method of claim 9, further comprising subjecting the at least one nucleic acid template to one or more additional rounds of sequencing, wherein the one or more additional rounds of sequencing comprise the steps of : (i) removing a previously annealed sequencing primer and previously generated sequencing read from the at least one nucleic acid template; (ii) annealing an additional sequencing primer to the at least one nucleic acid template; and (iii) subjecting the at least one nucleic acid template to an additional round of sequencing using the additional sequencing primer, thereby producing an additional sequencing read of the at least one nucleic acid template.

25. The method of claim 24, wherein the additional sequencing primer comprises a 3' terminal nucleotide which hybridizes to a binding site that is more than 5 nt away from a binding site of the 3' terminal nucleotide of the previously annealed sequencing primer.

26. The method of claim 24, wherein a first allele detected by the first sequencing read, a second allele detected by the second sequencing read, and an additional allele detected by the additional sequencing read are determined to be in phase.

27. The method of claim 24, wherein the additional sequencing primer and the previously annealed sequencing primer are designed to hybridize to genomic locations that map to a single chromosome.

28. The method of claim 27, wherein the genomic locations that map to a single chromosome span a distance that is less than a length of the at least one nucleic acid template.

29. The method of claim 9, wherein the at least one nucleic acid template is a member of a sequencing library.

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30. The method of claim 29, wherein the sequencing library is a whole-genome sequencing library.

31. The method of claim 29, wherein the sequencing library is a target-enriched sequencing library.

32. The method of claim 29, wherein the sequencing library comprises nucleic acid fragments of an average length.

33. The method of claim 32, wherein the average length is about 50 nucleotides to about 10 kb.

34. The method of any of claims 9-33, wherein the first, second, and optionally additional sequencing reads comprise sequence information from a plurality of loci.

35. The method of claim 34, wherein phasing of alleles detected at the plurality of loci is determined based upon the first, second, and optionally additional sequencing reads.

36. The method of any of claims 9-33, wherein the at least one nucleic acid template comprises a plurality of nucleic acid templates.

37. The method of any of claims 9-33, wherein the at least one nucleic acid template comprises at least 10,000 nucleic acid templates.

38. The method of claim 37, wherein the at least one nucleic acid template comprises 3 billion nucleic acid templates.

39. The method of any of claims 9-33, wherein the at least one nucleic acid template is coupled to a unique addressable location of the sequencing platform.

40. The method of claim 39, comprising compiling sequencing reads from the unique addressable location into one data file.

41. The method of claim 39, comprising determining phasing of a plurality of alleles detected across two or more unique addressable locations.

42. The method of claim 41, wherein the two or more unique addressable locations each comprise two or more detected alleles, wherein the two or more detected alleles of each of the two or more unique addressable locations are determined to be in phase if at least one common detected allele is shared by the two or more unique addressable locations.

43. The method of any of claims 9-33, wherein step (d) comprises subjecting the at least one nucleic acid template to a second round of sequencing using a mixture of second sequencing primers.

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44. The method of claim 43, wherein the mixture of second sequencing primers comprises a pair of primers, wherein each primer in the primer pair is designed to sequence a forward or reverse complement strand containing a locus.

45. The method of claim 43, wherein primers within the mixture are designed and/or selected to have minimal potential to hybridize to the same nucleic acid template.

46. A kit, comprising:

a) one or more oligonucleotide primers for performing a sequencing reaction; and

b) instructions for practicing a method of any of the preceding claims using the one or more oligonucleotide primers.

47. A computer readable medium comprising computer executable code that, upon execution by a computer processor, implements a method comprising:

a) accessing a data file in computer memory that comprises a plurality of sequencing reads corresponding to a first addressable location of a sequencing platform, wherein the sequencing reads are generating by subjecting a first nucleic acid template coupled to the addressable location to a first and at least a second round of sequencing, wherein the first and at least second rounds of sequencing are performed using a first and second sequencing primer, respectively;

b) using the data file, detecting a first detected allele at a first locus of the nucleic acid template and detecting a second detected allele at a second locus of the nucleic acid template; and

c) determining the first and second detected allele to be in phase based upon the detecting.

48. The computer readable medium of claim 47, wherein the method further comprises:

d) accessing a second data file in computer memory that comprises a plurality of sequencing reads corresponding to a second addressable location of a sequencing platform, wherein the sequencing reads are generated by subjecting a second nucleic acid template coupled to the addressable location to the first and the at least second round of sequencing;

e) using the second data file, detecting a first detected allele at a first locus of the second nucleic acid template and detecting a second detected allele at a second locus of the second nucleic acid template; and

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f) determining the first and second detected alleles of the second nucleic acid template to be in phase with the first and second detected alleles of the first nucleic acid template if the first or second detected allele of the second nucleic acid template is shared in common with the first nucleic acid template.

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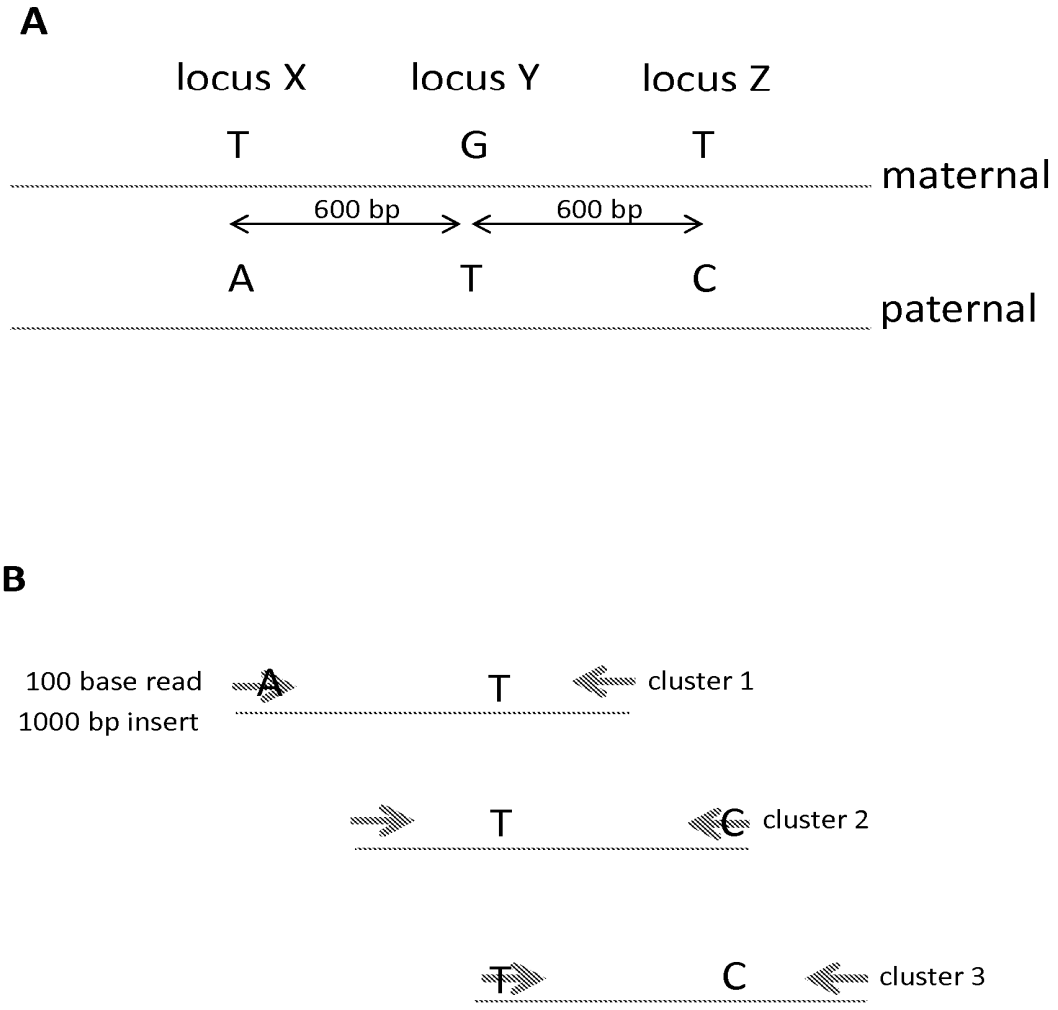

A
locus X
locus Y
locus Z
T
G
T
maternal
600 bp
600 bp
A
T
C
paternal
B
100 base read
1000 bp insert
A
T
cluster 1
T
C
cluster 2
T
C
cluster 3

FIG. 1

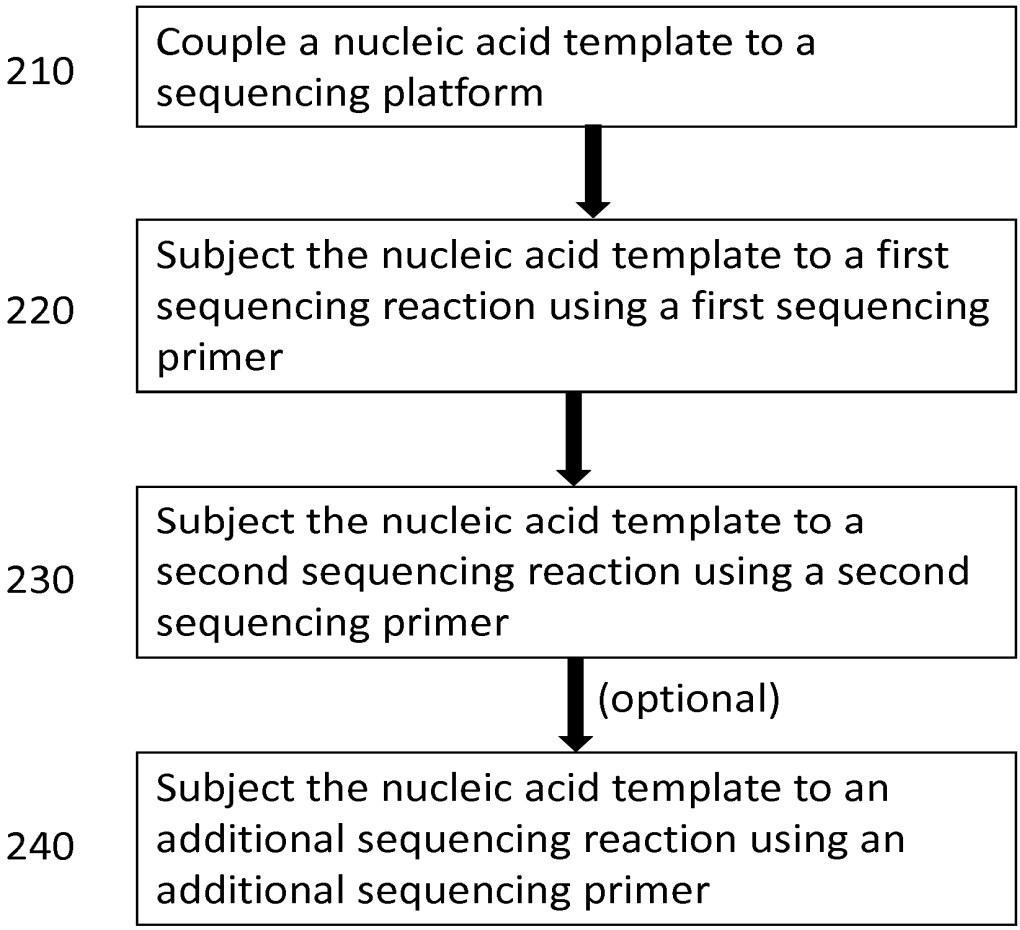

210
Couple a nucleic acid template to a sequencing platform
220
Subject the nucleic acid template to a first sequencing reaction using a first sequencing primer
230
Subject the nucleic acid template to a second sequencing reaction using a second sequencing primer
(optional)
240
Subject the nucleic acid template to an additional sequencing reaction using an additional sequencing primer

FIG. 2

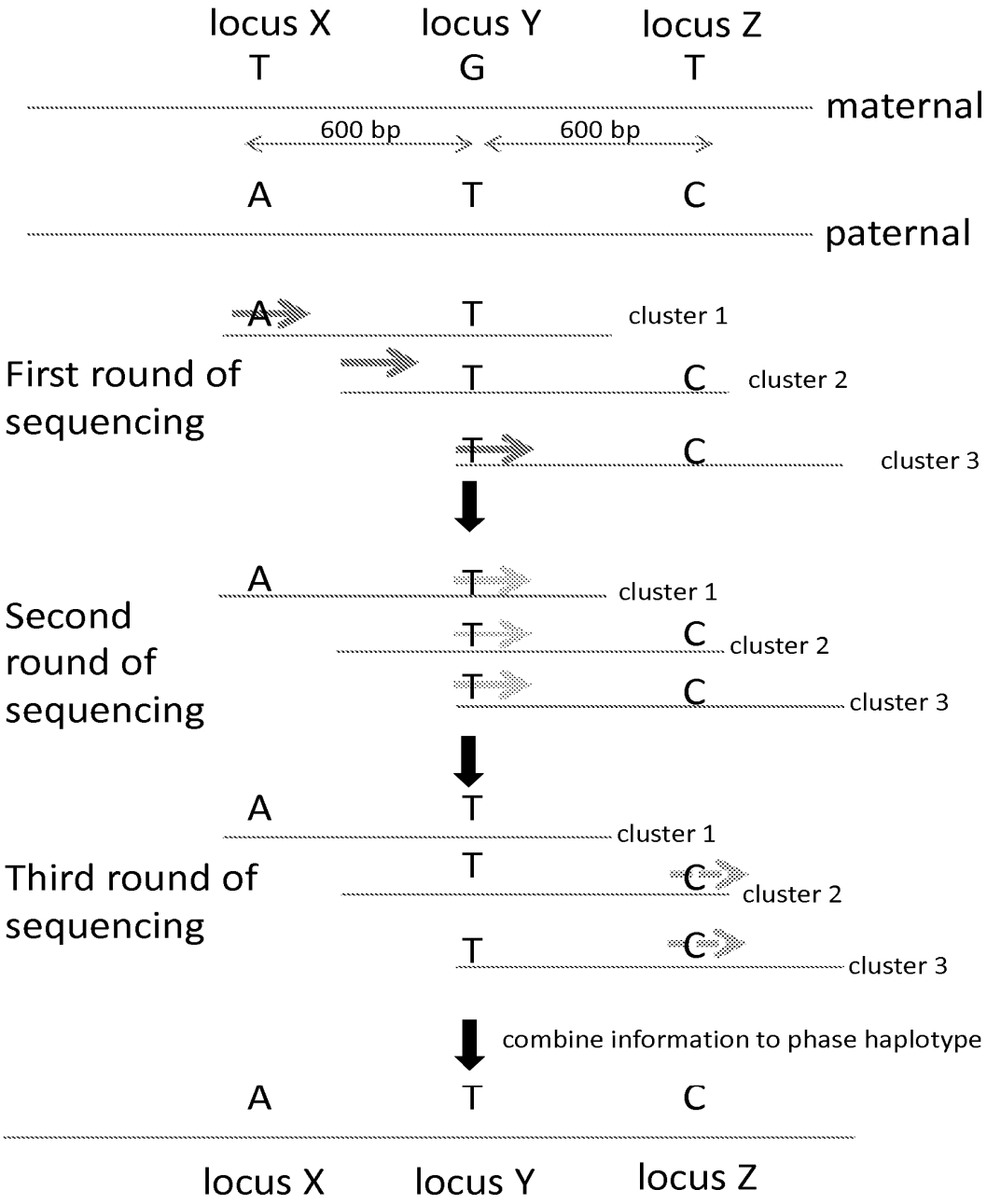

locus X
locus Y
locus Z
T
G
T
maternal
600 bp
600 bp
A
T
C
paternal
First round of sequencing
A
T
cluster 1
T
C
cluster 2
T
C
cluster 3
Second round of sequencing
A
T
cluster 1
T
C
cluster 2
T
C
cluster 3
Third round of sequencing
A
T
cluster 1
T
C
cluster 2
T
C
cluster 3
combine information to phase haplotype
A
T
C
locus X
locus Y
locus Z

FIG. 3

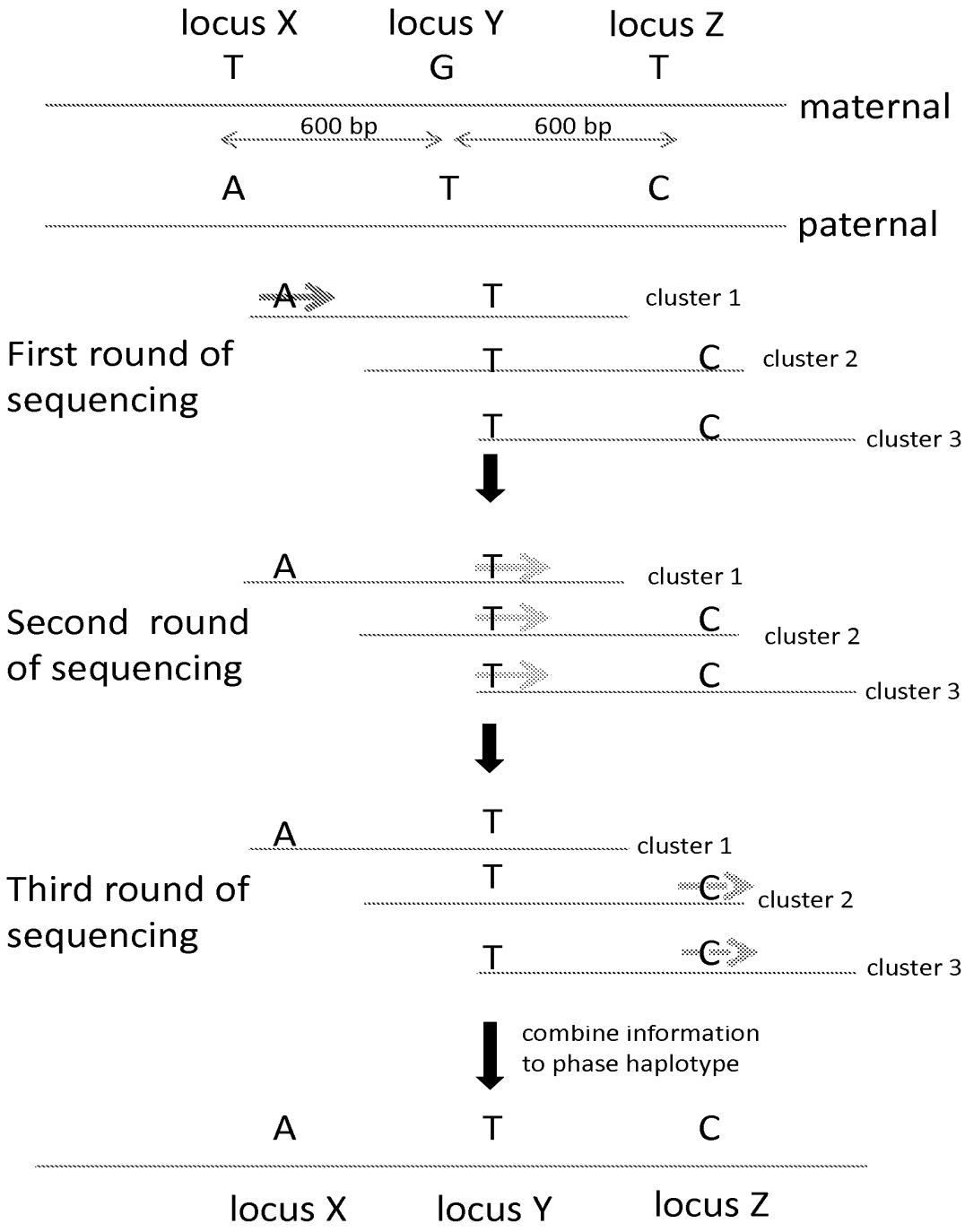

locus X
T
locus Y
G
locus Z
T
maternal
600 bp
600 bp
A
T
C
paternal
First round of sequencing
A
T
cluster 1
T
C
cluster 2
T
C
cluster 3
Second round of sequencing
A
T
cluster 1
T
C
cluster 2
T
C
cluster 3
Third round of sequencing
A
T
cluster 1
T
C
cluster 2
T
C
cluster 3
combine information to phase haplotype
A
T
C
locus X
locus Y
locus Z

FIG. 4

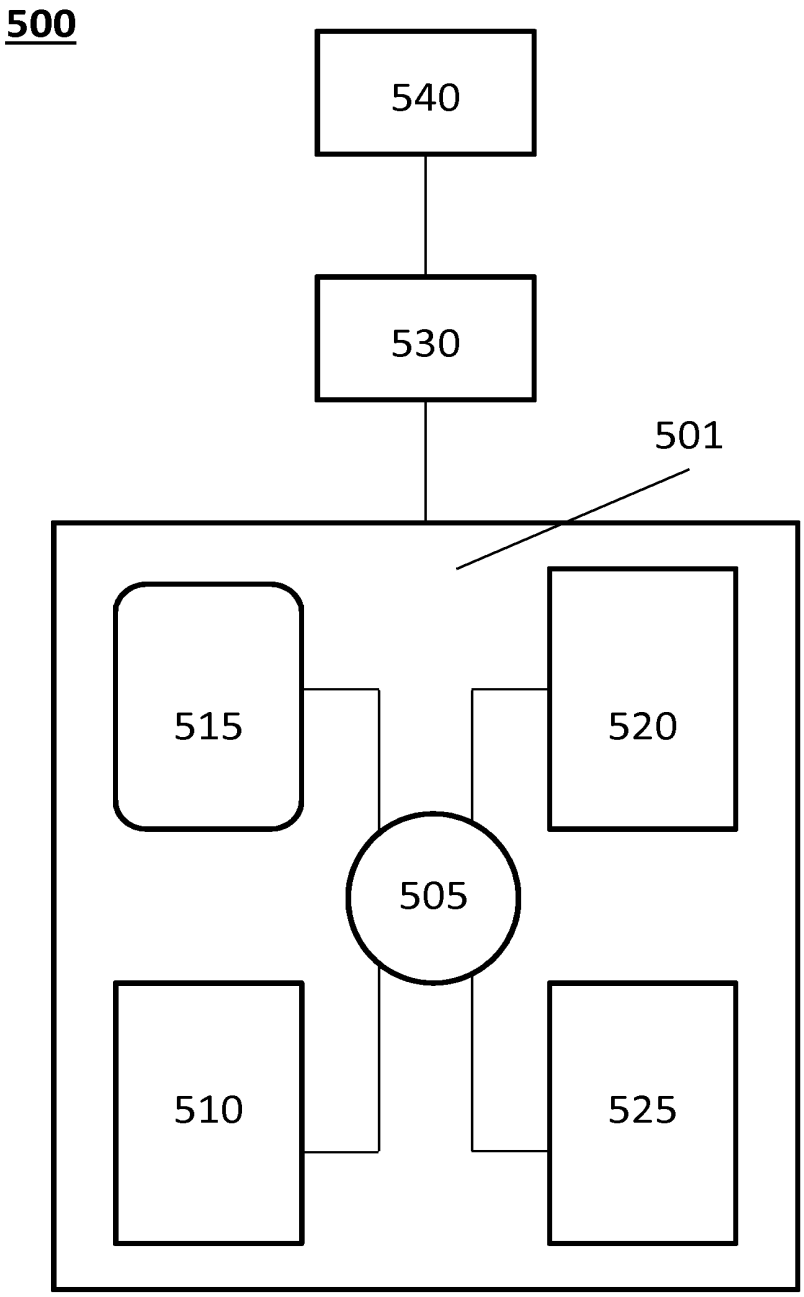


FIG. 5

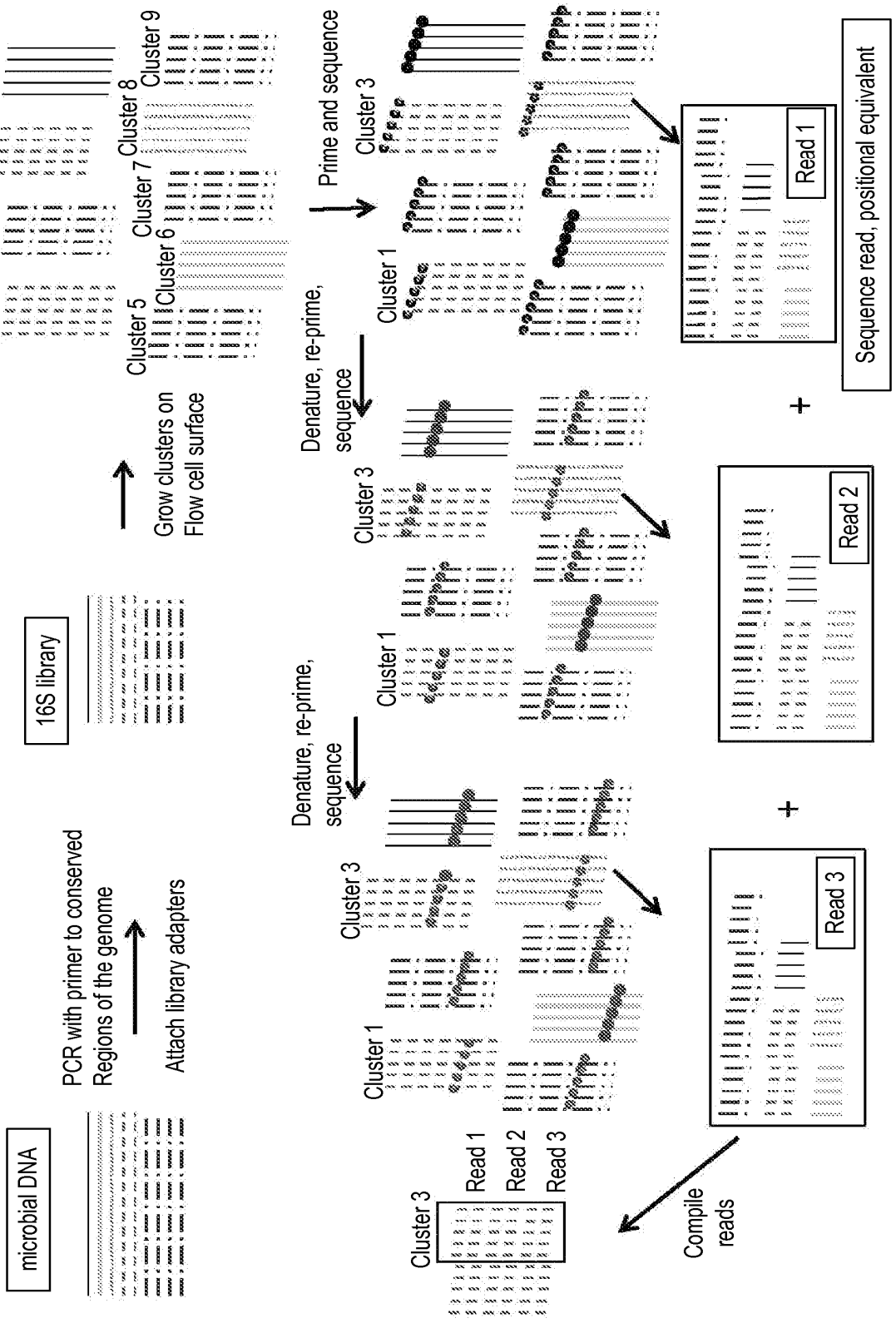

microbial DNA
16S library
PCR with primer to conserved Regions of the genome
Attach library adapters
Grow clusters on Flow cell surface
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Denature, re-prime, sequence
Prime and sequence
Read 1
Read 2
Read 3
Compile reads
Sequence read, positional equivalent

FIG. 6

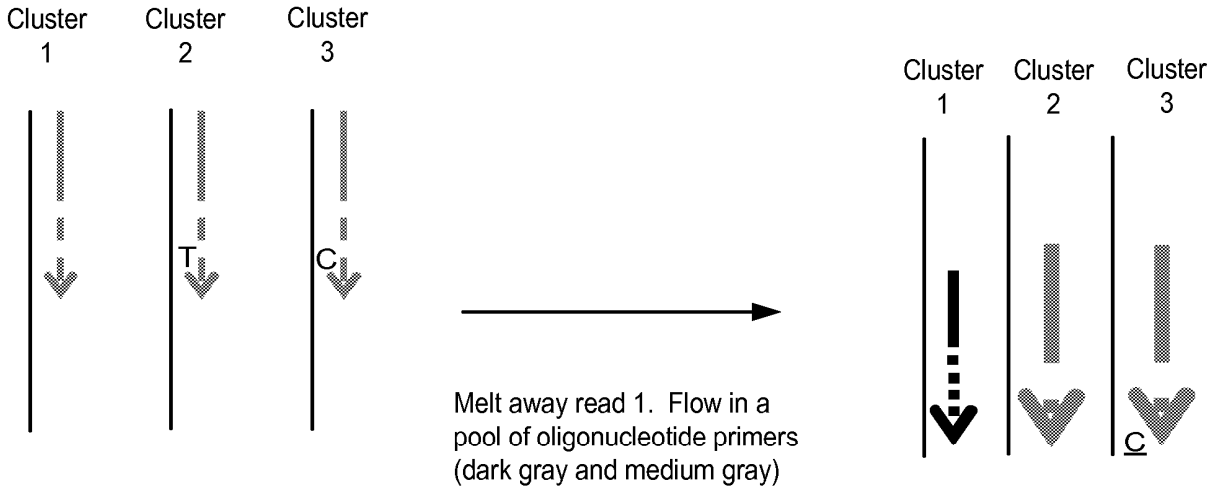


FIG. 7

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/028356

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2014.01)
USPC - 435/6.11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/68; C40B 20/02, 20/04, 20/06, 20/08, 30/00, 30/10, 40/06, 50/06 (2014.01)
USPC - 435/6.11, 6.12, 6.15; 506/4, 5, 6, 7, 16, 26; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 38/00; B01J 19/0046, 2219/00585, 2219/00596, 2219/00707, 2219/00722; C12Q 1/6827, 1/6883, 1/689 (2014.06)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2009/0298075 A1 (TRAVERS et al) 03 December 2009 (03.12.2009) entire document	1-36, 39-45, 47, 48 --- 37, 38
Y	US 2010/0105052 A1 (DRMANAC et al) 29 April 2010 (29.04.2010) entire document	37, 38
A	US 2012/0071331 A1 (CASBON et al) 22 March 2012 (22.03.2012) entire document	1-45, 47, 48
A	US 2011/0319290 A1 (RAYMOND et al) 29 December 2011 (29.12.2011) entire document	1-45, 47, 48
A	US 2011/0105364 A1 (KURN et al) 05 May 2011 (05.05.2011) entire document	1-45, 47, 48

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2014	15 JUL 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/028356

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 46
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

PTO/SB/08a (07-09)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	14/540,917
Filing Date	November 13, 2014
First Named Inventor	AMORESE, Douglas
Art Unit	1637
Examiner Name	STRZELECKA, Teresa E.
Attorney Docket Number	NUGN-001/01US 301735-2003

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	2012/0208705 A1	08-16-2012	STEEMERS et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] "Number[4] "Kind Code[5] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
	2.	WO 2008/093098 A2	08-07-2008	SOLEXA LIMITED		
	3.	WO 2012/061832 A1	05-10-2012	ILLUMINA, INC.		
	4.	WO 2014/144092 A1	09-18-2014	NUGEN TECHNOLOGIES, INC.		

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	5.	MYERS, R., "Protocol for Creating Multiplexed miRNA Libraries for Use in Illumina Sequencing", *Myers lab microRNA-seq Protocol, Hudson Alpha Institute for Biotechnology,* May 2, 2013, pages 1-15. Retrieved from the Internet:<https://www.encodeproject.org/documents/9fb8e06c-d145-447a-a606-e7f3dc44b549/@@download/attachment/HA_Myers_miRNA-seq_Protocol_2013-05-02.pdf> on February 28, 2015. entire document	
	6.	International Search Report and Written Opinion in international Application No. PCT/US2014/065530, mailed March 5, 2015, 14 pages.	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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(74) Agents: **WHITE, Nina, Louise** et al.; BOULT WADE TENNANT, Verulam Gardens, 70 Gray's Inn Road, London WC1X 8BT (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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(54) Title: METHODS FOR INDEXING SAMPLES AND SEQUENCING MULTIPLE NUCLEOTIDE TEMPLATES

(57) Abstract: The invention relates to methods for indexing samples during the sequencing of polynucleotide templates, resulting in the attachment of tags specific to the source of each nucleic acid sample such that after a sequencing run, both the source and sequence of each polynucleotide can be determined. Thus, the present invention pertains to analysis of complex genomes (e.g., human genomes), as well as multiplexing less complex genomes, such as those of bacteria, viruses, mitochondria, and the like.

Methods for indexing samples and sequencing multiple polynucleotide templates

Field of the invention

The invention relates to methods for indexing samples during the sequencing of polynucleotide templates, resulting in the attachment of tags specific to the source of each nucleic acid sample such that after a sequencing run, both the source and sequence of each polynucleotide can be determined. Thus, the present invention pertains to analysis of complex genomes (e.g., human genomes), as well as multiplexing less complex genomes, such as those of bacteria, viruses, mitochondria, and the like.

Background to the invention

Several publications and patent documents are referenced in this application in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications and documents is incorporated by reference herein.

Advances in the study of biological molecules have been led, in part, by improvement in technologies used to characterise the molecules or their biological reactions. In particular, the study of the nucleic acids DNA and RNA has benefited from developing technologies used for sequence analysis.

The study of complex genomes, in particular, the search for the genetic basis of disease in humans requires genotyping on a massive scale. Screens for numerous genetic markers performed on populations large enough to yield statistically significant data are needed before associations can be made between a given genotype and a

particular disease. However large-scale genotyping is demanding in terms of the cost of both materials and labour involved, and the time taken to perform the study, especially if the methodology employed involves separate serial analysis of individual DNA samples. One shortcut is to pool DNA from many individuals and to determine parameters such as the ratio of changes at certain positions in the genome. Such measurements of 'allele frequency' in the pool of samples can be used to correlate the relationship between the changes in the genome sequence and the occurrence of a disease. Hence, an association study involving 1000 patients would in theory only necessitate a 'one-pot' reaction for each genetic change. Pooling therefore represents an effective technique for analysing large quantities of samples in a facile manner.

One disadvantage of pooling samples prior to analysis is that information pertaining to individual DNA samples is lost; only global information such as allele frequencies is gathered, as there is no easy method for discerning which individuals gave rise to a particular genotype. An ability to genotype large populations in a small number of reactions, while retaining the information relating to the source of the individual samples, would yield the information content of a full non-pooled population screen in the time and at the cost of a pooled reaction.

Several of the new methods employed for high throughput DNA sequencing (*Nature*. 437, 376-380 (2005); *Science*. 309, 5741, 1728-1732 (2005)) rely on a universal amplification reaction, whereby a DNA sample is randomly fragmented, then treated such that the ends of the different fragments all contain the same DNA sequence. Fragments with universal ends can be amplified in a single reaction with a single pair of

amplification primers. Separation of the library of fragments to the single molecule level prior to amplification ensures that the amplified molecules form discrete populations that can then be further analysed. Such separations can be performed either in emulsions (*Nature*. 437, 376-380 (2005); *Science*. 309, 5741, 1728-1732 (2005)), or on a surface (Nucleic Acids Research 27, e34 (1999); Nucleic Acids Research 15, e87 (2000)).

WO 98/44151 and WO 00/18957 both describe methods of forming polynucleotide arrays based on 'solid-phase' nucleic acid amplification, which is a bridging amplification reaction wherein the amplification products are immobilised on a solid support in order to form arrays comprised of nucleic acid clusters or 'colonies'. Each cluster or colony on such an array is formed from a plurality of identical immobilised polynucleotide strands and a plurality of identical immobilised complementary polynucleotide strands. The arrays so-formed are generally referred to herein as 'clustered arrays' and their general features will be further understood by reference to WO 98/44151 or WO 00/18957, the contents of both documents being incorporated herein in their entirety by reference.

In common with all amplification techniques, solid-phase bridging amplification requires the use of forward and reverse amplification primers which include 'template-specific' nucleotide sequences which are capable of annealing to sequences in the template to be amplified, or the complement thereof, under the conditions of the annealing steps of the amplification reaction. The sequences in the template to which the primers anneal under conditions of the amplification reaction may be referred to herein as 'primer-binding' sequences.

Certain embodiments of the methods described in WO 98/44151 and WO 00/18957 make use of 'universal' primers to amplify templates comprising a variable template portion that it is desired to amplify flanked 5' and 3' by common or 'universal' primer binding sequences. The 'universal' forward and reverse primers include sequences capable of annealing to the 'universal' primer binding sequences in the template construct. The variable template portion, or 'target' may itself be of known, unknown or partially known sequence. This approach has the advantage that it is not necessary to design a specific pair of primers for each target sequence to be amplified; the same primers can be used for amplification of different templates provided that each template is modified by addition of the same universal primer-binding sequences to its 5' and 3' ends. The variable target sequence can therefore be any DNA fragment of interest. An analogous approach can be used to amplify a mixture of templates (targets with known ends), such as a plurality or library of target nucleic acid molecules (e.g. genomic DNA fragments), using a single pair of universal forward and reverse primers, provided that each template molecule in the mixture is modified by the addition of the same universal primer-binding sequences.

DNA from more than one source can be sequenced on an array if each DNA sample is first tagged to enable its identification after it has been sequenced. Many low scale DNA-tag methodologies already exist, for example fluorescent labelling (Haughland, Handbook of Fluorescent Probes and Research Products, Invitrogen/Molecular Probes), but these are limited in scope to less than 10 or so reactions in parallel. DNA tags can be added to the ends of DNA fragments by cloning, of example as described in US 5604097. The tags

consist of eight four base 'words', where each word uses only three bases (A,T and C) in various combinations resulting in a total of 16,777,216 different tags that all have the same base pair composition and melting points. Such tags are used to label target molecules in a sample so that after an amplification reaction, each original molecule in the sample has a unique tag. The tags can then be used to 'sort' the sample onto beads containing sequences complementary to the tags such that each bead contains multiple copies of a single amplified target sequence (Brenner et al., (2000) Nature Biotechnology, 18, 630). In this application the tags are not sequenced, so the method does not provide a method of analysing targets from multiple samples, but rather a method of sorting a mixture of amplified templates from a single sample. The problem with enabling the method for individual samples rather than individual molecules is that the tags are synthesised in a combinatorial manner, meaning that all 16,777,216 different sequences are obtained in a single mixture in the same tube. Whilst this is ideal for treating one sample such that each individual molecule in the sample carries a different tag, it does not permit attachment of the same tag to every molecule in the sample.

DNA samples from multiple sources can, however, be tagged with different nucleic acid tags such that the source of the sample can be identified. Previous application WO05068656 describes the generic concept of indexing samples. In order to utilise this invention on arrays of amplified single molecule templates, for example as described in WO9844151, WO06099579 or WO04069849, it is advantageous to prepare the nucleic acids using the novel method described herein. The optimised DNA sample

preparation techniques described herein are applicable to any method where the samples are amplified prior to sequencing. The DNA sample preparation techniques presented herein describe in detail the optimal placements of the sequencing primers and indexed tags within the DNA constructs to be sequenced.

Summary of the invention

The present inventors have developed methods for indexing samples, wherein the samples are amplified from isolated single template molecules. Using the techniques of the invention, it is possible to prepare a nucleic acid sample for a sequencing reaction wherein both the target sequence and the tag sequence can be determined.

In a first embodiment of the invention, a method is presented for sequencing nucleic acid sequences on an array and identifying subsets of nucleic acid sequences on an array, wherein each subset of nucleic acid sequences is isolated from a different source, wherein the method comprises the steps of:

(a) providing at least two samples of randomly fragmented double stranded nucleic acid targets, wherein each of said randomly fragmented double stranded nucleic acid targets is isolated from a different source;

(b) ligating a universal adaptor to the ends of each target fragment of each sample to generate adaptor-target-adaptors of each sample, wherein each of said adaptor-target-adaptors comprises a target fragment flanked by universal adaptor sequences and said universal adaptor comprises a region of double stranded nucleic acid and at least one region of single stranded nucleic acid;

(c) amplifying adaptor-target-adaptors of each sample with two or more sample specific amplification primers to generate amplified nucleic acids, wherein one of said amplification primers comprises a sample specific tag sequence, and wherein amplified nucleic acids of each sample comprise said sample specific tag sequence and said sample specific tag sequence differentiates amplified nucleic acids originating from different samples;

(d) pooling the amplified nucleic acids of different samples;

(e) immobilising the amplified nucleic acids of different samples on an array to generate an array of immobilised fragments;

(f) sequencing the immobilised fragments on the array to determine a sequence read of each immobilised target fragment and identify the sample specific tag sequence of each immobilised fragment, thereby determining both a nucleic acid sequence of said immobilized fragment and identifying the immobilized fragment as a member of a subset of nucleic acids on the array.

In a second embodiment of the invention, a method is presented for sequencing nucleic acid sequences on an array and identifying subsets of nucleic acid sequences on an array, wherein each subset of nucleic acid sequences is isolated from a different source, wherein the method comprises the steps of:

(a) providing at least two samples of randomly fragmented double stranded nucleic acid targets, wherein each of said randomly fragmented double stranded nucleic acid targets is isolated from a different source;

(b) ligating a sample specific tagged adaptor to the ends of each target fragment of each sample to generate adaptor-

target-adaptors of each sample, wherein each of said adaptor-target-adaptors comprises a target fragment flanked by tagged adaptor sequences and said tagged adaptor comprises a region of double stranded nucleic acid and at least one region of single stranded nucleic acid and a sample specific tag that differentiates adaptor-target-adaptors originating from different samples;

(c) pooling the adaptor-target-adaptors of different samples to generate a pooled sample of adaptor-target-adaptors;

(d) amplifying the pooled sample of adaptor-target-adaptors with a pair of universal amplification primers to generate an amplified pooled sample;

(e) immobilising the amplified pooled sample on an array to generate an array of immobilised fragments;

(f) sequencing the array to determine a sequence read of each immobilised target fragment and identify the sample specific tag sequence of each immobilised fragment, thereby determining both a nucleic acid sequence of said immobilized fragment and identifying the immobilized fragment as a member of a subset of nucleic acids on the array.

In a third embodiment of the invention, a method is presented for sequencing nucleic acid sequences on an array and identifying subsets of nucleic acid sequences on an array, wherein each subset of nucleic acid sequences is isolated from a different source, wherein the method comprises the steps of:

(a) providing at least two samples of randomly fragmented double stranded nucleic acid targets, wherein each of said randomly fragmented double stranded nucleic acid targets is isolated from a different source;

(b) ligating a sample specific tagged adaptor to the ends of each target fragment of each sample to generate adaptor-target-adaptors of each sample, wherein each of said adaptor-target-adaptors comprises a target fragment flanked by tagged adaptor sequences and said tagged adaptor comprises a region of double stranded nucleic acid and at least one region of single stranded nucleic acid and a sample specific tag that differentiates adaptor-target-adaptors originating from different samples, and wherein the sample specific tag sequence is attached directly to the target fragment in each adaptor-target-adaptor with no intervening nucleotides between the sample specific tag sequence and target fragment sequences;

(c) amplifying adaptor-target-adaptors of each sample with a pair of amplification primers complementary to the sample specific tag sequence to generate amplified nucleic acids from each sample;

(d) pooling the amplified nucleic acids from each sample to generate a pool of amplified nucleic acid samples;

(e) immobilising the pool of amplified nucleic acid samples on an array to generate an array of immobilised fragments;

(f) sequencing the array to determine a sequence read of each immobilised target fragment and identify the sample specific tag sequence of each immobilised fragment, thereby determining both a nucleic acid sequence of said immobilized fragment and identifying the immobilized fragment as a member of a subset of nucleic acids on the array.

In accordance with the first or second embodiment of the invention, sequencing can be performed in two separate reads. In accordance with the third embodiment of the invention, sequencing of the target fragment and the sample

specific tag sequence can be performed in a single sequencing read.

With respect to the first, second, or third embodiments, the method encompasses a step wherein the pooled samples are amplified on a solid surface prior to sequencing. In a particular aspect, the solid surface on which amplification is performed is a collection of beads. In a more particular aspect, the collection of beads may be isolated as single beads in an emulsion.

With respect to the first, second, or third embodiments, the method encompasses a step wherein the array of immobilized fragments is amplified prior to sequencing to generate an amplified array. In a particular aspect, an amplified array so produced is a clustered array of amplified single molecules. Such clustered arrays may be formed, for example, by solid-phase nucleic acid amplification or isothermal solid-phase nucleic acid amplification.

The present method encompasses sequencing that involves cycles of ligation with labelled oligonucleotides or cycles of addition of nucleotides. In a particular aspect of the invention, the nucleotides are labelled. In a more particular aspect the nucleotides are labelled with fluorophores.

In an aspect of any of the first, second, or third embodiments of the invention, the sequencing step may be carried out on an array of immobilised beads.

A further aspect of the invention comprises a kit comprising a double stranded nucleic acid adapter and at least two different primer polynucleotides that carry a different tag sequence that does not hybridise to the adapter.

Brief description of the figures

Figure 1 shows a schematic of an indexed sample construct using the first method of the invention, using universal adaptors. Common adaptors are used for all tags, tags are added using one unique amplification primer and sequencing is carried out in two reads.

Figure 2 shows a schematic of an indexed sample construct using the second method of the invention, using tagged adaptors. Tagged adaptors are used for each sample and amplified with one pair of universal primers. Sequencing is carried out in two reads.

Figure 3 shows a schematic of an indexed sample construct using the third method of the invention, using adjacent tag-targets. Two adaptors and two amplification primers are used per tag and sequencing carried out in a single read.

Figure 4 shows numerical data on two sequencing reads obtained from a sample prepared according to the first method of the invention, amplified into an array of clusters and sequenced with two sequencing reads, one for the target and one for the tag.

Figure 5 shows the concept of tagging a sample for use in a paired end reading process. Libraries are amplified at the PCR step using a nested PCR reaction with 3 primers (2 universal and 1 tag oligo.

Figure 6a shows a nested PCR reaction using three amplification primers, one of which carries the tag.

Figure 6b shows the same nested PCR concept as figure 6, but with the benefit of the sequences of the various adapters and primers.

Figure 7 shows the embodiment of the method where the first sequencing read is performed, followed by removing the first sequencing primer, hybridising a second sequencing primer to determine the tag sequence, inverting the strand on the surface using a strand resynthesis protocol, and performing a second read at the opposite end of the fragment to the first read to generate a pair of reads from each fragment along with the tag read.

Figure 8 shows some exemplary data obtained using the method shown in figure 7.

Figure 9 shows the sequence of the various oligonucleotides used in the process

Figure 10 shows a schematic of MJ Research thermocycler, coupled with an 8-way peristaltic pump Ismatec IPC ISM931 equipped with Ismatec tubing (orange/yellow, 0.51 mm ID).

Detailed description of the invention

The present invention is directed to methods that advance techniques for nucleic acid analysis, such that target nucleic acids from a plurality of sources can be tagged with a unique, identifiable sequence of nucleic acid bases and then sequenced on a single array. As described

herein, the presence of a characteristic nucleic acid sequence tag on an immobilised target molecule permits identification of the source of the target, concurrent with a sequence read from the target. This is a dramatic improvement over pre-existing array technologies which generally require a two-step process involving an initial sequencing step for sequencing the pooled nucleic acid targets, followed by a second analysis step wherein the source of the nucleic acid is determined.

The limitations of such pre-existing array technologies are evident in WO 98/44151 and WO 00/18957, for example, which both describe methods of nucleic acid amplification which allow amplification products to be immobilised on a solid support in order to form arrays comprised of clusters or "colonies" formed from a plurality of identical immobilised polynucleotide strands and a plurality of identical immobilised complementary strands. The nucleic acid molecules present in DNA colonies on the clustered arrays prepared according to these methods can provide templates for sequencing reactions, but to date only a single sequencing read can be obtained from each immobilised strand in each colony. The methods described herein allow for two or more reads on a single amplified strand, thereby allowing analysis of the tag sequence independently of the target sequence, and also allowing the possibility of a paired read from the opposite end of each fragment as well as a third read for the tag sequence. The methods also allow the preparation of constructs where the tag sequence is adjacent to the target sequence, and the tag and target are read in a single read, which again can be turned into a paired read methodology if desired.

As will be apparent to the skilled reader, references herein to a particular nucleic acid sequence may, depending on the context, also refer to nucleic acid molecules which comprise the nucleic acid sequence. Sequencing of a target fragment means that a read of the chronological order of bases is established. The bases do not need to be contiguous, although this is preferred, nor does every base on the entire fragment have to be sequenced.

The following passages describe different aspects of the invention in greater detail. Each aspect of the invention may be combined with any other aspect or aspects of the invention unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature of features indicated as being preferred or advantageous.

The terms 'target nucleic acid sequence', 'target nucleic acid molecule', 'target nucleic acid' and 'target nucleic acid fragment' may be used interchangeably to refer to nucleic acid molecules that it is desired to sequence on an array according to the invention. The target nucleic acid may be essentially any nucleic acid of known or unknown sequence. It may be, for example, a fragment of genomic DNA or cDNA. Sequencing may result in determination of the sequence of the whole, or a part of the target molecule. The targets can be derived from a primary nucleic acid sample that has been randomly fragmented. The targets can be processed into templates suitable for amplification by the placement of universal amplification sequences at the ends of each target fragment. The targets can also be obtained from a primary RNA sample by reverse transcription into cDNA.