ABSTRACT OF THE DISCLOSURE

Disclosed herein in are methods and systems for determining genetic variants (e.g., copy number variation) in a polynucleotide sample. A method for determining copy number variations includes tagging double-stranded polynucleotides with duplex tags, sequencing polynucleotides from the sample and estimating total number of polynucleotides mapping to selected genetic loci. The estimate of total number of polynucleotides can involve estimating the number of double-stranded polynucleotides in the original sample for which no sequence reads are generated. This number can be generated using the number of polynucleotides for which reads for both complementary strands are detected and reads for which only one of the two complementary strands is detected.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	42534-708.306
	Application Number	
Title of Invention	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	

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
	AmirAli		TALASAZ	

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

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

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Inventor 3 Remove

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Prefix	Given Name	Middle Name	Family Name	Suffix
	Stefanie	Ann Ward	MORTIMER	

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

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City	Morgan Hill	State/Province	CA	Country of Residence	US

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Address 1	2000 Willow Springs Road		
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City	Morgan Hill	**State/Province**	CA
Postal Code	95037	**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	115823		
Email Address	patents@guardanthealth.com	Add Email	Remove Email
Email Address	patentdocket@wsgr.com	Add Email	Remove Email

Application Information:

Title of the Invention	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		
Attorney Docket Number	42534-708.306	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	11	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete 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

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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.

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	115823		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim 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" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	16601168	2019-10-14
Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
16601168	Continuation of	15892178	2018-02-08
Prior Application Status	Patented		Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
15892178	Continuation of	14861989	2015-09-22	9920366	2018-03-20

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
14861989	a 371 of international	PCTUS2014072383	2014-12-24

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Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2014072383	Claims benefit of provisional	61948509	2014-03-05
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2014072383	Claims benefit of provisional	61921456	2013-12-28
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.			Add

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. 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(i)(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)
Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.			Add

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.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	42534-708.306
	Application Number	
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Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is 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 State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	42534-708.306
	Application Number	
Title of Invention	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	

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.

Applicant 1 — Remove

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	GUARDANT HEALTH, INC.		
Mailing Address Information For Applicant:			
Address 1	505 Penobscot Drive		
Address 2			
City	Redwood City	**State/Province**	CA
Country	US	Postal Code	94063
Phone Number		Fax Number	
Email Address	patents@guardanthealth.com		

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

Assignee Information including Non-Applicant Assignee 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	42534-708.306
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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.

Remove

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

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 i		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. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Timothy A. Hott/			Date (YYYY-MM-DD)	2020-07-31
First Name	Timothy	Last Name	Hott	Registration Number	67740

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	42534-708.306
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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.**

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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 records.

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 CooperationTreaty.

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.

CLAIMS

WHAT IS CLAIMED IS:

1. A method, comprising:

(a) providing a population of cell-free deoxyribonucleic acid (cfDNA) molecules having first and second complementary strands;

(b) tagging a plurality of the cfDNA molecules in the population with duplex tags comprising molecular barcodes from a set of molecular barcodes to produce tagged parent polynucleotides, wherein the duplex tags are attached at both ends of a molecule of the cfDNA molecules;

(c) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(d) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(e) reducing and/or tracking redundancy in a plurality of sequence reads from the set of sequence reads to determine distinct cfDNA molecules from among the tagged parent polynucleotides, wherein the distinct cfDNA molecules are determined based on (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides and/or (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides.

2. The method of claim 1, wherein the sample is obtained from a subject having cancer.

3. The method of claim 1, wherein the plurality of cfDNA molecules comprises 1 nanogram (ng) to 100 ng of cfDNA molecules.

4. The method of claim 1, wherein the molecular barcodes are ligated to the cfDNA molecules using more than a 10X molar excess of duplex tags as compared to the cfDNA molecules.

5. The method of claim 1, wherein the molecular barcodes are ligated to the cfDNA molecules using more than an 80X molar excess of duplex tags as compared to the cfDNA molecules.

6. The method of claim 5, wherein at least 40% of the cfDNA molecules from the population are tagged with the duplex tags.

7. The method of claim 1, wherein tagging comprises non-uniquely tagging the plurality of the cfDNA molecules, wherein the cfDNA molecules that map to a mappable base position of a reference sequence are tagged with a number of different molecular barcodes ranging from at least 2 to fewer than a number of cfDNA molecules that map to the mappable base position.

8. The method of claim 1, wherein the molecular barcodes in the set of molecular barcodes have 5 to 10,000 different molecular barcode sequences and are 5 to 20 base pairs in length.

9. The method of claim 1, further comprising enriching the amplified progeny polynucleotides for target regions of interest prior to sequencing.

10. The method of claim 9, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

11. The method of claim 10, further comprising amplifying a plurality of enriched progeny polynucleotides prior to sequencing.

12. The method of claim 1, wherein the duplex tags are part of sequencing adapters.

13. The method of claim 12, wherein the sequencing adapters are Y-shaped adapters.

14. The method of claim 1, wherein reducing and/or tracking redundancy in the set of sequence reads comprises mapping a plurality of the sequence reads to a reference sequence.

15. The method of claim 14, further comprising:

(f) determining quantitative measures of (i) paired reads and (ii) unpaired reads that map to a locus of a reference sequence.

16. The method of claim 15, further comprising:

(g) estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides that map to the locus based on the quantitative measures of the paired reads and the unpaired reads.

17. The method of claim 16, further comprising:

(h) detecting copy number variation in the sample by determining a normalized quantitative measure determined in step (g) at each of one or more loci.

18. A method, comprising:

(a) tagging a population of double-stranded cell-free deoxyribonucleic acid (cfDNA) molecules from a sample of a subject with a set of tags comprising molecular barcodes to generate tagged parent polynucleotides;

(b) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(c) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(d) sorting a plurality of sequence reads from the set of sequence reads into (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides, and (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides.

19. The method of claim 18, wherein the sample is blood, plasma, or serum.

20. The method of claim 18, wherein the plurality of double-stranded cfDNA molecules comprises 1 nanogram (ng) to 100 ng of double-stranded cfDNA molecules.

21. The method of claim 18, wherein the tagging is performed by ligating the molecular barcodes to double-stranded cfDNA molecules.

22. The method of claim 18, wherein the molecular barcodes in the set have 2 to 10,000 different molecular barcode sequences. from a set of molecular barcodes having a plurality of different molecular barcode sequences

23. The method of claim 18, further comprising enriching the amplified progeny polynucleotides for target regions of interest prior to sequencing.

24. The method of claim 23, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

25. The method of claim 18, further comprising, prior to (d), reducing and/or tracking redundancy for a plurality of sequence reads that map to the reference sequence.

26. The method of claim 25, wherein reducing and/or tracking redundancy comprises mapping a plurality of the sequence reads to a reference sequence.

27. The method of claim 26, wherein reducing and/or tracking redundancy comprises making a base call at one or more genomic loci for a plurality of sequence reads that map to the one or more loci on the reference sequence.

28. The method of claim 26, wherein reducing and/or tracking redundancy comprises collapsing the plurality of the sequence reads to generate consensus sequences representative of original double-stranded cfDNA molecules from among the tagged parent polynucleotides.

29. The method of claim 18, further comprising determining quantitative measures of at least two of (i) paired reads, (ii) unpaired reads, (iii) read depth of the paired reads and (iv) read depth of the unpaired reads.

30. The method of claim 29, further comprising estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides mapping to a locus of a reference sequence based on the quantitative measures of at least two of (i) paired reads, (ii)

unpaired reads, (iii) read depth of the paired reads and (iv) read depth of the unpaired reads at the locus.

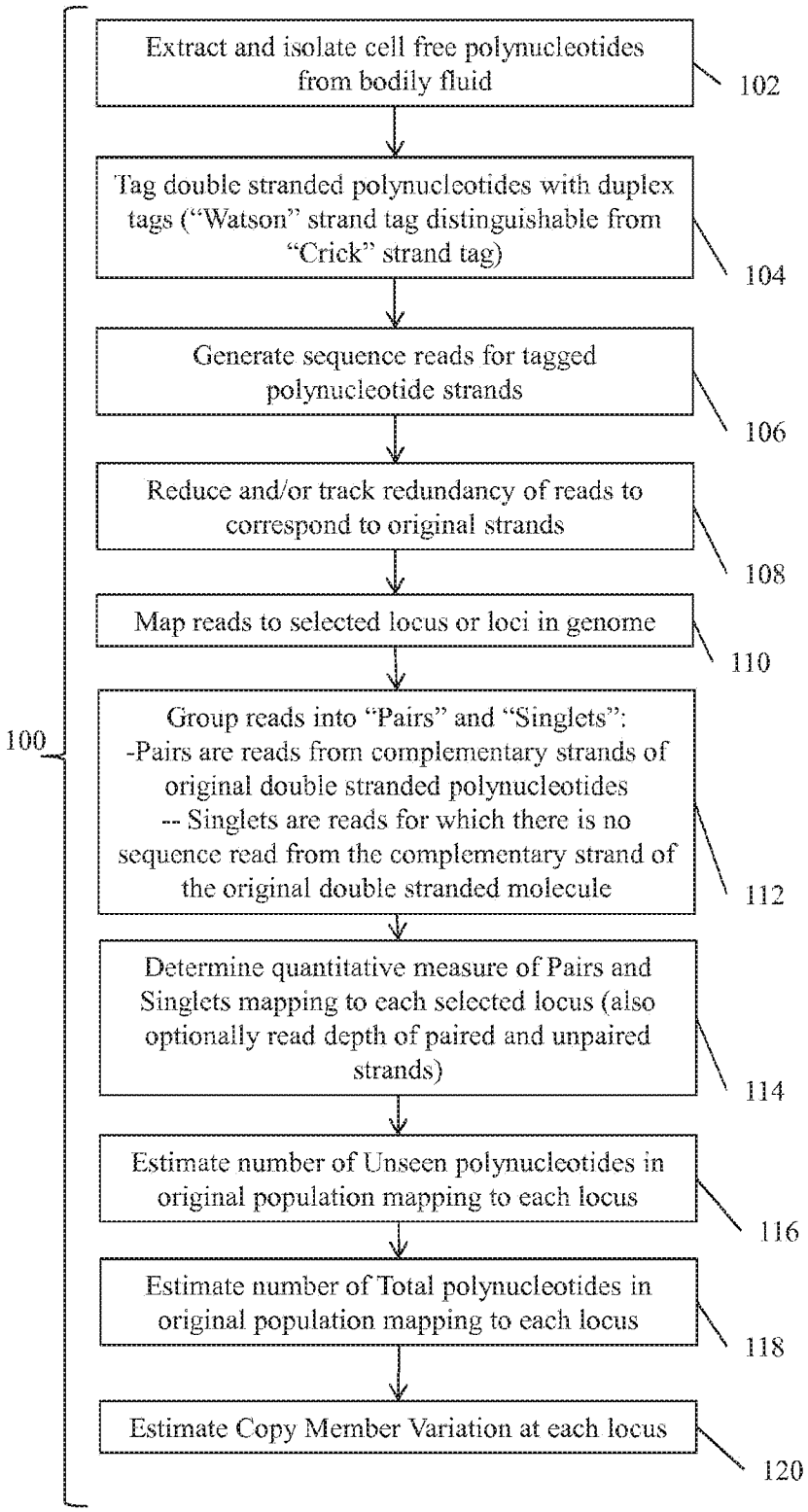

Extract and isolate cell free polynucleotides from bodily fluid
102
Tag double stranded polynucleotides with duplex tags ("Watson" strand tag distinguishable from "Crick" strand tag)
104
Generate sequence reads for tagged polynucleotide strands
106
Reduce and/or track redundancy of reads to correspond to original strands
108
Map reads to selected locus or loci in genome
110
Group reads into "Pairs" and "Singlets":
-Pairs are reads from complementary strands of original double stranded polynucleotides
-- Singlets are reads for which there is no sequence read from the complementary strand of the original double stranded molecule
112
Determine quantitative measure of Pairs and Singlets mapping to each selected locus (also optionally read depth of paired and unpaired strands)
114
Estimate number of Unseen polynucleotides in original population mapping to each locus
116
Estimate number of Total polynucleotides in original population mapping to each locus
118
Estimate Copy Member Variation at each locus
120
100

Fig. 1

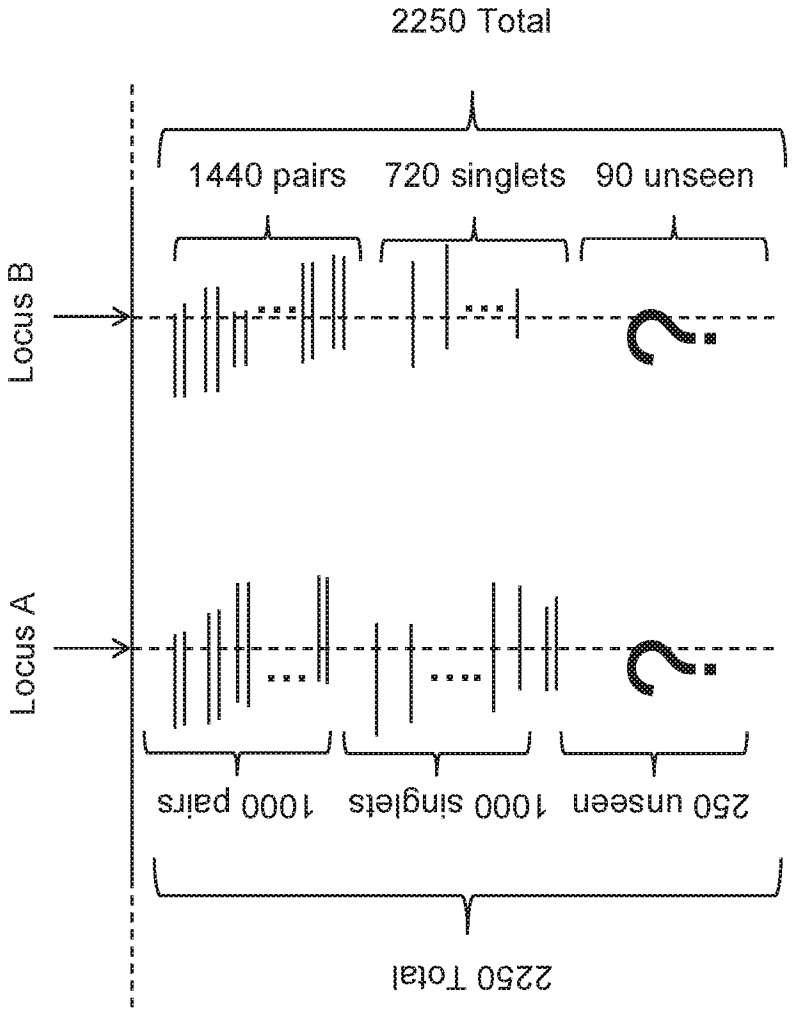

2250 Total
1440 pairs
720 singlets
90 unseen
Locus B
Locus A
1000 pairs
1000 singlets
250 unseen
2250 Total

Fig. 2

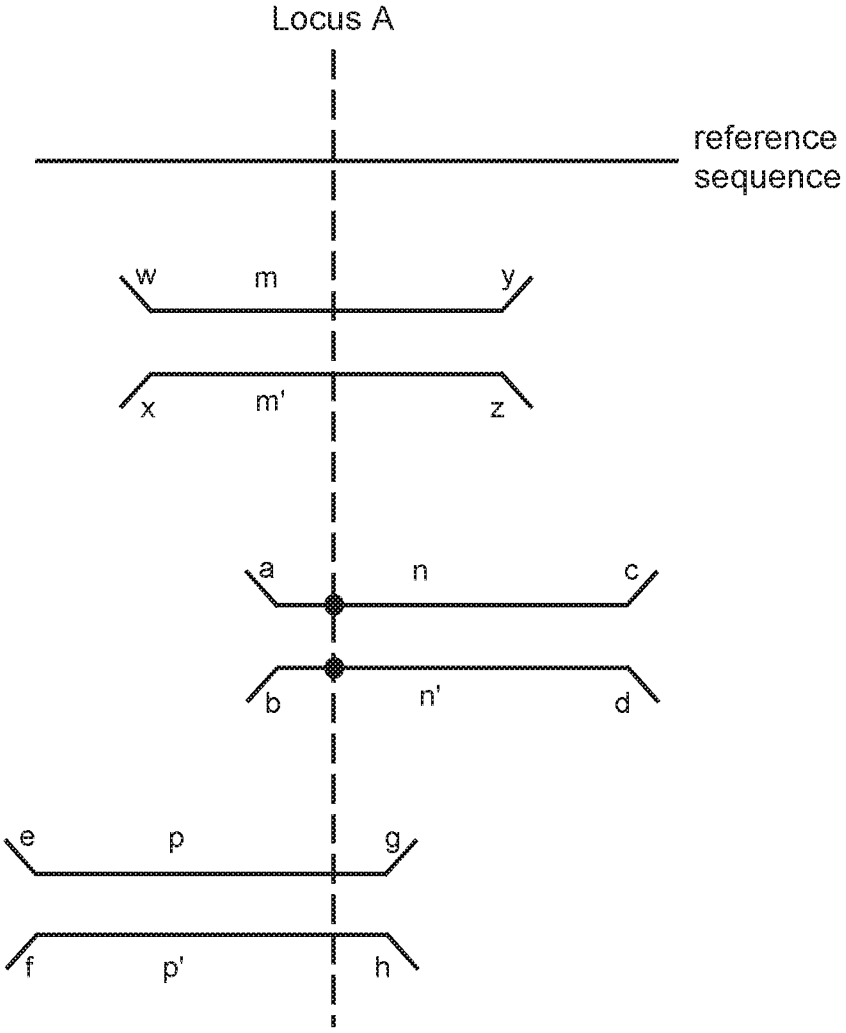


Fig. 3

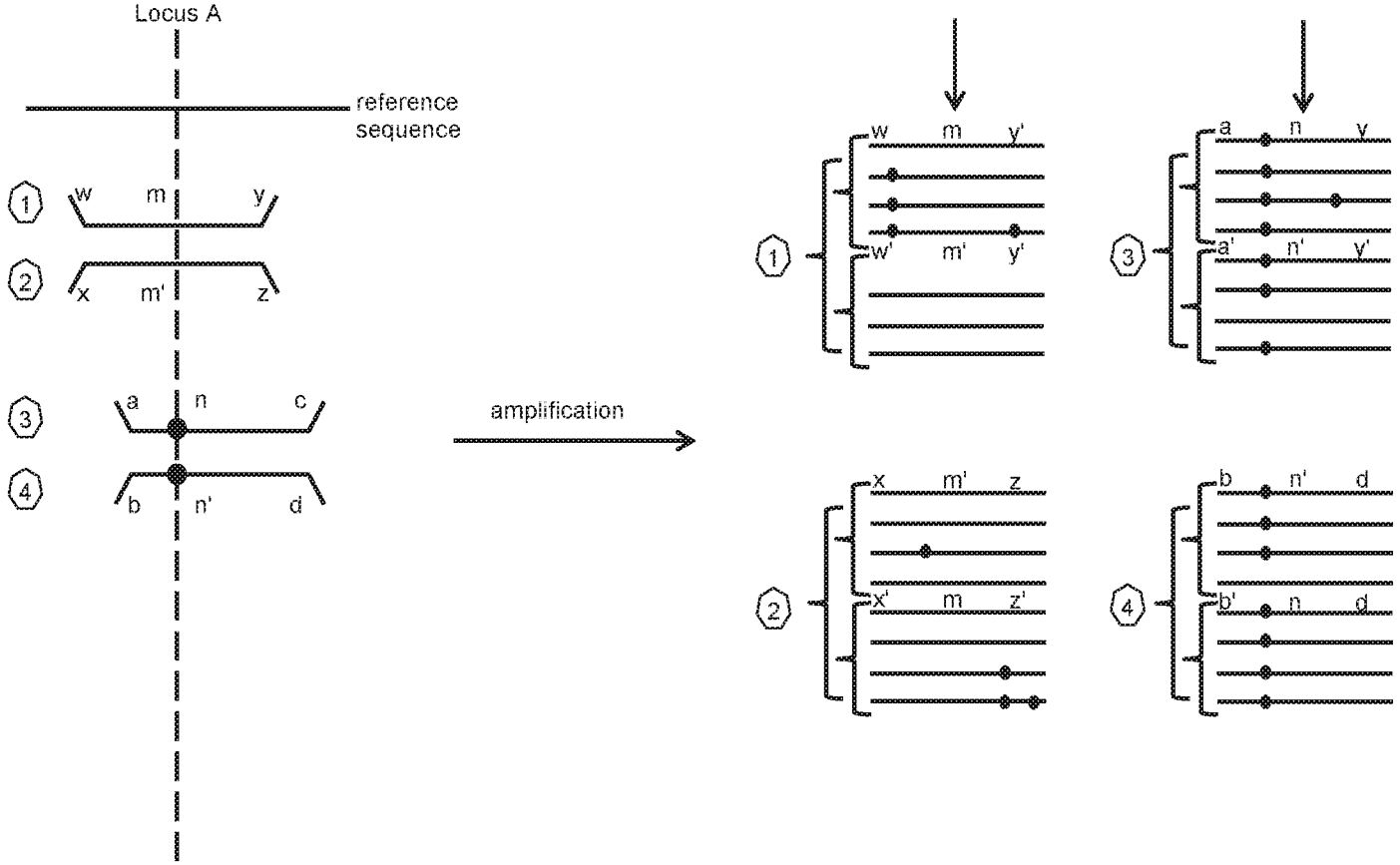

Locus A
reference sequence
1
w m y
2
x m' z
3
a n c
4
b n' d
amplification
w m y'
w' m' y'
a n y
a' n' y'
x m' z
x' m z'
b n' d
b' n d

Fig. 4A

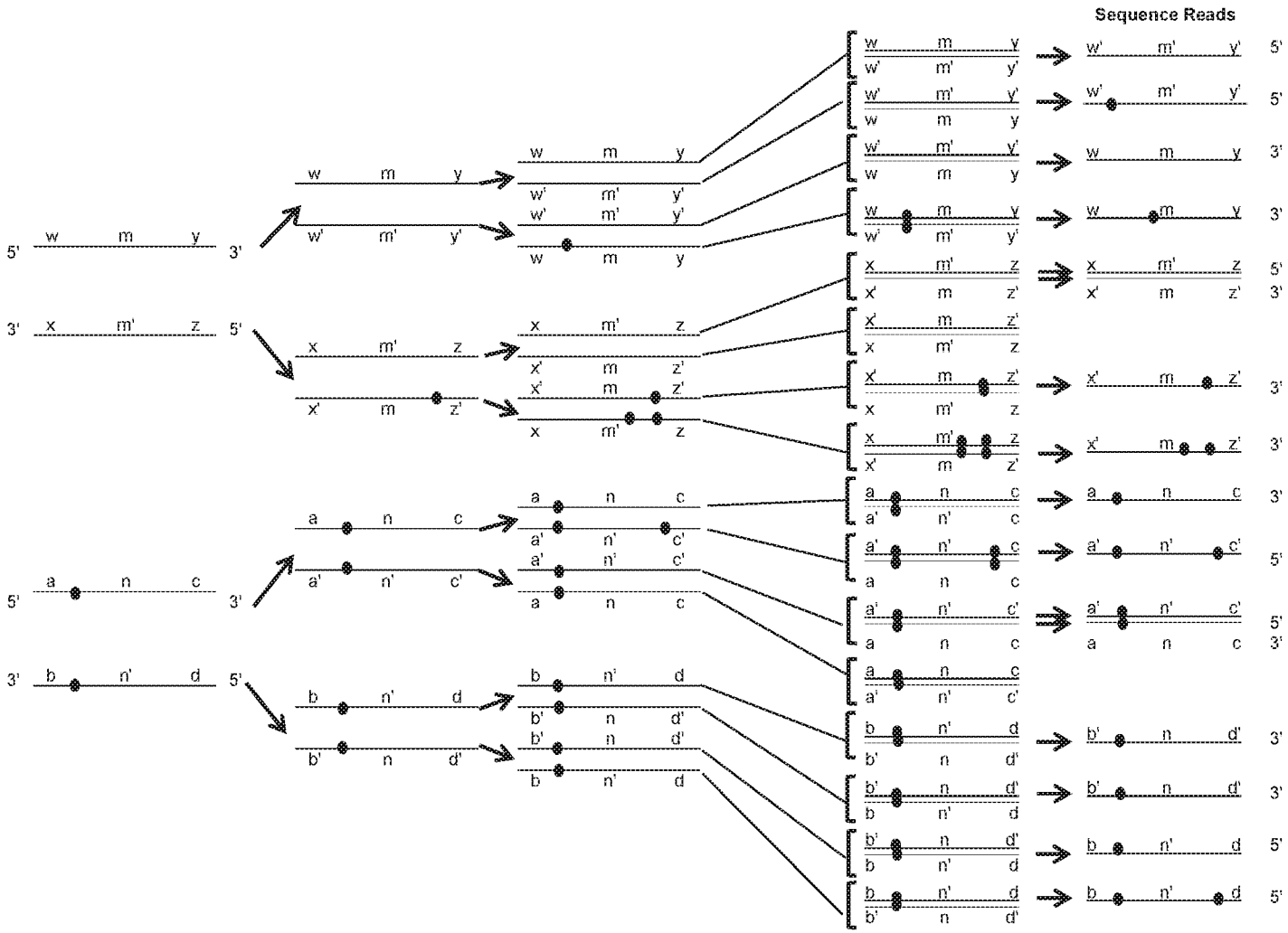


Fig. 4B

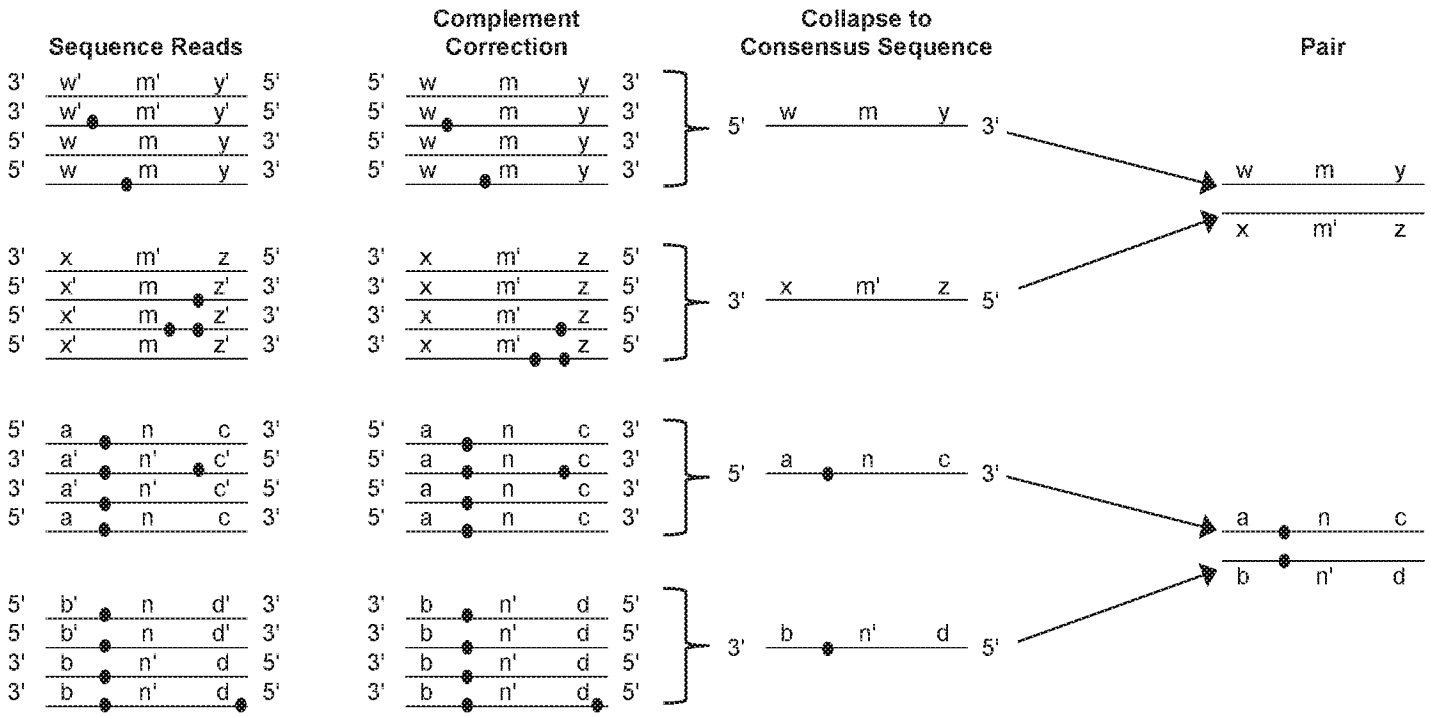

Sequence Reads
Complement Correction
Collapse to Consensus Sequence
Pair
3' w' m' y' 5'
3' w' m' y' 5'
5' w m y 3'
5' w m y 3'
5' w m y 3'
5' w m y 3'
5' w m y 3'
3' x m' z 5'
5' x' m z' 3'
5' x' m z' 3'
5' x' m z' 3'
3' x m' z 5'
3' x m' z 5'
3' x m' z 5'
3' x m' z 5'
3' x m' z 5'
5' a n c 3'
3' a' n' c' 5'
3' a' n' c' 5'
5' a n c 3'
5' a n c 3'
5' a n c 3'
5' a n c 3'
5' a n c 3'
5' b' n d' 3'
5' b' n d' 3'
3' b n' d 5'
3' b n' d 5'
3' b n' d 5'
3' b n' d 5'
3' b n' d 5'

Fig. 4C

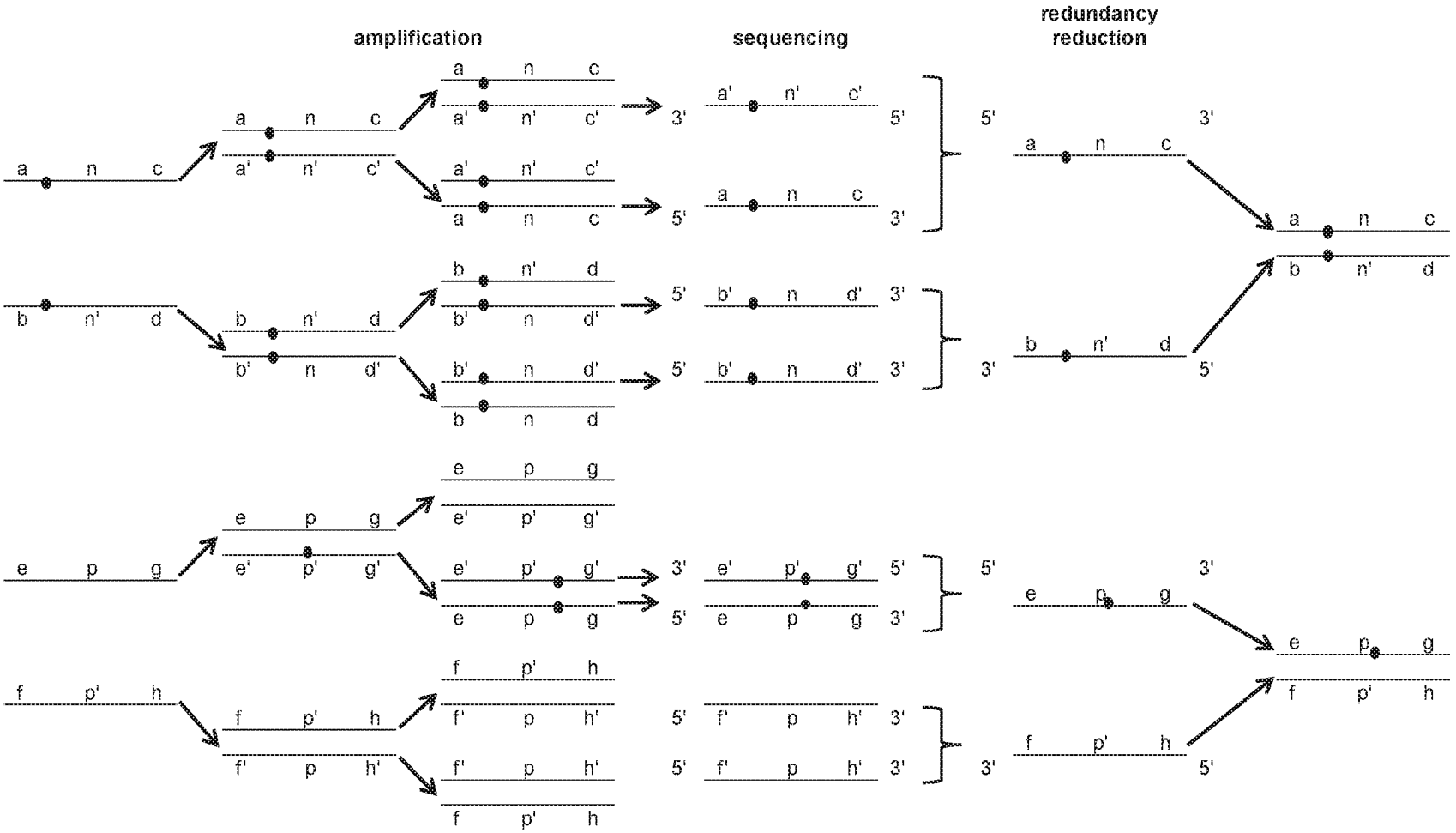

amplification
sequencing
redundancy reduction
a n c
a' n' c'
b n' d
b' n d'
e p g
e' p' g'
f p' h
f' p h'
3'
5'

Fig. 5

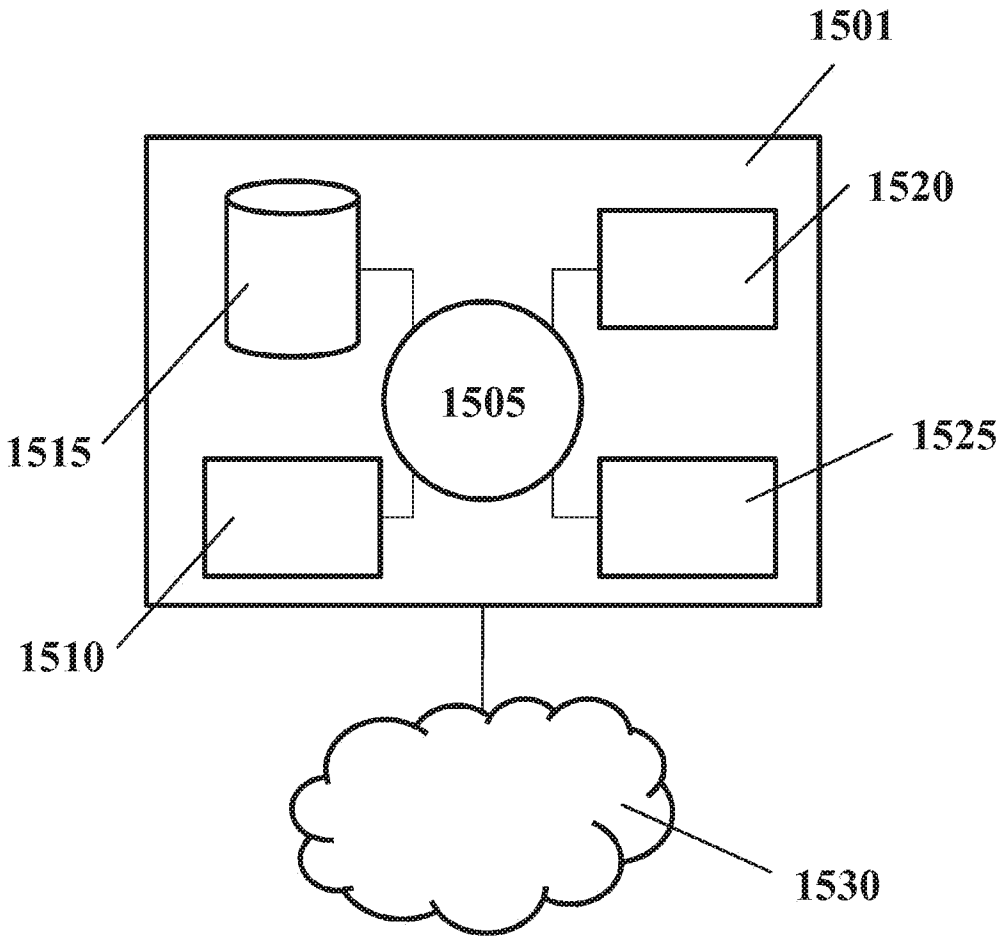


Fig. 6

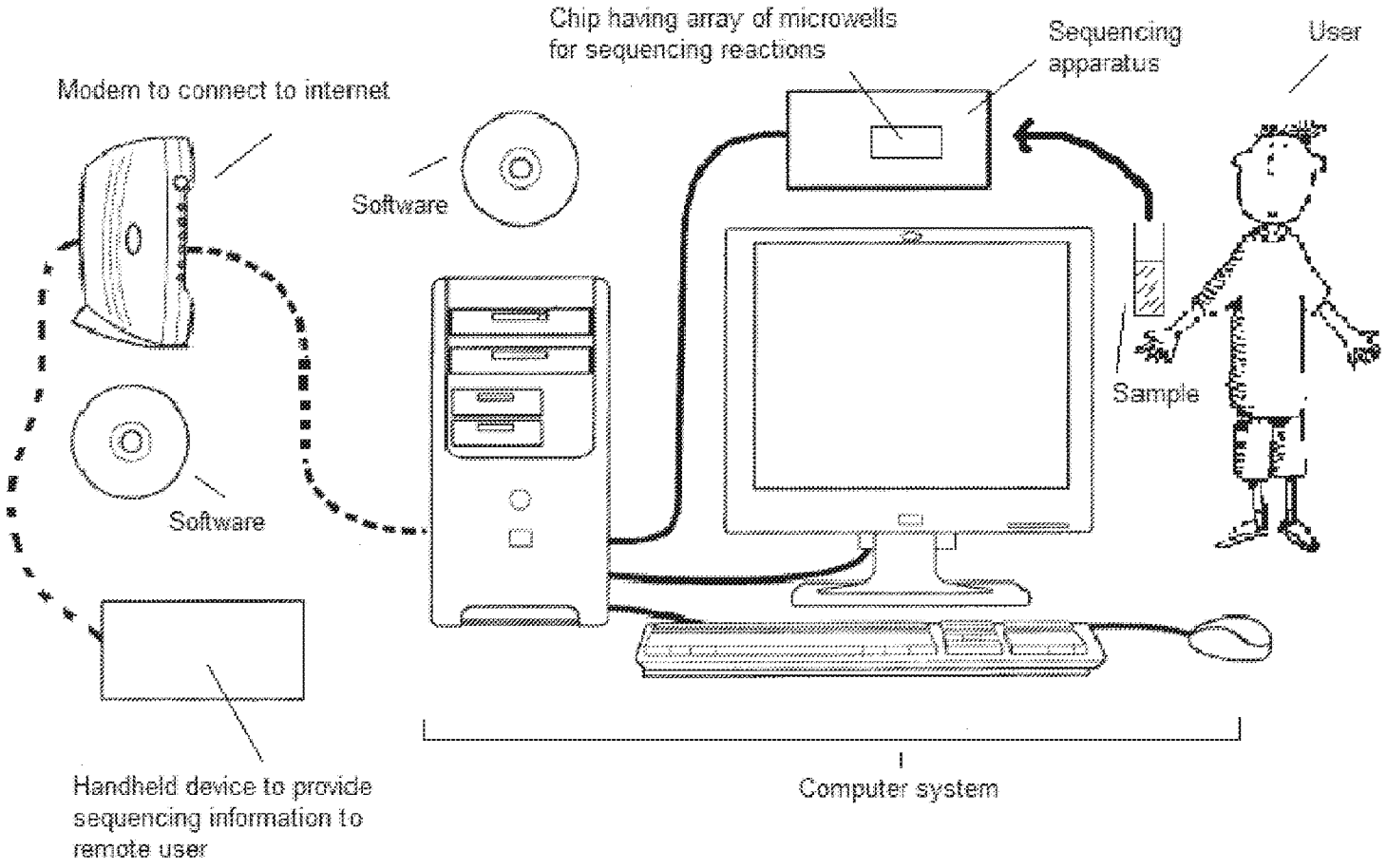

Chip having array of microwells for sequencing reactions
Sequencing apparatus
User
Modem to connect to internet
Software
Sample
Software
Handheld device to provide sequencing information to remote user
Computer system

Fig. 7

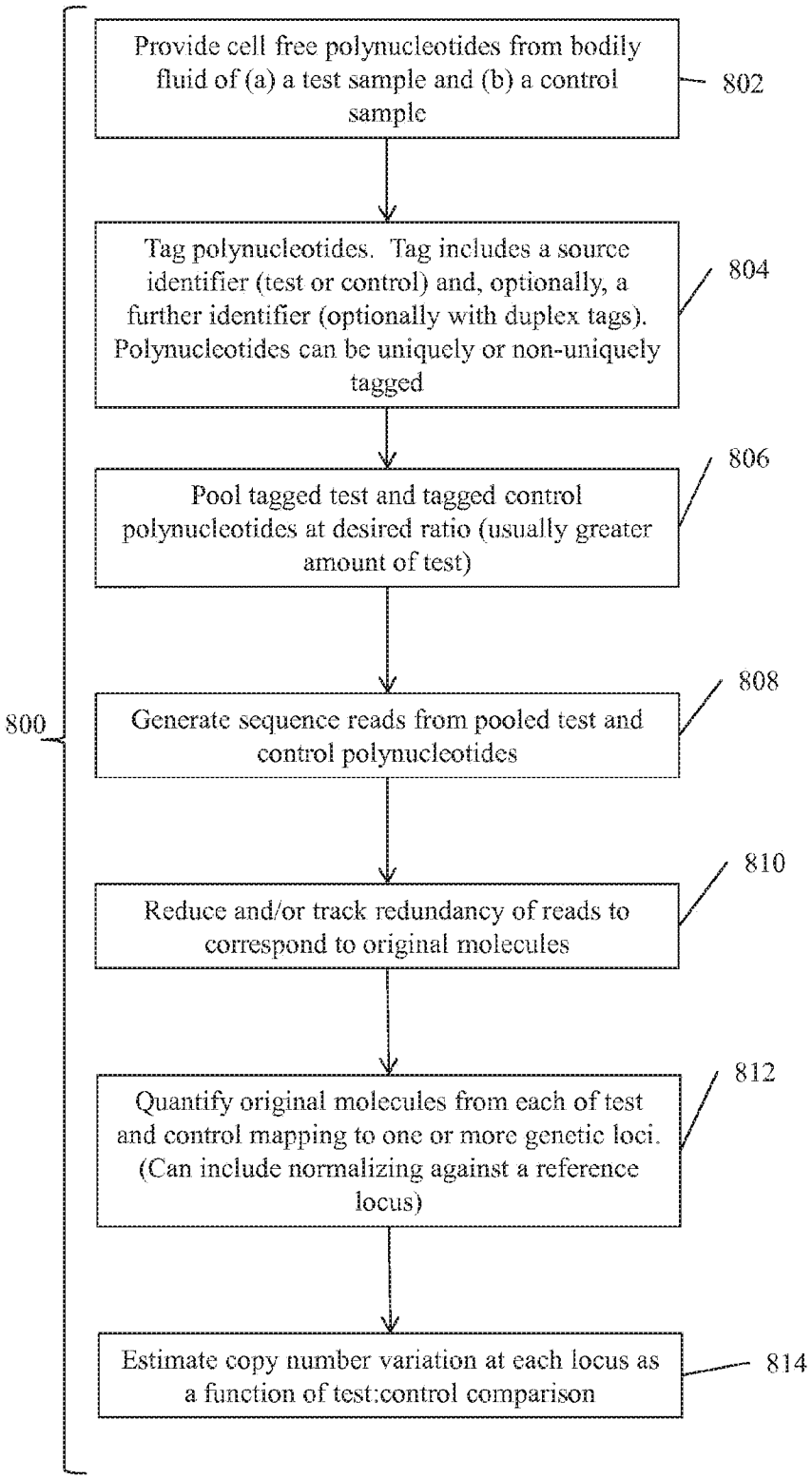

Provide cell free polynucleotides from bodily fluid of (a) a test sample and (b) a control sample
802
Tag polynucleotides. Tag includes a source identifier (test or control) and, optionally, a further identifier (optionally with duplex tags). Polynucleotides can be uniquely or non-uniquely tagged
804
Pool tagged test and tagged control polynucleotides at desired ratio (usually greater amount of test)
806
Generate sequence reads from pooled test and control polynucleotides
808
800
Reduce and/or track redundancy of reads to correspond to original molecules
810
Quantify original molecules from each of test and control mapping to one or more genetic loci. (Can include normalizing against a reference locus)
812
Estimate copy number variation at each locus as a function of test:control comparison
814

Fig. 8

Fig. 9A

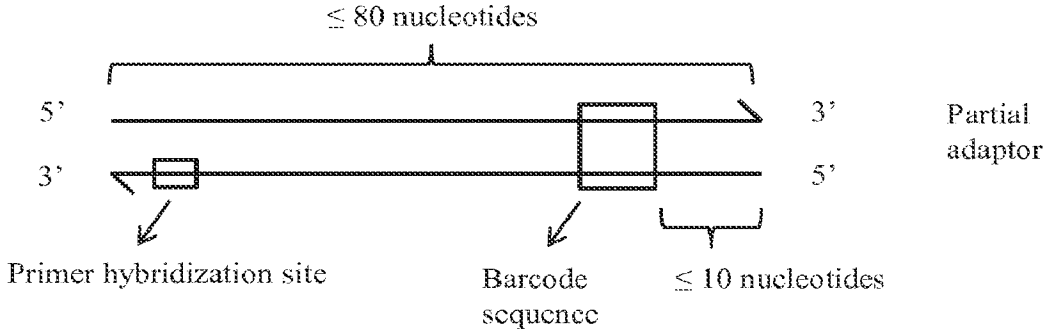


Fig. 9B

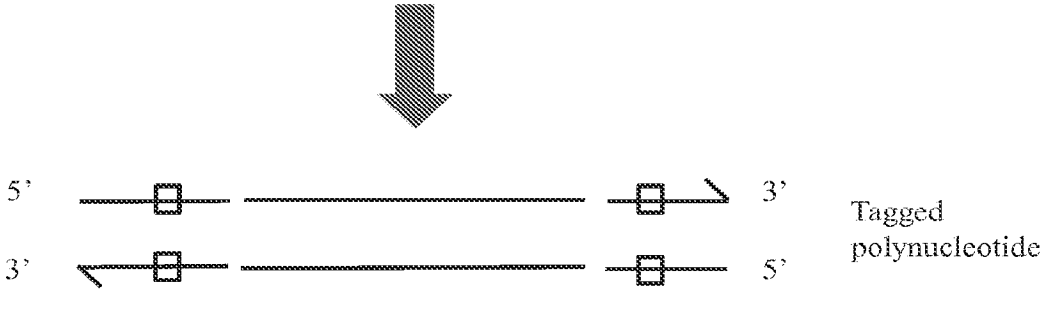
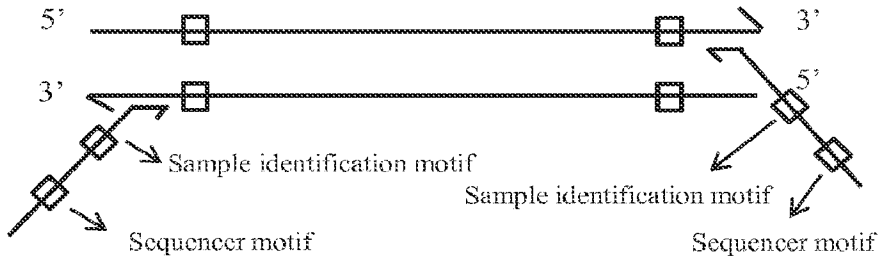


Fig. 9C



Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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Application Number	
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First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

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	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	1	ZHANG et al. "Comprehensive One-Step Molecular Analysis of Mitochondrial Genome by Massively Parallel Sequencing" Clinical Chem (2012) 58(9):1322-1331	
	2	ZHANG, et al. The impact of next-generation sequencing on genomics. J Genet Genomics. 2011 Mar 20;38(3):95-109. doi: 10.1016/j.jgg.2011.02.003. Epub 2011 Mar 15.	
	3	ZHAO, et al. Homozygous Deletions and Chromosome Amplifications in Human Lung Carcinomas Revealed by Single Nucleotide Polymorphism Array Analysis. Cancer Research, 65: 5561-5570 (2005).	
	4	ZHOU, et al. Counting alleles reveals a connection between chromosome 18q loss and vascularinvasion. Nature Biotechnology, 19: 78-81 (2001).	

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	Examiner Name	
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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.

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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First Named Inventor	AmirAli TALASAZ
Art Unit	
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Attorney Docket Number	42534-708.306

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	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	1	MOHAN, et al.,Changes in colorectal carcinoma genomes under anti-EGFR therapy identified by whole-genome plasma DNA sequencing.,PLoS Genet,doi: 10.1371/journal.pgen.1004271. eCollection 2014.,2014 Mar 27,10(3), e1004271.	
	2	MORI, et al. Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. J Clin Oncol. 2005 Dec 20;23(36):9351-8.	
	3	MORRISSY, A.S. et al. "Next-generation tag sequencing for cancer gene expression profiling" Genome Research (2009) 19(10): 1825–1835	
	4	MORTAZAVI, et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods. 2008; 5, 621-628	
	5	NARAYAN, et al. Ultrasensitive measurement of hotspot mutations in tumor DNA in blood using error-suppressed multiplexed deep sequencing. Cancer Res. 2012 Jul 15;72(14):3492-8. doi: 10.1158/0008-5472.CAN-11-4037. Epub 2012 May 10.	
	6	NAWROZ, et al. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med. 1996 Sep;2 (9):1035-7.	
	7	NEWMAN, A. et al. "Integrated digital error suppression for improved detection of circulating tumor DNA" Nature Biotech (2016) 34(5):547-555	
	8	NEWMAN, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014 May;20(5):548-54. doi: 10.1038/nm.3519. Epub 2014 Apr 6.	
	9	NG, S.B., et al., "Targeted capture and massively parallel sequencing of 12 human exomes," Nature 2009, 461(7261), 272-276	
	10	NIELSEN, R. et al. "Genotype and SNP calling from next-generation sequencing data" Nature Reviews Genetics (2011) 12(6):443-451	
	11	NORD, et al. Accurate and exact CNV identification from targeted high-throughput sequence data. BMC Genomics. 2011 Apr 12;12:184.	

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	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	12	Notice of Allowance dated 01/31/2017 for U.S. Patent Application No. 14/712,754.	
	13	Notice of allowance dated 03/21/2014 for US Application No. 12/969,581.	
	14	Notice of allowance dated 06/15/2017 for US Application No.15/076,565.	
	15	Notice of allowance dated 06/19/2014 for US Application No. 12/969,581.	
	16	Notice of allowance dated 08/01/2017 for US Application No. 15/492,659	
	17	Notice of allowance dated 08/04/2017 for US Application No.15/467,570	
	18	Notice of allowance dated 08/22/2014 for US Application No. 12/969,581.	
	19	Notice of allowance dated 08/29/2017 for US Application No. 15/492,659	
	20	Notice of allowance dated 09/11/2017 for US Application No. 15/076,565	
	21	Notice of allowance dated 10/03/2017 for US Application No. 15/076,565.	
	22	Notice of allowance dated 10/25/2017 for US Application No. 14/861,989	

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	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	23	Notice of allowance dated 12/28/2017 for US Application No. 14/861,989.	
	24	ODEGAARD, J.I. et al. "Validation of a Plasma-Based Comprehensive Cancer Genotyping Assay Utilizing Orthogonal Tissue- and Plasma-Based Methodologies" Clin Canc Res (2018) 24(15):3539-3549	
	25	Office Action dated 02/09/2017 for U.S. Patent Application No. 15/076,565.	
	26	Office action dated 05/13/2019 for US Application No. 15/669,779.	
	27	Office action dated 05/20/2016 for US Application No. 14/855,301.	
	28	Office action dated 05/31/2016 for US Application No. 14/712,754.	
	29	Office action dated 06/01/2017 for US Application No. 15/467,570	
	30	Office action dated 06/03/2016 for US Application No. 14/861,989.	
	31	Office action dated 06/12/2017 for US Application No. 15/492,659.	
	32	Office action dated 07/18/2017 for US Application No. 14/861,989	
	33	Office Action dated 07/30/2019 for U.S. Application No. 16/283,635.	

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	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	34	Office action dated 08/06/2019 for US Application No. 14/855,301.	
	35	Office action dated 10/03/2013 for US Application No. 12/969,581.	
	36	Office Action dated 10/12/2016 for U.S. Patent Application No. 14/712,754.	
	37	Office action dated 10/20/2017 for US Application No. 14/425,189.	
	38	Office Action dated 11/21/2016 for U.S. Patent Application No. 14/855,301.	
	39	Office action dated 12/04/2015 for US Application No. 14/712,754.	
	40	Office action dated 12/07/2017 for US Application No. 14/855,301	
	41	Office Action dated 12/09/2016 for U.S. Patent Application No. 14/861,989.	
	42	Office action dated 12/21/2018 for US Application No. 15/978,848.	
	43	OGINO, et al. Quantification of PCR bias caused by a single nucleotide polymorphism in SMN gene dosage analysis. J Mol Diagn. 2002 Nov;4(4):185-90.	
	44	Opposition Brief dated August 7, 2018 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	X

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	45	Opposition Decision dated January 15, 2019 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	☒
	46	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Foundation Medicine Inc.	☐
	47	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Grunecker Patent-und Rechtsanwalte PartGmbB	☐
	48	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Personal Genome Diagnostics, Inc.	☐
	49	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Strawman Limited	☐
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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.

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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	1	STUMM, M. et al. "Noninvasive prenatal detection of chromosomal aneuploidies using different next generation sequencing strategies and algorithms" Prenatal Diagnosis (2012) 32:569-577	
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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).

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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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	3	Guardant Health vs. FMI Invalidity Exhibit C-4, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

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).

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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.

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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	12	IPR2018-00130, Petition for Inter Partes Review of U.S. Patent 9,598,731, dated November 7, 2018	
	13	IPR2019-00130, Decision Denying Inter Partes Review U.S. 9,598,731, issued June 5, 2019	
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	17	IPR2019-00636 & 637, Exhibit 3001 Memo for Inter Partes Review of U.S. Patent 9,902,992, dated July 1, 2019	
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	22	IPR2019-00652, Decision Granting Inter Partes Review U.S.9,834,822, issued August 19, 2019	

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	23	IPR2019-00652, Petition for Inter Partes Review of U.S. Patent 9,834,822, dated February 1, 2019	
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	45	KIRSCH, S. et al. "Sequence error storms and the landscape of mutations in cancer," Proc. Natl. Acad. Sci. USA 2012, 109(36), 14289-14290	
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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
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	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

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).

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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Examiner Name	
Attorney Docket Number	42534-708.306

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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
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

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	40	CHIU, et al. Quantitative analysis of circulating mitochondrial DNA in plasma. Clin Chem. 2003 May;49(5):719-26.	
	41	CHIU, R.W.K. et al. "Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma" PNAS (2008) 105(51):20458-20463	
	42	CHURCH, et al. "Multiplex DNA sequencing." Science, 240: 185-188 (1988).	
	43	CLARK, T.A. et al. "Analytical Validation of a Hybrid Capture Based Next-Generation Sequencing Clinical Assay for Genomic Profiling of Cell-Free Circulating Tumor DNA," J. Mol. Diagnostics (2018) 20(5):686-702	
	44	COOK, et al. Methylated DNA labels for marking objects. Biotechnol Lett. 2003 Jan;25(1):89-94.	
	45	COSTELLO, et al. Discovery and characterization of artifactual mutations in deep coverage targeted capture sequencing data due to oxidative DNA damage during sample preparation. Nucleic Acids Res. 2013 Apr 1;41(6):e67. doi: 10.1093/nar/gks1443. Epub 2013 Jan 8.	
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	47	COX, J. "Bar coding objects with DNA." Analyst. May 2001;126(5):545-7.	
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	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

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	First Named Inventor	AmirAli TALASAZ
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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

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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EFS ID:	40166741
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	GH0004US-C5/42534-708.306
Receipt Date:	31-JUL-2020
Filing Date:	
Time Stamp:	16:40:36
Application Type:	Utility under 35 USC 111(a)

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Payment Type	DA
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Warnings:					
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2	Application Data Sheet	2020-07-31_GH0004US-CON5_ADS.pdf	1874538 5b8045e3c7f2d668593eccfa2e5ad9d754b446e4	no	9
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3	Oath or Declaration filed	2020-07-31_GH0004US-CON5_ParentDec.pdf	2645663 6151a53b79f64998d395a7870b542cde2c2be6f2	no	3
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4		2020-07-31_GH0004US-CON5_SpecAsFiled.pdf	693021 58e1810627ab76447a4ff88633439c0563412f34	yes	92

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	86
Claims	87	91
Abstract	92	92

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5	Drawings-only black and white line drawings	2020-07-31_GH0004US-CON5_Figs.pdf	2550181 97cf1eb2e8fae70ef9de5a96fb471e6f046d9975	no	11

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Title of Invention	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

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 14/861,989

filed on September 22, 2015.

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.

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LEGAL NAME OF INVENTOR

Inventor: AmirAli Talasaz Date (Optional): 11/10/15

Signature: [signature]

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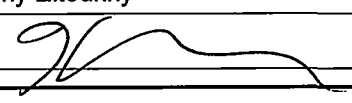
LEGAL NAME OF INVENTOR

Inventor: Helmy Eltoukhy Date (Optional): 11/10/15

Signature: 

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☐ The attached application, or

☒ United States application or PCT international application number 16/601,168

filed on 2019-10-14.

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.

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LEGAL NAME OF INVENTOR

Inventor: Stefanie Ann Ward MORTIMER Date (Optional): _____

Signature: _____

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I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

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Assignee
Guardant Health, Inc.
2686 Middlefield Rd, Suite D
Redwood City, CA 94063

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SIGNATURE of Assignee of Record
The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature	/[signature]/	Date *	9/5/13
Name	Helmy Eltoukhy	Telephone	
Title	CEO		

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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Guardant Health, Inc.

Application No./Patent No.: 14/861,989 Filed/Issue Date: September 22, 2015

Titled: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

Guardant Health, Inc., a corporation of the State of Delaware

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

☐ The extent (by percentage) of its ownership interest is ___%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

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The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

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☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

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[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Ali Alemozafar/	February 22, 2016
Signature	Date
Ali R. Alemozafar	68,180
Printed or Typed Name	Title or Registration Number

METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

CROSS-REFERENCE

[0001] This application is a continuation of U.S. Application No. 16/601,168, filed October 14, 2019, which is a continuation of U.S. Application No. 15/892,178, filed February 8, 2018, which is a continuation of U.S. Application No. 14/861,989, filed September 22, 2015 (now U.S. Patent 9,920,366), which is a continuation application of International Application No. PCT/US2014/072383, filed December 24, 2014, which application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/921,456, filed December 28, 2013, and U.S. Provisional Application No. 61/948,509, filed March 5, 2014, each of which is entirely incorporated herein by reference.

BACKGROUND

[0002] The detection and quantification of polynucleotides is important for molecular biology and medical applications, such as diagnostics. Genetic testing is particularly useful for a number of diagnostic methods. For example, disorders that are caused by rare genetic alterations (*e.g.*, sequence variants) or changes in epigenetic markers, such as cancer and partial or complete aneuploidy, may be detected or more accurately characterized with DNA sequence information.

[0003] Early detection and monitoring of genetic diseases, such as cancer, is often useful and needed in the successful treatment or management of the disease. One approach may include the monitoring of a sample derived from cell-free nucleic acids, a population of polynucleotides that can be found in different types of bodily fluids. In some cases, disease may be characterized or detected based on detection of genetic aberrations, such as copy number variation and/or sequence variation of one or more nucleic acid sequences, or the development of other certain rare genetic alterations. Cell-free DNA (cfDNA) may contain genetic aberrations associated with a particular disease. With improvements in sequencing and techniques to manipulate nucleic acids, there is a need in the art for improved methods and systems for using cell-free DNA to detect and monitor disease.

[0004] In particular, many methods have been developed for accurate copy number variation estimation, especially for heterogeneous genomic samples, such as tumor-derived gDNA or for

cfDNA for many applications (*e.g.*, prenatal, transplant, immune, metagenomics or cancer diagnostics). Most of these methods include sample preparation whereby the original nucleic acids are converted into a sequenceable library, followed by massively parallel sequencing, and finally bioinformatics to estimate copy number variation at one or more loci.

SUMMARY

[0005] Although many of these methods are able to reduce or combat the errors introduced by the sample preparation and sequencing processes for all molecules that are converted and sequenced, these methods are not able to infer the counts of molecules that were converted but not sequenced. Since this count of converted by unsequenced molecules can be highly variable from genomic region to region, these counts can dramatically and adversely affect the sensitivity that can be achieved.

[0006] To address this issue, input double-stranded deoxyribonucleic acid (DNA) can be converted by a process that tags both halves of the individual double-stranded molecule, in some cases differently. This can be performed using a variety of techniques, including ligation of hairpin, bubble, or forked adapters or other adaptors having double-stranded and single stranded segments (the unhybridized portion of a bubble, forked or hairpin adapter are deemed single-stranded herein). If tagged correctly, each original Watson and Crick (i.e., strand) side of the input double-stranded DNA molecule can be differently tagged and identified by the sequencer and subsequent bioinformatics. For all molecules in a particular region, counts of molecules where both Watson and Crick sides were recovered ("Pairs") versus those where only one half was recovered ("Singlets") can be recorded. The number of unseen molecules can be estimated based on the number of Pairs and Singlets detected.

[0007] An aspect of the present disclosure provides a method for detecting and/or quantifying rare deoxyribonucleic acid (DNA) in a heterogeneous population of original DNA fragments, comprising tagging the original DNA fragments in a single reaction using a library of a plurality of different tags such that greater than 30% of the fragments are tagged at both ends, wherein each of the tags comprises a molecular barcode. The single reaction can be in a single reaction vessel. Greater than 50% of the fragments can be tagged at both ends. The plurality of different tags can be no more than any of 100, 500, 1000, 10,000 or 100,000 different tags.

[0008] Another aspect provides a set of library adaptors that can be used to tag the molecules of interest (e.g., by ligation, hybridization, etc.). The set of library adaptors can comprise plurality of polynucleotide molecules with molecular barcodes, wherein the plurality of polynucleotide molecules are less than or equal to 80 nucleotide bases in length, wherein the molecular barcodes are at least 4 nucleotide bases in length, and wherein (a) the molecular barcodes are different from one another and have an edit distance of at least 1 between one another; (b) the molecular barcodes are located at least one nucleotide base away from a terminal end of their respective polynucleotide molecules; (c) optionally, at least one terminal base is identical in all of the polynucleotide molecules; and (d) none of the polynucleotide molecules contains a complete sequencer motif.

[0009] In some embodiments, the library adaptors (or adapters) are identical to one another but for the molecular barcodes. In some embodiments, each of the plurality of library adaptors comprises at least one double-stranded portion and at least one single-stranded portion (e.g., a non-complementary portion or an overhang). In some embodiments, the double-stranded portion has a molecular barcode selected from a collection of different molecular barcodes. In some embodiments, the given molecular barcode is a randomer. In some embodiments, each of the library adaptors further comprises a strand-identification barcode on the at least one single-stranded portion. In some embodiments, the strand-identification barcode includes at least 4 nucleotide bases. In some embodiments, the single-stranded portion has a partial sequencer motif. In some embodiments, the library adaptors do not include a complete sequencer motif.

[0010] In some embodiments, none of the library adaptors contains a sequence for hybridizing to a flow cell or forming a hairpin for sequencing.

[0011] In some embodiments, all of the library adaptors have a terminal end with nucleotide(s) that are the same. In some embodiments, the identical terminal nucleotide(s) are over two or more nucleotide bases in length.

[0012] In some embodiments, each of the library adapters is Y-shaped, bubble shaped or hairpin shaped. In some embodiments, none of the library adapters contains a sample identification motif. In some embodiments, each of the library adapters comprises a sequence that is selectively hybridizable to a universal primer. In some embodiments, each of the library adapters comprises a molecular barcode that is at least 5, 6, 7, 8, 9 and 10 nucleotide bases in

length. In some embodiments, each of the library adapters is from 10 nucleotide bases to 80 in length, or 30 to 70 nucleotide bases in length, or 40 to 60 nucleotide bases in length. In some embodiments, at least 1, 2, 3, or 4 terminal bases are identical in all of the library adaptors. In some embodiments, at least 4 terminal bases are identical in all of the library adaptors.

[0013] In some embodiments, the edit distance of the molecular barcodes of the library adapters is a Hamming distance. In some embodiments, the edit distance is at least 1, 2, 3, 4 or 5. In some embodiments, the edit distance is with respect to individual bases of the plurality of polynucleotide molecules. In some embodiments, the molecular barcodes are located at least 10 nucleotide base away from a terminal end of an adapter. In some embodiments, the plurality of library adapters includes at least 2, 4, 6, 8, 10, 20, 30, 40 or 50 different molecular barcodes, or from 2-100, 4-80, 6-60 or 8-40 different molecular barcodes. In any of the embodiments herein, there are more polynucleotides (e.g., cfDNA fragments) to be tagged than there are different molecular barcodes such that the tagging is not unique.

[0014] In some embodiments, the terminal end of an adaptor is configured for ligation (e.g., to a target nucleic acid molecule). In some embodiments, the terminal end of an adaptor is a blunt end.

[0015] In some embodiments, the adaptors are purified and isolated. In some embodiments, the library comprises one or more non-naturally occurring bases.

[0016] In some embodiments, the polynucleotide molecules comprise a primer sequence positioned 5' with respect to the molecular barcodes.

[0017] In some embodiments, the set of library adaptors consists essentially of the plurality of polynucleotide molecules.

[0018] In another aspect, a method comprises (a) tagging a collection of polynucleotides with a plurality of polynucleotide molecules from a library of adaptors to create a collection of tagged polynucleotides; and (b) amplifying the collection of tagged polynucleotides in the presence of sequencing adaptors, wherein the sequencing adaptors have primers with nucleotide sequences that are selectively hybridizable to complementary sequences in the plurality of polynucleotide molecules. The library of adaptors may be as described above or elsewhere herein. In some embodiments, each of the sequencer adaptors further comprises an index tag, which can be a sample identification motif.

[0019] Another aspect, provides a method for detecting and/or quantifying rare DNA in a heterogeneous population of original DNA fragments, wherein the rare DNA has a concentration that is less than 1%, the method comprising (a) tagging the original DNA fragments in a single reaction such that greater than 30% of the original DNA fragments are tagged at both ends with library adaptors that comprise molecular barcodes, thereby providing tagged DNA fragments; (b) performing high-fidelity amplification on the tagged DNA fragments; (c) optionally, selectively enriching a subset of the tagged DNA fragments; (d) sequencing one or both strands of the tagged, amplified and optionally selectively enriched DNA fragments to obtain sequence reads comprising nucleotide sequences of the molecular barcodes and at least a portion of the original DNA fragments; (e) from the sequence reads, determining consensus reads that are representative of single-strands of the original DNA fragments; and (f) quantifying the consensus reads to detect and/or quantify the rare DNA at a specificity that is greater than 99.9%.

[0020] In some embodiments, (e) comprises comparing sequence reads having the same or similar molecular barcodes and the same or similar end of fragment sequences. In some embodiments, the comparing further comprises performing a phylogentic analysis on the sequence reads having the same or similar molecular barcodes. In some embodiments, the molecular barcodes include a barcode having an edit distance of up to 3. In some embodiments, the end of fragment sequence includes fragment sequences having an edit distance of up to 3.

[0021] In some embodiments, the method further comprises sorting sequence reads into paired reads and unpaired reads, and quantifying a number of paired reads and unpaired reads that map to each of one or more genetic loci.

[0022] In some embodiments, the tagging occurs by having an excess amount of library adaptors as compared to original DNA fragments. In some embodiments, n the excess is at least a 5-fold excess. In some embodiments, the tagging comprises using a ligase. In some embodiments, the tagging comprises attachment to blunt ends.

[0023] In some embodiments, the method further comprises binning the sequence reads according to the molecular barcodes and sequence information from at least one end of each of the original DNA fragments to create bins of single stranded reads. In some embodiments, the method further comprises, in each bin, determining a sequence of a given original DNA fragment among the original DNA fragments by analyzing sequence reads. In some embodiments, the

method further comprises detecting and/or quantifying the rare DNA by comparing a number of times each base occurs at each position of a genome represented by the tagged, amplified, and optionally enriched DNA fragments.

[0024] In some embodiments, the library adaptors do not contain complete sequencer motifs. In some embodiments, the method further comprises selectively enriching a subset of the tagged DNA fragments. In some embodiments, the method further comprises, after enriching, amplifying the enriched tagged DNA fragments in the presence of sequencing adaptors comprising primers. In some embodiments, (a) provides tagged DNA fragments having from 2 to 1000 different combinations of molecular barcodes.

[0025] In some embodiments, the DNA fragments are tagged with polynucleotide molecules from a library of adaptors as described above or elsewhere herein.

[0026] In another aspect, a method for processing and/or analyzing a nucleic acid sample of a subject comprises (a) exposing polynucleotide fragments from the nucleic acid sample to a set of library adaptors to generate tagged polynucleotide fragments; and (b) subjecting the tagged polynucleotide fragments to nucleic acid amplification reactions under conditions that yield amplified polynucleotide fragments as amplification products of the tagged polynucleotide fragments. The set of library adaptors comprises a plurality of polynucleotide molecules with molecular barcodes, wherein the plurality of polynucleotide molecules are less than or equal to 80 nucleotide bases in length, wherein the molecular barcodes are at least 4 nucleotide bases in length, and wherein (1) the molecular barcodes are different from one another and have an edit distance of at least 1 between one another; (2) the molecular barcodes are located at least one nucleotide base away from a terminal end of their respective polynucleotide molecules; (3) optionally, at least one terminal base is identical in all of the polynucleotide molecules; and (4) none of the polynucleotide molecules contains a complete sequencer motif.

[0027] In some embodiments, the method further comprises determining nucleotide sequences of the amplified tagged polynucleotide fragments. In some embodiments, the nucleotide sequences of the amplified tagged polynucleotide fragments are determined without polymerase chain reaction (PCR). In some embodiments, the method further comprises analyzing the nucleotide sequences with a programmed computer processor to identify one or more genetic variants in the nucleotide sample of the subject. In some embodiments, the one or

more genetic variants are selected from the group consisting of base change(s), insertion(s), repeat(s), deletion(s), copy number variation(s) and transversion(s). In some embodiments, the one or more genetic variants include one or more tumor associated genetic alterations.

[0028] In some embodiments, the subject has or is suspected of having a disease. In some embodiments, the disease is cancer. In some embodiments, the method further comprises collecting the nucleic acid sample from the subject. In some embodiments, the nucleic acid sample is collected from a location selected from the group consisting of blood, plasma, serum, urine, saliva, mucosal excretions, sputum, stool, cerebral spinal fluid and tears of the subject. In some embodiments, the nucleic acid sample is a cell-free nucleic acid sample. In some embodiments, the nucleic acid sample is collected from no more than 100 nanograms (ng) of double-stranded polynucleotide molecules of the subject.

[0029] In some embodiments, the polynucleotide fragments comprise double-stranded polynucleotide molecules. In some embodiments, in (a), the plurality of polynucleotide molecules couple to the polynucleotide fragments via blunt end ligation, sticky end ligation, molecular inversion probes, PCR, ligation-based PCR, multiplex PCR, single stranded ligation, and single stranded circularization. In some embodiments, exposing the polynucleotide fragments of the nucleic acid sample to the plurality of polynucleotide molecules yields the tagged polynucleotide fragments with a conversion efficiency of at least 10%. In some embodiments, any of at least 5%, 6%, 7%, 8%, 9%, 10%, 20%, or 25% of the tagged polynucleotide fragments share a common polynucleotide molecule or sequence. In some embodiments, the method further comprises generating the polynucleotide fragments from the nucleic acid sample.

[0030] In some embodiments, the subjecting comprises amplifying the tagged polynucleotide fragments from sequences corresponding to genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1,

CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

[0031] In another aspect, a method comprises (a) generating a plurality of sequence reads from a plurality of polynucleotide molecules, wherein the plurality of polynucleotide molecules cover genomic loci of a target genome, wherein the genomic loci correspond to a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1; (b) grouping with a computer processor the plurality of sequence reads into families, wherein each family comprises sequence reads from one of the template polynucleotides; (c) for each of the families, merging sequence reads to generate a consensus sequence; (d) calling the consensus sequence at a given genomic locus among the genomic loci; and (e) detecting at the given genomic locus any of genetic variants among the calls, frequency of a genetic alteration among the calls, total number of calls, and total number of alterations among the calls.

[0032] In some embodiments, each family comprises sequence reads from only one of the template polynucleotides. In some embodiments, the given genomic locus comprises at least one nucleic acid base. In some embodiments, the given genomic locus comprises a plurality of nucleic acid bases. In some embodiments, the calling comprises calling at least one nucleic acid base at the given genomic locus. In some embodiments, the calling comprises calling a plurality of nucleic acid bases at the given genomic locus. In some embodiments, the calling comprises any one of phylogenetic analysis, voting, weighing, assigning a probability to each read at the locus in a family and calling the base with the highest probability.

[0033] In some embodiments, the method further comprises performing (d)-(e) at an additional genomic locus among the genomic loci. In some embodiments, the method further comprises determining a variation in copy number at one of the given genomic locus and

additional genomic locus based on counts at the given genomic locus and additional genomic locus.

[0034] In some embodiments, the grouping comprises classifying the plurality of sequence reads into families by identifying (i) different molecular barcodes coupled to the plurality of polynucleotide molecules and (ii) similarities between the plurality of sequence reads, wherein each family includes a plurality of nucleic acid sequences that are associated with a different combination of molecular barcodes and similar or identical sequence reads. Different molecular barcodes have different sequences.

[0035] In some embodiments, the consensus sequence is generated by evaluating a quantitative measure or a statistical significance level for each of the sequence reads. In some embodiments, the quantitative measure comprises use of a binomial distribution, exponential distribution, beta distribution, or empirical distribution. In some embodiments, the method further comprises mapping the consensus sequence to the target genome. In some embodiments, the plurality of genes includes at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50 or all of the plurality of genes selected from the group.

[0036] Another aspect of the present disclosure provides a method, comprising (a) providing template polynucleotide molecules and a set of library adaptors in a single reaction vessel, wherein the library adaptors are polynucleotide molecules that have different molecular barcodes (e.g., from 2 to 1,000 different molecular barcodes), and wherein none of the library adaptors contains a complete sequencer motif; (b) in the single reaction vessel, coupling the library adaptors to the template polynucleotide molecules at an efficiency of at least 10%, thereby tagging each template polynucleotide with a tagging combination that is among a plurality of different tagging combinations (e.g., 4 to 1,000,000 different tagging combinations), to produce tagged polynucleotide molecules; (c) subjecting the tagged polynucleotide molecules to an amplification reaction under conditions that yield amplified polynucleotide molecules as amplification products of the tagged polynucleotide molecules; and (d) sequencing the amplified polynucleotide molecules.

[0037] In some embodiments, the template polynucleotide molecules are blunt ended or sticky-ended. In some embodiments, the library adaptors are identical but for the molecular barcodes. In some embodiments, each of the library adaptors has a double stranded portion and

at least one single-stranded portion. In some embodiments, the double-stranded portion has a molecular barcode among the molecular barcodes. In some embodiments, each of the library adaptors further comprises a strand-identification barcode on the at least one single-stranded portion. In some embodiments, the single-stranded portion has a partial sequencer motif. In some embodiments, the library adaptors have a sequence of terminal nucleotides that are the same. In some embodiments, the template polynucleotide molecules are double-stranded. In some embodiments, the library adaptors couple to both ends of the template polynucleotide molecules.

[0038] In some embodiments, subjecting the tagged polynucleotide molecules to the amplification reaction comprises non-specifically amplifying the tagged polynucleotide molecules.

[0039] In some embodiments, the amplification reaction comprises use of a priming site to amplify each of the tagged polynucleotide molecules. In some embodiments, the priming site is a primer. In some embodiments, the primer is a universal primer. In some embodiments, the priming site is a nick.

[0040] In some embodiments, the method further comprises, prior to (e), (i) separating polynucleotide molecules comprising one or more given sequences from the amplified polynucleotide molecules, to produce enriched polynucleotide molecules; and (ii) amplifying the enriched polynucleotide molecules with sequencing adaptors.

[0041] In some embodiments, the efficiency is at least 30%, 40%, or 50%. In some embodiments, the method further comprises identifying genetic variants upon sequencing the amplified polynucleotide molecules. In some embodiments, the sequencing comprises (i) subjecting the amplified polynucleotide molecules to an additional amplification reaction under conditions that yield additional amplified polynucleotide molecules as amplification products of the amplified polynucleotide molecules, and (ii) sequencing the additional amplified polynucleotide molecules. In some embodiments, the additional amplification is performed in the presence of sequencing adaptors.

[0042] In some embodiments, (b) and (c) are performed without aliquoting the tagged polynucleotide molecules. In some embodiments, the tagging is non-unique tagging.

[0043] Another aspect, provides a system for analyzing a target nucleic acid molecule of a subject, comprising a communication interface that receives nucleic acid sequence reads for a plurality of polynucleotide molecules that cover genomic loci of a target genome; computer memory that stores the nucleic acid sequence reads for the plurality of polynucleotide molecules received by the communication interface; and a computer processor operatively coupled to the communication interface and the memory and programmed to (i) group the plurality of sequence reads into families, wherein each family comprises sequence reads from one of the template polynucleotides, (ii) for each of the families, merge sequence reads to generate a consensus sequence, (iii) call the consensus sequence at a given genomic locus among the genomic loci, and (iv) detect at the given genomic locus any of genetic variants among the calls, frequency of a genetic alteration among the calls, total number of calls; and total number of alterations among the calls, wherein the genomic loci correspond to a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

[0044] In another aspect, a set of oligonucleotide molecules that selectively hybridize to at least 5 genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

[0045] In some embodiments, the oligonucleotide molecules are from 10-200 bases in length. In some embodiments, the oligonucleotide molecules selectively hybridize to exon

regions of the at least 5 genes. In some embodiments, the oligonucleotide molecules selectively hybridize to at least 30 exons in the at least 5 genes. In some embodiments, multiple oligonucleotide molecules selectively hybridize to each of the at least 30 exons. In some embodiments, the oligonucleotide molecules that hybridize to each exon have sequences that overlap with at least 1 other oligonucleotide molecule.

[0046] In another aspect, a kit comprises a first container containing a plurality of library adaptors each having a different molecular barcode; and a second container containing a plurality of sequencing adaptors, each sequencing adaptor comprising at least a portion of a sequencer motif and optionally a sample barcode. The library adaptors can be as described above or elsewhere herein.

[0047] In some embodiments, the sequencing adaptor comprises the sample barcode. In some embodiments, the library adaptors are blunt ended and Y-shaped, and are less than or equal to 80 nucleic acid bases in length. In some embodiments, the sequencing adaptor is up to 70 bases from end to end.

[0048] In another aspect, a method for detecting sequence variants in a cell free DNA sample, comprising detecting rare DNA at a concentration less than 1% with a specificity that is greater than 99.9%.

[0049] In another aspect, a method comprises detecting genetic variants in a sample comprising DNA with a detection limit of at least 1% and specificity greater than 99.9%. In some embodiments, the method further comprises converting cDNA (e.g. cfDNA) into adaptor tagged DNA with a conversion efficiency of at least 30%, 40%, or 50% and reducing sequencing noise (or distortion) by eliminating false positive sequence reads.

[0050] Another aspect provides a method, comprising (a) providing a sample comprising a set of double-stranded polynucleotide molecules, each double-stranded polynucleotide molecule including first and second complementary strands; (b) tagging the double-stranded polynucleotide molecules with a set of duplex tags, wherein each duplex tag differently tags the first and second complementary strands of a double-stranded polynucleotide molecule in the set; (c) sequencing at least some of the tagged strands to produce a set of sequence reads; (d) reducing and/or tracking redundancy in the set of sequence reads; (e) sorting sequence reads into paired reads and unpaired reads, wherein (i) each paired read corresponds to sequence reads

generated from a first tagged strand and a second differently tagged complementary strand derived from a double-stranded polynucleotide molecule in the set, and (ii) each unpaired read represents a first tagged strand having no second differently tag complementary strand derived from a double-stranded polynucleotide molecule represented among the sequence reads in the set of sequence reads; (f) determining quantitative measures of (i) the paired reads and (ii) the unpaired reads that map to each of one or more genetic loci; and (g) estimating with a programmed computer processor a quantitative measure of total double-stranded polynucleotide molecules in the set that map to each of the one or more genetic loci based on the quantitative measure of paired reads and unpaired reads mapping to each locus.

[0051] In some embodiments, the method further comprises (h) detecting copy number variation in the sample by determining a normalized total quantitative measure determined in step (g) at each of the one or more genetic loci and determining copy number variation based on the normalized measure. In some embodiments, the sample comprises double-stranded polynucleotide molecules sourced substantially from cell-free nucleic acids. In some embodiments, the duplex tags are not sequencing adaptors.

[0052] In some embodiments, reducing redundancy in the set of sequence reads comprises collapsing sequence reads produced from amplified products of an original polynucleotide molecule in the sample back to the original polynucleotide molecule. In some embodiments, the method further comprises determining a consensus sequence for the original polynucleotide molecule. In some embodiments, the method further comprises identifying polynucleotide molecules at one or more genetic loci comprising a sequence variant. In some embodiments, the method further comprises determining a quantitative measure of paired reads that map to a locus, wherein both strands of the pair comprise a sequence variant. In some embodiments, the method further comprises determining a quantitative measure of paired molecules in which only one member of the pair bears a sequence variant and/or determining a quantitative measure of unpaired molecules bearing a sequence variant. In some embodiments, the sequence variant is selected from the group consisting of a single nucleotide variant, an indel, a transversion, a translocation, an inversion, a deletion, a chromosomal structure alteration, a gene fusion, a chromosome fusion, a gene truncation, a gene amplification, a gene duplication and a chromosomal lesion.

[0053] Another aspect provides a system comprising a computer readable medium comprising machine-executable code that, upon execution by a computer processor, implements a method comprising (a) receiving into memory a set of sequence reads of polynucleotides tagged with duplex tags; (b) reducing and/or tracking redundancy in the set of sequence reads; (c) sorting sequence reads into paired reads and unpaired reads, wherein (i) each paired read corresponds to sequence reads generated from a first tagged strand and a second differently tagged complementary strand derived from a double-stranded polynucleotide molecule in the set, and (ii) each unpaired read represents a first tagged strand having no second differently tag complementary strand derived from a double-stranded polynucleotide molecule represented among the sequence reads in the set of sequence reads; (d) determining quantitative measures of (i) the paired reads and (ii) the unpaired reads that map to each of one or more genetic loci; and (e) estimating a quantitative measure of total double-stranded polynucleotide molecules in the set that map to each of the one or more genetic loci based on the quantitative measure of paired reads and unpaired reads mapping to each locus.

[0054] Another aspect provides a method, comprising (a) providing a sample comprising a set of double-stranded polynucleotide molecules, each double-stranded polynucleotide molecule including first and second complementary strands; (b) tagging the double-stranded polynucleotide molecules with a set of duplex tags, wherein each duplex tag differently tags the first and second complementary strands of a double-stranded polynucleotide molecule in the set; (c) sequencing at least some of the tagged strands to produce a set of sequence reads; (d) reducing and/or tracking redundancy in the set of sequence reads; (e) sorting sequence reads into paired reads and unpaired reads, wherein (i) each paired read corresponds to sequence reads generated from a first tagged strand and a second differently tagged complementary strand derived from a double-stranded polynucleotide molecule in the set, and (ii) each unpaired read represents a first tagged strand having no second differently tag complementary strand derived from a double-stranded polynucleotide molecule represented among the sequence reads in the set of sequence reads; and (f) determining quantitative measures of at least two of (i) the paired reads, (ii) the unpaired reads that map to each of one or more genetic loci, (iii) read depth of the paired reads and (iv) read depth of unpaired reads.

[0055] In some embodiments, (f) comprises determining quantitative measures of at least three of (i)-(iv). In some embodiments, (f) comprises determining quantitative measures of all of (i)-(iv). In some embodiments, the method further comprises (g) estimating with a programmed computer processor a quantitative measure of total double-stranded polynucleotide molecules in the set that map to each of the one or more genetic loci based on the quantitative measure of paired reads and unpaired reads and their read depths mapping to each locus.

[0056] In another aspect, a method comprises (a) tagging control parent polynucleotides with a first tag set to produce tagged control parent polynucleotides, wherein the first tag set comprises a plurality of tags, wherein each tag in the first tag set comprises a same control tag and an identifying tag, and wherein the tag set comprises a plurality of different identifying tags; (b) tagging test parent polynucleotides with a second tag set to produce tagged test parent polynucleotides, wherein the second tag set comprises a plurality of tags, wherein each tag in the second tag set comprises a same test tag that is distinguishable from the control tag and an identifying tag, and wherein the second tag set comprises a plurality of different identifying tags; (c) mixing tagged control parent polynucleotides with tagged test parent polynucleotides to form a pool; (d) amplifying tagged parent polynucleotides in the pool to form a pool of amplified, tagged polynucleotides; (e) sequencing amplified, tagged polynucleotides in the amplified pool to produce a plurality of sequence reads; (f) grouping sequence reads into families, each family comprising sequence reads generated from a same parent polynucleotide, which grouping is optionally based on information from an identifying tag and from start/end sequences of the parent polynucleotides, and, optionally, determining a consensus sequence for each of a plurality of parent polynucleotides from the plurality of sequence reads in a group; (g) classifying each family or consensus sequence as a control parent polynucleotide or as a test parent polynucleotide based on having a test tag or a control tag; (h) determining a quantitative measure of control parent polynucleotides and control test polynucleotides mapping to each of at least two genetic loci; and (i) determining copy number variation in the test parent polynucleotides at at least one locus based on relative quantity of test parent polynucleotides and control parent polynucleotides mapping to the at least one locus.

[0057] In another aspect, a method comprises (a) generating a plurality of sequence reads from a plurality of template polynucleotides, each polynucleotide mapped to a genomic locus;

(b) grouping the sequence reads into families, each family comprising sequence reads generated from one of the template polynucleotides; (c) calling a base (or sequence) at the genomic locus for each of the families; (d) detecting at the genomic locus any of genomic alterations among the calls, frequency of a genetic alteration among the calls, total number of calls and total number of alterations among the calls.

[0058] In some embodiments, calling comprises any of phylogenetic analysis, voting, weighing, assigning a probability to each read at the locus in a family, and calling the base with the highest probability. In some embodiments, the method is performed at two loci, comprising determining CNV at one of the loci based on counts at each of the loci.

[0059] Another aspect provides a method for determining a quantitative measure indicative of a number of individual double-stranded DNA fragments in a sample comprising (a) determining a quantitative measure of individual DNA molecules for which both strands are detected; (b) determining a quantitative measure of individual DNA molecules for which only one of the DNA strands are detected; (c) inferring from (a) and (b) above a quantitative measure of individual DNA molecules for which neither strand was detected; and (d) using (a)-(c) determining the quantitative measure indicative of a number of individual double-stranded DNA fragments in the sample.

[0060] In some embodiments, the method further comprises detecting copy number variation in the sample by determining a normalized quantitative measure determined in step (d) at each of one or more genetic loci and determining copy number variation based on the normalized measure. In some embodiments, the sample comprises double-stranded polynucleotide molecules sourced substantially from cell-free nucleic acids.

[0061] In some embodiments, determining the quantitative measure of individual DNA molecules comprises tagging the DNA molecules with a set of duplex tags, wherein each duplex tag differently tags complementary strands of a double-stranded DNA molecule in the sample to provide tagged strands. In some embodiments, the method further comprises sequencing at least some of the tagged strands to produce a set of sequence reads. In some embodiments, the method further comprises sorting sequence reads into paired reads and unpaired reads, wherein (i) each paired read corresponds to sequence reads generated from a first tagged strand and a second differently tagged complementary strand derived from a double-stranded polynucleotide

molecule in the set, and (ii) each unpaired read represents a first tagged strand having no second differently tag complementary strand derived from a double-stranded polynucleotide molecule represented among the sequence reads in the set of sequence reads. In some embodiments, the method further comprises determining quantitative measures of (i) the paired reads and (ii) the unpaired reads that map to each of one or more genetic loci to determine a quantitative measure of total double-stranded DNA molecules in the sample that map to each of the one or more genetic loci based on the quantitative measure of paired reads and unpaired reads mapping to each locus.

[0062] In another aspect, a method for reducing distortion in a sequencing assay, comprises (a) tagging control parent polynucleotides with a first tag set to produce tagged control parent polynucleotides; (b) tagging test parent polynucleotides with a second tag set to produce tagged test parent polynucleotides; (c) mixing tagged control parent polynucleotides with tagged test parent polynucleotides to form a pool; (d) determining quantities of tagged control parent polynucleotides and tagged test parent polynucleotides; and (e) using the quantities of tagged control parent polynucleotides to reduce distortion in the quantities of tagged test parent polynucleotides.

[0063] In some embodiments, the first tag set comprises a plurality of tags, wherein each tag in the first tag set comprises a same control tag and an identifying tag, and wherein the first tag set comprises a plurality of different identifying tags. In some embodiments, the second tag set comprises a plurality of tags, wherein each tag in the second tag set comprises a same test tag and an identifying tag, wherein the test tag is distinguishable from the control tag, and wherein the second tag set comprises a plurality of different identifying tags. In some embodiments, (d) comprises amplifying tagged parent polynucleotides in the pool to form a pool of amplified, tagged polynucleotides, and sequencing amplified, tagged polynucleotides in the amplified pool to produce a plurality of sequence reads. In some embodiments, the method further comprises grouping sequence reads into families, each family comprising sequence reads generated from a same parent polynucleotide, which grouping is optionally based on information from an identifying tag and from start/end sequences of the parent polynucleotides, and, optionally, determining a consensus sequence for each of a plurality of parent polynucleotides from the plurality of sequence reads in a group.

[0064] In some embodiments, (d) comprises determining copy number variation in the test parent polynucleotides at greater than or equal to one locus based on relative quantity of test parent polynucleotides and control parent polynucleotides mapping to the locus.

[0065] Another aspect provides a method comprising (a) ligating adaptors to double-stranded DNA polynucleotides, wherein ligating is performed in a single reaction vessel, and wherein the adaptors comprise molecular barcodes, to produce a tagged library comprising an insert from the double-stranded DNA polynucleotides, and having between 4 and 1 million different tags; (b) generating a plurality of sequence reads for each of the double-stranded DNA polynucleotides in the tagged library; (c) grouping sequence reads into families, each family comprising sequence reads generated from a single DNA polynucleotide among the double-stranded DNA polynucleotides, based on information in a tag and information at an end of the insert; and (d) calling bases at each position in the double-stranded DNA molecule based on bases at the position in members of a family. In some embodiments, (b) comprises amplifying each of the double-stranded DNA polynucleotide molecules in the tagged library to generate amplification products, and sequencing the amplification products. In some embodiments, the method further comprises sequencing the double-stranded DNA polynucleotide molecules a plurality of times. In some embodiments, (b) comprises sequencing the entire insert. In some embodiments, (c) further comprises collapsing sequence reads in each family to generate a consensus sequence. In some embodiments, (d) comprises calling a plurality of sequential bases from at least a subset of the sequence reads to identify single nucleotide variations (SNV) in the double-stranded DNA molecule.

[0066] Another aspect provides a method of detecting disease cell heterogeneity from a sample comprising polynucleotides from somatic cells and disease cells. The method comprises quantifying polynucleotides in the sample bearing a nucleotide sequence variant at each of a plurality of genetic loci; determining copy number variation (CNV) at each of the plurality of genetic loci, wherein the CNV indicates a genetic dose of a locus in the disease cell polynucleotides; determining with a programmed computer processor a relative measure of quantity of polynucleotides bearing a sequence variant at a locus per the genetic dose at the locus for each of a plurality of the loci; and comparing the relative measures at each of the plurality of loci, wherein different relative measures is indicative of tumor heterogeneity.

[0067] In another aspect, a method comprises subjecting a subject to one or more pulsed therapy cycles, each pulsed therapy cycle comprising (a) a first period during which a drug is administered at a first amount; and (b) a second period during which the drug is administered at a second, reduced amount, wherein (i) the first period is characterized by a tumor burden detected above a first clinical level; and (ii) the second period is characterized by a tumor burden detected below a second clinical level.

[0068] Additional aspects and advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description, wherein only illustrative embodiments of the present disclosure are shown and described. As will be realized, the present disclosure is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the disclosure. Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

INCORPORATION BY REFERENCE

[0069] 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

[0001] 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 detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings (also "figure" and "FIG." herein), of which:

[0070] **FIG. 1** is a flowchart representation of a method of the present disclosure for determining copy number variation (CNV);

[0071] **FIG. 2** depicts mapping of pairs and singlets to Locus A and Locus B in a genome;

[0072] **FIG. 3** shows a reference sequence encoding a genetic Locus A;

[0073] **FIGs. 4A-C** shows amplification, sequencing, redundancy reduction and pairing of complementary molecules;

[0074] **FIG. 5** shows increased confidence in detecting sequence variants by pairing reads from Watson and Crick strands;

[0075] **FIG. 6** shows a computer system that is programmed or otherwise configured to implement various methods of the present disclosure;

[0076] **FIG. 7** is schematic representation of a system for analyzing a sample comprising nucleic acids from a user, including a sequencer; bioinformatic software and internet connection for report analysis by, for example, a hand held device or a desk top computer;

[0077] **FIG. 8** is a flowchart representation of a method of this invention for determining CNV using pooled test and control pools; and

[0078] **FIGs. 9A-9C** schematically illustrate a method for tagging a polynucleotide molecule with a library adaptor and subsequently a sequencing adaptor.

DETAILED DESCRIPTION

[0079] While various embodiments of the 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 may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

[0080] The term "genetic variant," as used herein, generally refers to an alteration, variant or polymorphism in a nucleic acid sample or genome of a subject. Such alteration, variant or polymorphism can be with respect to a reference genome, which may be a reference genome of the subject or other individual. Single nucleotide polymorphisms (SNPs) are a form of polymorphisms. In some examples, one or more polymorphisms comprise one or more single nucleotide variations (SNVs), insertions, deletions, repeats, small insertions, small deletions, small repeats, structural variant junctions, variable length tandem repeats, and/or flanking sequences,. Copy number variants (CNVs), transversions and other rearrangements are also forms of genetic variation. A genomic alternation may be a base change, insertion, deletion, repeat, copy number variation, or transversion.

[0081] The term "polynucleotide," as used herein, generally refers to a molecule comprising one or more nucleic acid subunits. A polynucleotide can include one or more subunits selected from adenosine (A), cytosine (C), guanine (G), thymine (T) and uracil (U), or variants thereof. A

nucleotide can include A, C, G, T or U, or variants thereof. A nucleotide can include any subunit that can be incorporated into a growing nucleic acid strand. Such subunit can be an A, C, G, T, or U, or any other subunit that is specific to one or more complementary A, C, G, T or U, or complementary to a purine (i.e., A or G, or variant thereof) or a pyrimidine (i.e., C, T or U, or variant thereof). A subunit can enable individual nucleic acid bases or groups of bases (*e.g.*, AA, TA, AT, GC, CG, CT, TC, GT, TG, AC, CA, or uracil-counterparts thereof) to be resolved. In some examples, a polynucleotide is deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), or derivatives thereof. A polynucleotide can be single-stranded or double stranded.

[0082] The term "subject," as used herein, generally refers to an animal, such as a mammalian species (*e.g.*, human) or avian (*e.g.*, bird) species, or other organism, such as a plant. More specifically, the subject can be a vertebrate, a mammal, a mouse, a primate, a simian or a human. Animals include, but are not limited to, farm animals, sport animals, and pets. A subject can be a healthy individual, an individual that has or is suspected of having a disease or a pre-disposition to the disease, or an individual that is in need of therapy or suspected of needing therapy. A subject can be a patient.

[0083] The term "genome" generally refers to an entirety of an organism's hereditary information. A genome can be encoded either in DNA or in RNA. A genome can comprise coding regions that code for proteins as well as non-coding regions. A genome can include the sequence of all chromosomes together in an organism. For example, the human genome has a total of 46 chromosomes. The sequence of all of these together constitutes a human genome.

[0084] The terms "adaptor(s)", "adapter(s)" and "tag(s)" are used synonymously throughout this specification. An adaptor or tag can be coupled to a polynucleotide sequence to be "tagged" by any approach including ligation, hybridization, or other approaches.

[0085] The term "library adaptor" or "library adapter" as used herein, generally refers to a molecule (e.g., polynucleotide) whose identity (e.g., sequence) can be used to differentiate polynucleotides in a biological sample (also "sample" herein).

[0086] The term "sequencing adaptor," as used herein, generally refers to a molecule (e.g., polynucleotide) that is adapted to permit a sequencing instrument to sequence a target polynucleotide, such as by interacting with the target polynucleotide to enable sequencing. The sequencing adaptor permits the target polynucleotide to be sequenced by the sequencing

instrument. In an example, the sequencing adaptor comprises a nucleotide sequence that hybridizes or binds to a capture polynucleotide attached to a solid support of a sequencing system, such as a flow cell. In another example, the sequencing adaptor comprises a nucleotide sequence that hybridizes or binds to a polynucleotide to generate a hairpin loop, which permits the target polynucleotide to be sequenced by a sequencing system. The sequencing adaptor can include a sequencer motif, which can be a nucleotide sequence that is complementary to a flow cell sequence of other molecule (e.g., polynucleotide) and usable by the sequencing system to sequence the target polynucleotide. The sequencer motif can also include a primer sequence for use in sequencing, such as sequencing by synthesis. The sequencer motif can include the sequence(s) needed to couple a library adaptor to a sequencing system and sequence the target polynucleotide.

[0087] As used herein the terms "at least", "at most" or "about", when preceding a series, refers to each member of the series, unless otherwise identified.

[0088] The term *"about"* and its grammatical equivalents in relation to a reference numerical value can include a range of values up to plus or minus 10% from that value. For example, the amount *"about 10"* can include amounts from 9 to 11. In other embodiments, the term *"about"* in relation to a reference numerical value can include a range of values plus or minus 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% from that value.

[0089] The term "at least" and its grammatical equivalents in relation to a reference numerical value can include the reference numerical value and greater than that value. For example, the amount "at least 10" can include the value 10 and any numerical value above 10, such as 11, 100, and 1,000.

[0090] The term "at most" and its grammatical equivalents in relation to a reference numerical value can include the reference numerical value and less than that value. For example, the amount "at most 10" can include the value 10 and any numerical value under 10, such as 9, 8, 5, 1, 0.5, and 0.1.

[0091] **1. <u>Methods for processing and/or analyzing a nucleic acid sample</u>**

[0092] An aspect of the present disclosure provides methods for determining a genomic alternation in a nucleic acid sample of a subject. **FIG. 1** shows a method of determining copy

number variation (CNV). The method can be implemented to determine other genomic alternations, such as SNVs.

[0093] **A. Polynucleotide Isolation**

[0094] Methods disclosed herein can comprise isolating one or more polynucleotides. A polynucleotide can comprise any type of nucleic acid, for example, a sequence of genomic nucleic acid, or an artificial sequence (*e.g.*, a sequence not found in genomic nucleic acid). For example, an artificial sequence can contain non-natural nucleotides. Also, a polynucleotide can comprise both genomic nucleic acid and an artificial sequence, in any portion. For example, a polynucleotide can comprise 1 to 99% of genomic nucleic acid and 99% to 1% of artificial sequence, where the total adds up to 100%. Thus, fractions of percentages are also contemplated. For example, a ratio of 99.1% to 0.9% is contemplated.

[0095] A polynucleotide can comprise any type of nucleic acids, such as DNA and/or RNA. For example, if a polynucleotide is DNA, it can be genomic DNA, complementary DNA (cDNA), or any other deoxyribonucleic acid. A polynucleotide can also be cell-free DNA (cfDNA). For example, the polynucleotide can be circulating DNA. The circulating DNA can comprise circulating tumor DNA (ctDNA). A polynucleotide can be double-stranded or single-stranded. Alternatively, a polynucleotide can comprise a combination of a double-stranded portion and a single-stranded portion.

[0096] Polynucleotides do not have to be cell-free. In some cases, the polynucleotides can be isolated from a sample. For example, in step (102) (**FIG. 1**), double-stranded polynucleotides are isolated from a sample. A sample can be any biological sample isolated from a subject. For example, a sample can comprise, without limitation, bodily fluid, whole blood, platelets, serum, plasma, stool, red blood cells, white blood cells or leucocytes, endothelial cells, tissue biopsies, synovial fluid, lymphatic fluid, ascites fluid, interstitial or extracellular fluid, the fluid in spaces between cells, including gingival crevicular fluid, bone marrow, cerebrospinal fluid, saliva, mucous, sputum, semen, sweat, urine, or any other bodily fluids. A bodily fluid can include saliva, blood, or serum. For example, a polynucleotide can be cell-free DNA isolated from a bodily fluid, *e.g.*, blood or serum. A sample can also be a tumor sample, which can be obtained from a subject by various approaches, including, but not limited to, venipuncture, excretion,

ejaculation, massage, biopsy, needle aspirate, lavage, scraping, surgical incision, or intervention or other approaches.

[0097] A sample can comprise various amount of nucleic acid that contains genome equivalents. For example, a sample of about 30 ng DNA can contain about 10,000 (10^4) haploid human genome equivalents and, in the case of cfDNA, about 200 billion ($2x10^{11}$) individual polynucleotide molecules. Similarly, a sample of about 100 ng of DNA can contain about 30,000 haploid human genome equivalents and, in the case of cfDNA, about 600 billion individual molecules.

[0098] A sample can comprise nucleic acids from different sources. For example, a sample can comprise germline DNA or somatic DNA. A sample can comprise nucleic acids carrying mutations. For example, a sample can comprise DNA carrying germline mutations and/orsomatic mutations, . A sample can also comprise DNA carrying cancer-associated mutations (*e.g.*, cancer-associated somatic mutations).

[0099] **B. Tagging**

[00100] Polynucleotides disclosed herein can be tagged. For example, in step (104) (**FIG. 1**) the double-stranded polynucleotides are tagged with duplex tags, tags that differently label the complementary strands (*i.e.*, the "Watson" and "Crick" strands) of a double-stranded molecule. In one embodiment the duplex tags are polynucleotides having complementary and non-complementary portions.

[00101] Tags can be any types of molecules attached to a polynucleotide, including, but not limited to, nucleic acids, chemical compounds, florescent probes, or radioactive probes. Tags can also be oligonucleotides (*e.g.*, DNA or RNA). Tags can comprise known sequences, unknown sequences, or both. A tag can comprise random sequences, pre-determined sequences, or both. A tag can be double-stranded or single-stranded. A double-stranded tag can be a duplex tag. A double-stranded tag can comprise two complementary strands. Alternatively, a double-stranded tag can comprise a hybridized portion and a non-hybridized portion. The double-stranded tag can be Y-shaped, *e.g.*, the hybridized portion is at one end of the tag and the non-hybridized portion is at the opposite end of the tag. One such example are the "Y adapters" used in Illumina sequencing. Other examples include hairpin shaped adapters or bubble shaped

adapters. Bubble shaped adapters have non-complementary sequences flanked on both sides by complementary sequences.

[00102] Tagging disclosed herein can be performed using any method. A polynucleotide can be tagged with an adaptor by hybridization. For example, the adaptor can have a nucleotide sequence that is complementary to at least a portion of a sequence of the polynucleotide. As an alternative, a polynucleotide can be tagged with an adaptor by ligation.

[00103] For example, tagging can comprise using one or more enzymes. The enzyme can be a ligase. The ligase can be a DNA ligase. For example, the DNA ligase can be a T4 DNA ligase, *E. coli* DNA ligase, and/or mammalian ligase. The mammalian ligase can be DNA ligase I, DNA ligase III, or DNA ligase IV. The ligase can also be a thermostable ligase. Tags can be ligated to a blunt-end of a polynucleotide (blunt-end ligation). Alternatively, tags can be ligated to a sticky end of a polynucleotide (sticky-end ligation). Efficiency of ligation can be increased by optimizing various conditions. Efficiency of ligation can be increased by optimizing the reaction time of ligation. For example, the reaction time of ligation can be less than 12 hours, *e.g.*, less than 1, less than 2, less than 3, less than 4, less than 5, less than 6, less than 7, less than 8, less than 9, less than 10, less than 11, less than 12, less than 13, less than 14, less than 15, less than 16, less than 17, less than 18, less than 19, or less than 20 hours. In a particular example, reaction time of ligation is less than 20 hours. Efficiency of ligation can be increased by optimizing the ligase concentration in the reaction. For example, the ligase concentration can be at least 10, at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400, at least 500, or at least 600 unit/microliter. Efficiency can also be optimized by adding or varying the concentration of an enzyme suitable for ligation, enzyme cofactors or other additives, and/or optimizing a temperature of a solution having the enzyme. Efficiency can also be optimized by varying the addition order of various components of the reaction. The end of tag sequence can comprise dinucleotide to increase ligation efficiency. When the tag comprises a non-complementary portion (e.g., Y-shaped adaptor), the sequence on the complementary portion of the tag adaptor can comprise one or more selected sequences that promote ligation efficiency. Preferably such sequences are located at the terminal end of the tag. Such sequences can comprise 1, 2, 3, 4, 5, or 6 terminal bases. Reaction solution with high viscosity (*e.g.*, a low Reynolds number) can also be used to increase ligation efficiency. For example, solution can

have a Reynolds number less than 3000, less than 2000, less than 1000, less than 900, less than 800, less than 700, less than 600, less than 500, less than 400, less than 300, less than 200, less than 100, less than 50, less than 25, or less than 10. It is also contemplated that roughly unified distribution of fragments (*e.g.*, tight standard deviation) can be used to increase ligation efficiency. For example, the variation in fragment sizes can vary by less than 20%, less than 15%, less than 10%, less than 5%, or less than 1%. Tagging can also comprise primer extension, for example, by polymerase chain reaction (PCR). Tagging can also comprise any of ligation-based PCR, multiplex PCR, single strand ligation, or single strand circularization.

[00104] In some instances, the tags herein comprise molecular barcodes. Such molecular barcodes can be used to differentiate polynucleotides in a sample. Preferably molecular barcodes are different from one another. For example, molecular barcodes can have a difference between them that can be characterized by a predetermined edit distance or a Hamming distance. In some instances, the molecular barcodes herein have a minimum edit distance of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. To further improve efficiency of conversion (e.g., tagging) of untagged molecular to tagged molecules, one preferably utilizes short tags. For example, in some embodiments, a library adapter tag can be up to 65, 60, 55, 50, 45, 40, or 35 nucleotide bases in length. A collection of such short library barcodes preferably includes a number of different molecular barcodes, e.g., at least 2, 4, 6, 8, 10, 12, 14, 16, 18 or 20 different barcodes with a minimum edit distance of 1, 2, 3 or more.

[00105] Thus, a collection of molecules can include one or more tags. In some instances, some molecules in a collection can include an identifying tag ("identifier") such as a molecular barcode that is not shared by any other molecule in the collection. For example, in some instances of a collection of molecules, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the molecules in the collection

can include an identifier or molecular barcode that is not shared by any other molecule in the collection. As used herein, a collection of molecules is considered to be "uniquely tagged" if each of at least 95% of the molecules in the collection bears an identifier that is not shared by any other molecule in the collection ("unique tag" or "unique identifier"). A collection of molecules is considered to be "non-uniquely tagged" if each of at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or at least or about 50% of the molecules in the collection bears an identifying tag or molecular barcode that is shared by at least one other molecule in the collection ("non-unique tag" or "non-unique identifier"). Accordingly, in a non-uniquely tagged population no more than 1% of the molecules are uniquely tagged. For example, in a non-uniquely tagged population, no more than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% of the molecules can be uniquely tagged.

[00106] A number of different tags can be used based on the estimated number of molecules in a sample. In some tagging methods, the number of different tags can be at least the same as the estimated number of molecules in the sample. In other tagging methods, the number of different tags can be at least two, three, four, five, six, seven, eight, nine, ten, one hundred or one thousand times as many as the estimated number of molecules in the sample. In unique tagging, at least two times (or more) as many different tags can be used as the estimated number of molecules in the sample.

[00107] The molecules in the sample may be non-uniquely tagged. In such instances a fewer number of tags or molecular barcodes is used then the number of molecules in the sample to be tagged. For example, no more than 100, 50, 40, 30, 20 or 10 unique tags or molecular barcodes are used to tag a complex sample such as a cell free DNA sample with many more different fragments.

[00108] The polynucleotide to be tagged can be fragmented, such as either naturally or using other approaches, such as, for example, shearing. The polynucleotides can be fragmented by certain methods, including but not limited to, mechanical shearing, passing the sample through a syringe, sonication, heat treatment (*e.g.*, for 30 minutes at 90°C), and/or nuclease treatment (*e.g.*, using DNase, RNase, endonuclease, exonuclease, and/or restriction enzyme).

[00109] The polynucleotides fragments (prior to tagging) can comprise sequences of any length. For example, polynucleotide fragments (prior to tagging) can comprise at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000 or more nucleotides in length. The polynucleotide fragment are preferably about the average length of cell-free DNA. For example, the polynucleotide fragments can comprise about 160 bases in length. The polynucleotide fragment can also be fragmented from a larger fragment into smaller fragments about 160 bases in length.

[00110] Polynucleotides tagged can comprise sequences associated with cancer. The cancer-associated sequences can comprise single nucleotide variation (SNV), copy number variation (CNV), insertions, deletions, and/or rearrangements.

[00111] The polynucleotides can comprise sequences associated with cancer, such as acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), adrenocortical carcinoma, Kaposi Sarcoma, anal cancer, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, osteosarcoma, malignant fibrous histiocytoma, brain stem glioma, brain cancer, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloeptithelioma, pineal parenchymal tumor, breast cancer, bronchial tumor, Burkitt lymphoma, Non-Hodgkin lymphoma, carcinoid tumor, cervical cancer, chordoma, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), colon cancer, colorectal cancer, cutaneous T-cell lymphoma, ductal carcinoma in situ, endometrial cancer, esophageal cancer, Ewing Sarcoma, eye cancer, intraocular melanoma, retinoblastoma, fibrous histiocytoma, gallbladder cancer, gastric cancer, glioma, hairy cell leukemia, head and neck cancer, heart cancer, hepatocellular (liver) cancer, Hodgkin lymphoma, hypopharyngeal cancer, kidney cancer, laryngeal cancer, lip cancer, oral cavity cancer, lung cancer, non-small cell carcinoma, small cell carcinoma, melanoma, mouth cancer, myelodysplastic syndromes, multiple myeloma, medulloblastoma, nasal cavity cancer, paranasal sinus cancer, neuroblastoma, nasopharyngeal cancer, oral cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, papillomatosis, paraganglioma, parathyroid cancer, penile cancer, pharyngeal cancer, pituitary tumor, plasma

cell neoplasm, prostate cancer, rectal cancer, renal cell cancer, rhabdomyosarcoma, salivary gland cancer, Sezary syndrome, skin cancer, nonmelanoma, small intestine cancer, soft tissue sarcoma, squamous cell carcinoma, testicular cancer, throat cancer, thymoma, thyroid cancer, urethral cancer, uterine cancer, uterine sarcoma, vaginal cancer, vulvar cancer, Waldenstrom macroglobulinemia, and/or Wilms Tumor.

[00112] A haploid human genome equivalent has about 3 picograms of DNA. A sample of about 1 microgram of DNA contains about 300,000 haploid human genome equivalents. Improvements in sequencing can be achieved as long as at least some of the duplicate or cognate polynucleotides bear unique identifiers with respect to each other, that is, bear different tags. However, in certain embodiments, the number of tags used is selected so that there is at least a 95% chance that all duplicate molecules starting at any one position bear unique identifiers. For example, in a sample comprising about 10,000 haploid human genome equivalents of fragmented genomic DNA, *e.g.*, cfDNA, z is expected to be between 2 and 8. Such a population can be tagged with between about 10 and 100 different identifiers, for example, about 2 identifiers, about 4 identifiers, about 9 identifiers, about 16 identifiers, about 25 identifiers, about 36 different identifiers, about 49 different identifiers, about 64 different identifiers, about 81 different identifiers, or about 100 different identifiers.

[00113] Nucleic acid barcodes having identifiable sequences including molecular barcodes, can be used for tagging. For example, a plurality of DNA barcodes can comprise various numbers of sequences of nucleotides. A plurality of DNA barcodes having 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 or more identifiable sequences of nucleotides can be used. When attached to only one end of a polynucleotide, the plurality of DNA barcodes can produce 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 or more different identifiers. Alternatively, when attached to both ends of a polynucleotide, the plurality DNA barcodes can produce 4, 9, 16, 25, 36, 49, 64, 81, 100, 121, 144, 169, 196, 225, 256, 289, 324, 361, 400 or more different identifiers (which is the ^2 of when the DNA barcode is attached to only 1 end of a polynucleotide). In one example, a plurality of DNA barcodes having 6, 7, 8, 9 or 10 identifiable sequences of nucleotides can be used. When attached to both ends of a polynucleotide, they produce 36, 49, 64, 81 or 100 possible different identifiers, respectively. In

a particular example, the plurality of DNA barcodes can comprise 8 identifiable sequences of nucleotides. When attached to only one end of a polynucleotide, the plurality of DNA barcodes can produce 8 different identifiers. Alternatively, when attached to both ends of a polynucleotide, the plurality of DNA barcodes can produce 64 different identifiers. Samples tagged in such a way can be those with a range of about 10 ng to any of about 100 ng, about 1 μg, about 10 μg of fragmented polynucleotides, *e.g.*, genomic DNA, *e.g.*, cfDNA.

[00114] A polynucleotide can be uniquely identified in various ways. A polynucleotide can be uniquely identified by a unique DNA barcode. For example, any two polynucleotides in a sample are attached two different DNA barcodes. Alternatively, a polynucleotide can be uniquely identified by the combination of a DNA barcode and one or more endogenous sequences of the polynucleotide. For example, any two polynucleotides in a sample can be attached the same DNA barcode, but the two polynucleotides can still be identified by different endogenous sequences. The endogenous sequence can be on an end of a polynucleotide. For example, the endogenous sequence can be adjacent (*e.g.*, base in between) to the attached DNA barcode. In some instances the endogenous sequence can be at least 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 bases in length. Preferably, the endogenous sequence is a terminal sequence of the fragment/polynucleotides to be analyzed. The endogenous sequence may be the length of the sequence. For example, a plurality of DNA barcodes comprising 8 different DNA barcodes can be attached to both ends of each polynucleotide in a sample. Each polynucleotide in the sample can be identified by the combination of the DNA barcodes and about 10 base pair endogenous sequence on an end of the polynucleotide. Without being bound by theory, the endogenous sequence of a polynucleotide can also be the entire polynucleotide sequence.

[00115] Also disclosed herein are compositions of tagged polynucleotides. The tagged polynucleotide can be single-stranded. Alternatively, the tagged polynucleotide can be double-stranded (*e.g.*, duplex-tagged polynucleotides). Accordingly, this invention also provides compositions of duplex-tagged polynucleotides. The polynucleotides can comprise any types of nucleic acids (DNA and/or RNA). The polynucleotides comprise any types of DNA disclosed herein. For example, the polynucleotides can comprise DNA, *e.g.*, fragmented DNA or cfDNA. A set of polynucleotides in the composition that map to a mappable base position in a genome can be non-uniquely tagged, that is, the number of different identifiers can be at least 2 and fewer

than the number of polynucleotides that map to the mappable base position. The number of different identifiers can also be at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25and fewer than the number of polynucleotides that map to the mappable base position.

[00116] In some instances, as a composition goes from about 1 ng to about 10 μg or higher, a larger set of different molecular barcodes can be used. For example, between 5 and 100 different library adaptors can be used to tag polynucleotides in a cfDNA sample.

[00117] The systems and methods disclosed herein may be used in applications that involve the assignment of molecular barcodes. The molecular barcodes can be assigned to any types of polynucleotides disclosed in this invention. For example, the molecular barcodes can be assigned to cell-free polynucleotides (*e.g.*, cfDNAs). Often, an identifier disclosed herein can be a barcode oligonucleotide that is used to tag the polynucleotide. The barcode identifier may be a nucleic acid oligonucleotide (*e.g.*, a DNA oligonucleotide). The barcode identifier can be single-stranded. Alternatively, the barcode identifier can be double-stranded. The barcode identifier can be attached to polynucleotides using any method disclosed herein. For example, the barcode identifier can be attached to the polynucleotide by ligation using an enzyme. The barcode identifier can also be incorporated into the polynucleotide through PCR. In other cases, the reaction may comprise addition of a metal isotope, either directly to the analyte or by a probe labeled with the isotope. Generally, assignment of unique or non-unique identifiers or molecular barcodes in reactions of this disclosure may follow methods and systems described by, for example, U.S. patent applications 2001/0053519, 2003/0152490, 2011/0160078 and U.S. Patent No. 6,582,908, each of which is entirely incorporated herein by reference.

[00118] Identifiers or molecular barcodes used herein may be completely endogenous whereby circular ligation of individual fragments may be performed followed by random shearing or targeted amplification. In this case, the combination of a new start and stop point of the molecule and the original intramolecular ligation point can form a specific identifier.

[00119] Identifiers or molecular barcodes used herein can comprise any types of oligonucleotides. In some cases, identifiers may be predetermined, random, or semi-random sequence oligonucleotides. Identifiers can be barcodes. For example, a plurality of barcodes may be used such that barcodes are not necessarily unique to one another in the plurality.

Alternatively, a plurality of barcodes may be used such that each barcode is unique to any other barcode in the plurality. The barcodes can comprise specific sequences (*e.g.*, predetermined sequences) that can be individually tracked. Further, barcodes may be attached (*e.g.*, by ligation) to individual molecules such that the combination of the barcode and the sequence it may be ligated to creates a specific sequence that may be individually tracked. As described herein, detection of barcodes in combination with sequence data of beginning (start) and/or end (stop) portions of sequence reads can allow assignment of a unique identity to a particular molecule. The length or number of base pairs of an individual sequence read may also be used to assign a unique identity to such a molecule. As described herein, fragments from a single strand of nucleic acid having been assigned a unique identity, may thereby permit subsequent identification of fragments from the parent strand. In this way the polynucleotides in the sample can be uniquely or substantially uniquely tagged. A duplex tag can include a degenerate or semi-degenerate nucleotide sequence, *e.g.*, a random degenerate sequence. The nucleotide sequence can comprise any number of nucleotides. For example, the nucleotide sequence can comprise 1 (if using a non-natural nucleotide), 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 nucleotides. In a particular example, the sequence can comprise 7 nucleotides. In another example, the sequence can comprise 8 nucleotides. The sequence can also comprise 9 nucleotides. The sequence can comprise 10 nucleotides.

[00120] A barcode can comprise contiguous or non-contiguous sequences. A barcode that comprises at least 1, 2, 3, 4, 5 or more nucleotides is a contiguous sequence or non-contiguous sequence. if the 4 nucleotides are uninterrupted by any other nucleotide. For example, if a barcode comprises the sequence TTGC, a barcode is contiguous if the barcode is TTGC. On the other hand, a barcode is non-contiguous if the barcode is TTXGC, where X is a nucleic acid base.

[00121] An identifier or molecular barcode can have an n-mer sequence which may be 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 nucleotides in length. A tag herein can comprise any range of nucleotides in length. For example, the sequence can be between 2 to 100, 10 to 90, 20 to 80, 30 to 70, 40 to 60, or about 50 nucleotides in length.

[00122] The tag can comprise a double-stranded fixed reference sequence downstream of the identifier or molecular barcode. Alternatively, the tag can comprise a double-stranded fixed reference sequence upstream or downstream of the identifier or molecular barcode. Each strand of a double-stranded fixed reference sequence can be, for example, 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 nucleotides in length.

[00123] **C. Adaptors**

[00124] A library of polynucleotide molecules can be synthesized for use in sequencing. For example, a library of polynucleotides comprising a plurality of polynucleotide molecules that are each less than or equal to 100, 90, 80, 70, 60, 50, 45, 40, or 35 nucleic acid (or nucleotide) bases in length can be made. A plurality of polynucleotide molecules can be each less than or equal to 35 nucleic acid bases in length. A plurality of polynucleotide molecules can be each less than or equal to 30 nucleic acid bases in length. A plurality of polynucleotide molecules can also be less than or equal to 250, 200, 150, 100, or 50 nucleic acid bases. Additionally, the plurality of polynucleotide molecules can also be less than or equal to 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, or 10 nucleic acid bases.

[00125] A library of polynucleotides comprising a plurality of polynucleotide molecules can also have distinct (with respect to each other) molecular barcode sequences (or molecular barcodes) with respect to at least 4 nucleic acid bases. A molecular barcode (also "barcode" or "identifier" herein) sequence is a nucleotide sequence that distinguishes one polynucleotide from another. In other embodiments, the polynucleotide molecules can also have different barcode sequences with respect 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 or more nucleic acid bases.

[00126] A library of polynucleotides comprising a plurality of polynucleotide molecules can also have a plurality of different barcode sequences. For example, a plurality of polynucleotide molecules can have at least 4 different molecular barcode sequences. In some cases, the plurality

of polynucleotide molecules has from 2-100, 4-50, 4-30, 4-20, or 4-10 different molecular barcode sequences. The plurality of polynucleotides molecules can also have other ranges of different barcode sequences such as, 1-4, 2-5, 3-6, 4-7, 5-8, 6-9, 7-10, 8-11, 9-12, 10-13, 11-14, 12-15, 13-16, 14-17, 15-18, 16-19, 17-20, 18-21, 19-22, 20-23, 21-24, or 22-25 different barcode sequences. In other cases, a plurality of polynucleotide molecules can have at least 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, or 100 more different barcode sequences. In a particular example, the plurality library adapters comprise at least 8 different sequences.

[00127] The location of the different barcode sequences can vary within the plurality of polynucleotides. For example, the different barcode sequences can be within 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, or 2 nucleic acid bases from a terminal end of a respective one of the plurality of polynucleotide molecules. In an example, a plurality of polynucleotide molecules has distinct barcode sequences that are within 10 nucleic acid bases from the terminal end. In another example, a plurality of polynucleotide molecules has distinct barcode sequences that are within 5 or 1 nucleic acid bases from the terminal end. In other instances, the distinct barcode sequences can be at the terminal end of a respective one of the plurality of polynucleotide molecules. Other variations include that the distinct molecular barcode sequences can be within 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, or 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, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, or more nucleic acid bases from a terminal end of a respective one of the plurality of polynucleotide molecules.

[00128] The terminal end of the plurality of polynucleotide molecules can be adapted for ligation to a target nucleic acid molecule. For example, the terminal end can be a blunt end. In some other cases, the terminal end is adapted for hybridization to a complementary sequence of a target nucleic acid molecule.

[00129] A library of polynucleotides comprising a plurality of polynucleotide molecules can also have an edit distance of at least 1. In some cases, the edit distance is with respect to individual bases of the plurality of polynucleotide molecules. In other cases, the plurality of polynucleotide molecules can have an edit distance of at least 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. The edit distance can be a Hamming distance.

[00130] In some cases, the plurality of polynucleotides does not contain sequencing adaptors. A sequence adaptor can be a polynucleotide that comprises a sequence that hybridizes to one or more sequencing adaptors or primers. A sequencing adaptor can further comprise a sequence hybridizing to a solid support, *e.g.*, a flow cell sequence. The term "flow cell sequence" and its grammatical equivalents as used herein, refers to a sequence that permits hybridization to a substrate, for example, by way of a primer attached to the substrate. The substrate can be bead or a planar surface. In some embodiments, a flow cell sequence can allow a polynucleotide to attach to a flow cell or surface (e.g., surface of a bead, for example, an Illumina flow cell.

[00131] When a plurality of polynucleotide molecules does not contain sequencing adaptors or primers, each polynucleotide molecule of the plurality does not contain a nucleic acid sequence or other moiety that is adapted to permit sequencing of a target nucleic acid molecule with a given sequencing approach, such as Illumina, SOLiD, Pacific Biosciences, GeneReader, Oxford Nanopore, Complete Genomics, Gnu-Bio, Ion Torrent, Oxford Nanopore or Genia. In some examples, when a plurality of polynucleotide molecules does not contain sequencing adaptors or primers, the plurality of polynucleotide molecules does not contain flow cell sequences. For example, the plurality of polynucleotide molecules cannot bind to flow cells, such as used in Illumina flow cell sequencers. However, these flow cell sequences, if desired, can be added to the plurality of polynucleotide molecules by methods such as PCR amplification or ligation. At this point, Illumina flow cell sequencers can be used. Alternatively, when the

plurality of polynucleotide molecules does not contain sequencing adaptors or primers, the plurality of polynucleotide molecules does not contain hairpin shaped adaptors or adaptors for generating hairpin loops in a target nucleic acid molecule, such as Pacific Bioscience SMRTbell™ adaptors. However, these hairpin shaped adaptors, if desired, can be added to the plurality of polynucleotide molecules by methods such as PCR amplification or ligation. The plurality of polynucleotide molecules can be circular or linear.

[00132] A plurality of polynucleotide molecules can be double stranded. In some cases, the plurality of polynucleotide molecules can be single stranded, or can comprise hybridized and non-hybridized regions. A plurality of polynucleotide molecules can be non-naturally occurring polynucleotide molecules.

[00133] Adaptors can be polynucleotide molecules. The polynucleotide molecules can be Y-shaped, bubble-shaped or hairpin-shaped. A hairpin adaptor may contain a restriction site(s) or a Uracil containing base. Adaptors can comprise a complementary portion and a non-complementary portion. The non-complementary portion can have an edit distance (*e.g.*, Hamming distance). For example, the edit distance can be at least 1, at least 2, at least 3, at least 4, 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 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, or at least 30. The complementary portion of the adaptor can comprise sequences that are selected to enable and/or promote ligation to a polynucleotide, *e.g.*, a sequence to enable and/or promote ligation to a polynucleotide at a high yield.

[00134] A plurality of polynucleotide molecules as disclosed herein can be purified. In some cases, a plurality of polynucleotide molecules as disclosed herein can be isolated polynucleotide molecules. In other cases, a plurality of polynucleotide molecules as disclosed herein can be purified and isolated polynucleotide molecules.

[00135] In certain aspects, each of the plurality of polynucleotide molecules is Y-shaped or hairpin-shaped. Each of the plurality of polynucleotide molecules can comprise a different barcode. The different barcode can be a randomer in the complementary portion (*e.g.*, double stranded portion) of the Y-shaped or hairpin-shaped adaptor. Alternatively, the different barcode can be in one strand of the non-complementary portion (*e.g.*, one of the Y-shaped arms). As

discussed above, the different barcode can be at least 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, or more (or any length as described throughout) nucleic acid bases, *e.g.*, 7 bases. The barcode can be contiguous or non-contiguous sequences, as described above. The plurality of polynucleotide molecules is from 10 nucleic acid bases to 35 nucleic acid bases (or any length as described above) in length. Further, the plurality of polynucleotide molecules can comprise an edit distance (as described above), that is a Hamming distance. A plurality of polynucleotide molecules can have distinct barcode sequences that are within 10 nucleic acid bases from the terminal end.

[00136] In another aspect, a plurality of polynucleotide molecules can be sequencing adaptors. A sequencing adaptor can comprise a sequence hybridizing to one or more sequencing primers. A sequencing adaptor can further comprise a sequence hybridizing to a solid support, *e.g.*, a flow cell sequence. For example, a sequencing adaptor can be a flow cell adaptor. The sequencing adaptors can be attached to one or both ends of a polynucleotide fragment. In another example, a sequencing adaptor can be hairpin shaped. For example, the hairpin shaped adaptor can comprise a complementary double-stranded portion and a loop portion, where the double-stranded portion can be attached (*e.g.*, ligated) to a double-stranded polynucleotide. Hairpin shaped sequencing adaptors can be attached to both ends of a polynucleotide fragment to generate a circular molecule, which can be sequenced multiple times. A sequencing adaptor can be up to 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, or more bases from end to end. For example, a sequencing adaptor can be up to 70 bases from end to end. The sequencing adaptor can comprise 20-30, 20-40, 30-50, 30-60, 40-60, 40-70, 50-60, 50-70, bases from end to end. In a particular example, the sequencing adaptor can comprise 20-30 bases from end to end. In another example, the sequencing adaptor can comprise 50-60 bases from end to end. A sequencing adaptor can comprise one or more barcodes. For example, a sequencing adaptor can comprise a sample barcode. The sample barcode can comprise a pre-determined sequence. The sample barcodes can be used to identify the source of the polynucleotides. The sample barcode can be at least 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,

or more (or any length as described throughout) nucleic acid bases, *e.g.*, at least 8 bases. The barcode can be contiguous or non-contiguous sequences, as described above.

[00137] The plurality of polynucleotide molecules as described herein can be used as adaptors. Adaptors can comprise one or more identifiers. An adaptor can comprise an identifier with a random sequence. Alternatively, an adaptor can comprise an identifier with pre-determined sequences. Some adaptors can comprise an identifier with a random sequence and another identifier with a pre-determined sequence. The adaptors comprising identifiers can be double-stranded or single-stranded adaptors. The adaptors comprising identifiers can be Y-shaped adaptors. A Y-shaped adaptor can comprise one or more identifiers with a random sequence. The one or more identifiers can be on the hybrid portion and/or non-hybridized portion of the Y-shaped adaptor. A Y-shaped adaptor can comprise one or more identifiers with a pre-determined sequence. The one or more identifiers with pre-determined sequence can be on the hybridized portion and/or non- hybridized portion of the Y-shaped adaptor. A Y-shaped adaptor can comprise one or more identifiers with a random sequence and one or more identifiers with a pre-determined sequence. For example, the one or more identifiers with a random sequence can be on the hybridized portion of the Y-shaped adaptor and/or the non- hybridized portion of the Y-shaped adaptor. The one or more identifiers with a pre-determined sequence can be on the hybridized portion of the Y-shaped adaptor and/or the non- hybridized portion of the Y-shaped adaptor. In a particular example, a Y-shaped adaptor can comprise an identifier with a random sequence on its hybridized portion and an identifier with a pre-determined sequence on its non- hybridized portion. The identifiers can be in any length disclosed herein. For example, a Y-shaped adaptor can comprise an identifier with a random sequence of 7 nucleotides on its hybridized portion and an identifier with a pre-determined sequence of 8 nucleotides on its non- hybridized portion.

[00138] An adaptor can include a double-stranded portion with a molecular barcode and at least one or two single-stranded portion. For example, the adaptor can be Y-shaped and include a double-stranded portion and two single-stranded portions. The single-stranded portions can include sequences that are not complementary to one another.

[00139] The adaptor can include a terminal end that has a sequence that is selected to permit the adaptor to be efficiently (e.g., at an efficiency of at least about 20%, 30%, 40%, 50%) ligated

or otherwise coupled to a polynucleotide. In some examples, terminal nucleotides in a double-stranded portion of an adaptor are selected from a combination of purines and pyrimidines to provide for efficient ligation.

[00140] In some examples, a set of library adaptors comprises a plurality of polynucleotide molecules (library adaptors) with molecular barcodes. The library adaptors are less than or equal to 80, 70, 60, 50, 45, or 40 nucleotide bases in length. The molecular barcodes can be at least 4 nucleotide bases in length, but may be from 4 to 20 nucleotide bases in length. The molecular barcodes can be different from one another and have an edit distance of at least 1, 2, 3, 4, or 5 between one another. The molecular barcodes are located at least 1, 2, 3, 4, 5, 10, or 20 nucleotide bases away from a terminal end of their respective library adaptors. In some cases, the at least one terminal base is identical in all of the library adaptors.

[00141] The library adaptors can be identical but for the molecular barcodes. For example, the library adaptors can have identical sequences but differ only with respect to nucleotide sequences of the molecular barcodes.

[00142] Each of the library adaptors can have a double stranded portion and at least one single-stranded portion. By "single stranded portion" is meant an area of non-complementarity or an overhang. In some cases, each of the library adaptors has a double-stranded portion and two single-stranded portions. The double-stranded portion can have a molecular barcode. In some cases, the molecular barcode is a randomer. Each of the library adaptors can further include a strand-identification barcode on a single-stranded portion. The strand-identification barcode can include at least 4 nucleotide bases, in some cases from 4 to 20 nucleotide bases.

[00143] In some examples, each of the library adaptors has a double-stranded portion with a molecular barcode and two single-stranded portions. The single-stranded portions may not hybridize to one another. The single-stranded portions may not be completely complementary to one another.

[00144] The library adaptors can have a sequence of terminal nucleotides in a double-stranded portion that are the same. The sequence of terminal nucleotides can be at least 2, 3, 4, 5 or 6 nucleotide bases in length. For example, one strand of a double-stranded portion of the library adaptor can have the sequence ACTT, TCGC, or TACC at the terminal end, while the other strand can have a complementary sequence. In some cases, such a sequence is selected to

optimize the efficiency at which the library adaptors ligate to target polynucleotides. Such sequences can be selected to optimize a binding interaction between the ends of the library adaptors and the target polynucleotides.

[00145] In some cases, none of the library adaptors contains a sample identification motif (or sample molecular barcode). Such sample identification motif can be provided via sequencing adaptors. A sample identification motif can include a sequencer of at least 4, 5, 6, 7, 8, 9, 10, 20, 30, or 40 nucleotide bases that permits the identification of polynucleotide molecules from a given sample from polynucleotide molecules from other samples. For example, this can permit polynucleotide molecules from two subjects to be sequenced in the same pool and sequence reads for the subjects subsequently identified.

[00146] A sequencer motif includes nucleotide sequence(s) needed to couple a library adaptor to a sequencing system and sequence a target polynucleotide coupled to the library adaptor. The sequencer motif can include a sequence that is complementary to a flow cell sequence and a sequence (sequencing initiation sequence) that is selectively hybridizable to a primer (or priming sequence) for use in sequencing. For example, such sequencing initiation sequence can be complementary to a primer that is employed for use in sequence by synthesis (e.g., Illumina). Such primer can be included in a sequencing adaptor. A sequencing initiation sequence can be a primer hybridization site.

[00147] In some cases, none of the library adaptors contains a complete sequencer motif. The library adaptors can contain partial or no sequencer motifs. In some cases, the library adaptors include a sequencing initiation sequence. The library adaptors can include a sequencing initiation sequence but no flow cell sequence. The sequence initiation sequence can be complementary to a primer for sequencing. The primer can be a sequence specific primer or a universal primer. Such sequencing initiation sequences may be situated on single-stranded portions of the library adaptors. As an alternative, such sequencing initiation sequences may be priming sites (e.g., kinks or nicks) to permit a polymerase to couple to the library adaptors during sequencing.

[00148] In some cases, partial or complete sequencer motifs are provided by sequencing adaptors. A sequencing adaptor can include a sample molecular barcode and a sequencer motif. The sequencing adaptors can be provided in a set that is separate from the library adaptors. The

sequencing adaptors in a given set can be identical – i.e., they contain the same sample barcode and sequencer motif.

[00149] Sequencing adaptors can include sample identification motifs and sequencer motifs. Sequencer motifs can include primers that are complementary to a sequencing initiation sequence. In some cases, sequencer motifs also include flow cell sequences or other sequences that permit a polynucleotide to a configured or arranged in a manner that permits the polynucleotide to be sequenced by a sequencer.

[00150] Library adaptors and sequencing adaptors can each be partial adaptors, that is, containing part but not all of the sequences necessary to enable sequencing by a sequencing platform. Together they provide complete adaptors. For example, library adaptors can include partial or no sequencer motifs, but such sequencer motifs are provided by sequencing adaptors.

[00151] **FIGs. 9A-9C** schematically illustrate a method for tagging a target polynucleotide molecule with library adaptors. **FIG. 9A** shows a library adaptor as a partial adaptor containing a primer hybridization site on one of the strands and a molecular barcode towards another end. The primer hybridization site can be a sequencing initiation sequence for subsequent sequencing. The library adaptor is less than or equal to 80 nucleotide bases in length. In **FIG. 9B**, the library adaptors are ligated at both ends of the target polynucleotide molecule to provide a tagged target polynucleotide molecule. The tagged target polynucleotide molecule may be subjected to nucleic acid amplification to generate copies of the target. Next, in **FIG. 9C**, sequencing adaptors containing sequencer motifs are provided and hybridized to the tagged target polynucleotide molecule. The sequencing adaptors contain sample identification motifs. The sequencing adaptors can contain sequences to permit sequencing of the tagged target with a given sequencer.

[00152] **D. <u>Sequencing</u>**

[00153] Tagged polynucleotides can be sequenced to generate sequence reads (*e.g.*, as shown in step (106), **FIG. 1**). For example, a tagged duplex polynucleotide can be sequenced. Sequence reads can be generated from only one strand of a tagged duplex polynucleotide. Alternatively, both strands of a tagged duplex polynucleotide can generate sequence reads. The two strands of the tagged duplex polynucleotide can comprise the same tags. Alternatively, the two strands of the tagged duplex polynucleotide can comprise different tags. When the two

strands of the tagged duplex polynucleotide are differently tagged, sequence reads generated from one strand (*e.g.*, a Watson strand) can be distinguished from sequence reads generated from the other strands (*e.g.*, a Crick strand). Sequencing can involve generating multiple sequence reads for each molecule. This occurs, for example, as a result the amplification of individual polynucleotide strands during the sequencing process, *e.g.*, by PCR.

[00154] Methods disclosed herein can comprise amplifying of polynucleotides. Polynucleotides amplification can result in the incorporation of nucleotides into a nucleic acid molecule or primer thereby forming a new nucleic acid molecule complementary to a template nucleic acid. The newly formed polynucleotide molecule and its template can be used as templates to synthesize additional polynucleotides. The polynucleotides being amplified can be any nucleic acids, for example, deoxyribonucleic acids, including genomic DNAs, cDNAs (complementary DNA), cfDNAs, and circulating tumor DNAs (ctDNAs). The polynucleotides being amplified can also be RNAs. As used herein, one amplification reaction may comprise many rounds of DNA replication. DNA amplification reactions can include, for example, polymerase chain reaction (PCR). One PCR reaction may comprise 2-100 "cycles" of denaturation, annealing, and synthesis of a DNA molecule. For example, 2-7, 5-10, 6-11, 7-12, 8-13, 9-14, 10-15, 11-16, 12-17, 13-18, 14-19, or 15-20 cycles can be performed during the amplification step. The condition of the PCR can be optimized based on the GC content of the sequences, including the primers.

[00155] Nucleic acid amplification techniques can be used with the assays described herein. Some amplification techniques are the PCR methodologies which can include, but are not limited to, solution PCR and *in situ* PCR. For example, amplification may comprise PCR-based amplification. Alternatively, amplification may comprise non PCR-based amplification. Amplification of the template nucleic acid may comprise use of one or more polymerases. For example, the polymerase may be a DNA polymerase or an RNA polymerase. In some cases, high fidelity amplification is performed such as with the use of high fidelity polymerase (e.g., Phusion® High-Fidelity DNA Polymerase) or PCR protocols. In some cases, the polymerase may be a high fidelity polymerase. For example, the polymerase may be KAPA HiFi DNA polymerase. The polymerase may also be Phusion DNA polymerase. The polymerase may be

used under reaction conditions that reduce or minimize amplification biases, e.g., due to fragment length, GC content, etc.

[00156] Amplification of a single strand of a polynucleotide by PCR will generate copies both of that strand and its complement. During sequencing, both the strand and its complement will generate sequence reads. However, sequence reads generated from the complement of, for example, the Watson strand, can be identified as such because they bear the complement of the portion of the duplex tag that tagged the original Watson strand. In contrast, a sequence read generated from a Crick strand or its amplification product will bear the portion of the duplex tag that tagged the original Crick strand. In this way, a sequence read generated from an amplified product of a complement of the Watson strand can be distinguished from a complement sequence read generated from an amplification product of the Crick strand of the original molecule.

[00157] All amplified polynucleotides can be submitted to a sequencing device for sequencing. Alternatively, a sampling, or subset, of all of the amplified polynucleotides is submitted to a sequencing device for sequencing. With respect to any original double-stranded polynucleotide there can be three results with respect to sequencing. First, sequence reads can be generated from both complementary strands of the original molecule (that is, from both the Watson strand and from the Crick strand). Second, sequence reads can be generated from only one of the two complementary strands (that is, either from the Watson strand or from the Crick strand, but not both). Third, no sequence read may be generated from either of the two complementary strands. Consequently, counting unique sequence reads mapping to a genetic locus will underestimate the number of double-stranded polynucleotides in the original sample mapping to the locus. Described herein are methods of estimating the unseen and uncounted polynucleotides.

[00158] The sequencing method can be massively parallel sequencing, that is, simultaneously (or in rapid succession) sequencing any of at least 100, 1000, 10,000, 100,000, 1 million, 10 million, 100 million, or 1 billion polynucleotide molecules. Sequencing methods may include, but are not limited to: high-throughput sequencing, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, nanopore sequencing, semiconductor sequencing, sequencing-by-ligation, sequencing-by-hybridization, RNA-Seq (Illumina), Digital Gene Expression (Helicos), Next generation sequencing, Single Molecule Sequencing by Synthesis (SMSS)(Helicos),

massively-parallel sequencing, Clonal Single Molecule Array (Solexa), shotgun sequencing, Maxam-Gilbert or Sanger sequencing, primer walking, sequencing using PacBio, SOLiD, Ion Torrent, or Nanopore platforms and any other sequencing methods known in the art.

[00159] For example, duplex-tagged polynucleotides can be amplified, by for example PCR (*see e.g.*, **FIG. 4A** duplex-tagged polynucleotides are referred to as mm' and nn'). In **Fig. 4A**, the strand of the duplex polynucleotide including sequence m bears sequence tags w and y, while the strand of the duplex polynucleotide including sequence m' bears sequence tags x and z. Similarly, the strand of the duplex polynucleotide including sequence n bears sequence tags a and c, while the strand of the duplex polynucleotide including sequence n' bears sequence tags b and d. During amplification, each strand produces itself and its complementary sequence. However, for example, an amplification progeny of original strand m that includes the complementary sequence, m', is distinguishable from an amplification progeny of original strand m' because the progeny from original strand m will have the sequence 5'-y'm'w'-3' and the progeny of the original m' strand one strand will have the sequence 5'-zm'x-3'. **FIG. 4B** shows amplification in more detail. During amplification, errors can be introduced into the amplification progeny, represented by dots. The application progeny are sampled for sequencing, so that not all strands produce sequence reads, resulting in the sequence reads indicated. Because sequence reads can come from either of a strand or its complement, both sequences and complement sequences will be included in the set of sequence reads. It should be noted that it is possible that a polynucleotide would bear the same tag on each end. Thus, for a tag "a", and polynucleotide "m", a first strand could be tagged a-m-a', and the complement could be tagged a-m'-a.

[00160] **E. <u>Determining consensus sequence reads</u>**

[00161] Methods disclosed herein can comprise determining consensus sequence reads in sequence reads (*e.g.*, as shown in step (108), **FIG. 1**), such as by reducing or tracking redundancy. Sequencing of amplified polynucleotides can produce reads of the several amplification products from the same original polynucleotide, referred to as "redundant reads". By identifying redundant reads, unique molecules in the original sample can be determined. If the molecules in a sample are uniquely tagged, then reads generated from amplification of a

single unique original molecule can be identified based on their distinct barcode. Ignoring barcodes, reads from unique original molecules can be determined based on sequences at the beginning and end of a read, optionally in combination with the length of the read. In certain cases, however, a sample may be expected to have a plurality of original molecules having the same start stop sequences and the same length. Without barcoding, these molecules are difficult to distinguish from one another. However, if a collection of polynucleotides is non-uniquely tagged (that is, an original molecule shares the same identifier with at least one other original molecule), combining information from a barcode with start/stop sequence and/or polynucleotide length significantly increases the probability that any sequence read can be traced back to an original polynucleotide. This is because, in part, even without unique tagging, it is unlikely that any two original polynucleotides having the same start/stop sequence and length also will be tagged with the same identifier.

[00162] **F. Collapsing**

[00163] Collapsing allows for reduction in noise (*i.e.*, background) that is generated at each step of the process. Methods disclosed herein can comprise collapsing, *e.g.*, generating a consensus sequence by comparing multiple sequence reads. For example, sequence reads generated from a single original polynucleotide can be used to generate a consensus sequence of that original polynucleotide. Iterative rounds of amplification can introduce errors into progeny polynucleotides. Also, sequencing typically may not be performed with perfect fidelity so sequencing errors are introduced at this stage as well. However, comparison of sequence reads of molecules derived from a single original molecule, including those that have sequence variants, can be analyzed so as to determine the original, or "consensus" sequence. This can be done phylogenetically. Consensus sequences can be generated from families of sequence reads by any of a variety of methods. Such methods include, for example, linear or non-linear methods of building consensus sequences (such as voting (*e.g.*, biased voting), averaging, statistical, maximum a posteriori or maximum likelihood detection, dynamic programming, Bayesian, hidden Markov or support vector machine methods, etc.) derived from digital communication theory, information theory, or bioinformatics. For example, if all or most of the sequence reads tracking back to an original molecule bear the same sequence variant, that variant probably existed in the original molecule. On the other hand, if a sequence variant exists in a subset of

redundant sequence reads, that variant may have been introduced during amplification/sequencing and represents an artifact not existing in the original. Furthermore, if only sequence reads derived from the Watson or Crick strand of an original polynucleotide contain the variant, the variant may have been introduced through single-sided DNA damage, first-cycle PCR error or through contaminating polynucleotides that were amplified from a different sample.

[00164] After fragments are amplified and the sequences of amplified fragments are read and aligned, the fragments are subjected to base calling, *e.g.*, determining for each locus the most likely nucleotide. However, variations in the number of amplified fragments and unseen amplified fragments (*e.g.*, those without being read their sequences; reasons could be too many such as amplification errors, sequencing reading errors, too long, too short, being chopped, etc.) may introduce errors in base calling. If there are too many unseen amplified fragments with respect to the seen amplified fragments (amplified fragments actually being read), the reliability of base calling may be diminished.

[00165] Therefore, disclosed herein is a method to correct for the number of unseen fragments in base calling. For example, when base calling for locus A (an arbitrary locus), it is first assumed that there are N amplified fragments. The sequence readouts can come from two types of fragments: double-strand fragments and single-strand fragments. Therefore, we assign N1, N2, and N3 as the numbers of double-strands, single-strands, and unseen fragments, respectively. Thus, N=N1+N2+N3 (N1 and N2 are known from the sequence readouts, and N and N3 are unknown). If the formula is solved for N (or N3), then N3 (or N) will be inferred.

[00166] Probability is used to estimate N. For example, we assign "p" to be the probability of having detected (or having read) a nucleotide of locus A in a sequence readout of a single-strand.

[00167] For sequence readouts from double-strands, the nucleotide call from a double-strand amplified fragment has a probability of p * p=p^2, seeing all N1 double-strands has the following equation: N1=N * (p^2).

[00168] For sequence readouts from a single-strand. Assuming that one of the 2 strands is seen, and the other is unseen, the probability of seeing one strand is "p", but the probability of missing the other strand is (1-p). Furthermore, by not distinguishing the single strand sourcing from 5-primer and sourcing from 3-primer, there is a factor of 2. Therefore, the nucleotide call

from a single-strand amplified fragment has a probability 2×p×(1-p). Thus, seeing all N2 single-strands has the following equation: N2=N×2×p×(1-p).

[00169] "p" is also unknown. To solve p, the ratio of N1 to N2 is used to solve for "p":

$$R = \frac{N1}{N2} = \frac{Np^2}{2Np(1-p)} = \frac{p^2}{2p(1-p)} = \frac{p}{2(1-p)}$$

Once "p" is found, N can be found. After N is found, can be found N3 = N − N1 − N2.

[00170] Besides the ratio of paired versus unpaired strands (which is a measure post-collapsing), there is useful information in the pre-collapsing read depth at each locus. This information can be used to further improve the call for total molecule count and/or increase confidence of calling variants.

[00171] For example, **FIG. 4C** demonstrates sequence reads corrected for complementary sequences. Sequences generated from an original Watson strand or an original Crick strand can be differentiated on the basis of their duplex tags. Sequences generated from the same original strand can be grouped. Examination of the sequences can allow one to infer the sequence of the original strand (the "consensus sequence"). In this case, for example, the sequence variant in the nn' molecule is included in the consensus sequence because it included in every sequence read while other variants are seen to be stray errors. After collapsing sequences, original polynucleotide pairs can be identified based on their complementary sequences and duplex tags.

[00172] **FIG. 5** demonstrates increased confidence in detecting sequence variants by pairing reads from Watson and Crick strands. Sequence nn' can include a sequence variant indicated by a dot. In some cases, sequence pp' does not include a sequence variant. Amplification, sequencing, redundancy reduction and pairing can result in both Watson and Crick strands of the same original molecule including the sequence variant. In contrast, as a result of errors introduced during amplification and sampling during sequencing, the consensus sequence of the Watson strand p can contain a sequence variant, while the consensus sequence of the Crick strand p' does not. It is less likely that amplification and sequencing will introduce the same variant into both strands (nn' sequence) of a duplex than onto one strand (pp' sequence). Therefore, the variant in the pp' sequence is more likely to be an artifact, and the variant in the nn' sequence is more likely to exist in the original molecule.

[00173] Methods disclosed herein can be used to correct errors resulted from experiments, *e.g.*, PCR, amplification, and/or sequencing. For example, such a method can comprises attaching one or more double stranded adaptors to both ends of a double stranded polynucleotide, thereby providing a tagged double stranded polynucleotide; amplifying the double stranded tagged polynucleotide; sequencing both strands of the tagged polynucleotide; comparing the sequence of one strand with its complement to determine any errors introduced during sequencing; and correcting errors in the sequence based on (d). The adaptors used in this method can be any adaptors disclosed herein, *e.g.*, Y-shaped adaptors. The adaptor can comprise any barcodes (*e.g.*, distinct barcodes) disclosed herein.

[00174] **G. Mapping**

[00175] Sequence reads or consensus sequences can be mapped to one or more selected genetic loci (*e.g.*, as shown step (110), **FIG. 1**). A genetic locus can be, for example, a specific nucleotide position in the genome, a sequence of nucleotides (for example, an open reading frame), a fragment of a chromosome, a whole chromosome, or an entire genome. A genetic locus can be a polymorphic locus. Polymorphic locus can be a locus at which sequence variation exists in the population and/or exists in a subject and/or a sample. A polymorphic locus can be generated by two or more distinct sequences coexisting at the same location of the genome. The distinct sequences can differ from one another by one or more nucleotide substitutions, a deletion/insertion, and/or a duplication of any number of nucleotides, generally a relatively small number of nucleotides, such as less than 50, 45, 40, 35, 30, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 nucleotide(s), among others. A polymorphic locus can be created by a single nucleotide position that varies within the population, *e.g.* a single nucleotide variation (SNV) or a single nucleotide polymorphism (SNP).

[00176] A reference genome for mapping can include the genome of any species of interest. Human genome sequences useful as references can include the hg19 assembly or any previous or available hg assembly. Such sequences can be interrogated using the genome browser available at genome.ucsc.edu/index.html. Other species genomes include, for example PanTro2 (chimp) and mm9 (mouse).

[00177] In methods disclosed herein, collapsing can be performed before or after mapping. In some aspects, collapsing can be performed before mapping. For example, sequence reads can be

grouped into families based on their tags and one or more endogenous sequences, without regard to where the reads map in the genome. Then, the members of a family can be collapsed into a consensus sequence. The consensus sequence can be generated using any collapsing method disclosed herein. Then the consensus sequence can be mapped to locations in the genome. Reads mapped to a locus can be quantified (*e.g.*, counted). Percentage of reads carrying a mutation at a locus can also be determined. Alternatively, collapsing can be performed after mapping. For example, all reads can first be mapped to the genome. Then the reads can be grouped into families based on their tags and one or more endogenous sequences. Since the reads have been mapped to the genome, consensus bases can be determined for each family at each locus. In other aspects, consensus sequence can be generated for one strand of a DNA molecule (*e.g.*, for a Watson strand or a Crick strand). Mapping can be performed before or after the consensus sequence for one strand of the DNA molecule is determined. Numbers of Doublets and Singlets can be determined. These numbers can be used to calculate unseen molecules. For example, the unseen molecules can be calculated using the following equation: N= D+S+U; D=Np(2), S=N2pq, where p=1-q, where p is the probability of seeing; q is the probability of missing a strand.

[00178] **H. <u>Grouping</u>**

[00179] Methods disclosed herein can also comprise grouping sequence reads. Sequence reads can be grouped based on various types of sequences, *e.g.*, sequences of an oligonucleotide tag (*e.g.*, a barcode), sequence of a polynucleotide fragments, or any combinations. For example, as shown in step (112) (**FIG. 1**), sequence reads can be grouped as follows: Sequence reads generated from a "Watson" strand and those generated from a "Crick" strand of a double-stranded polynucleotide in the sample are identifiable based on the duplex tags that they bear. In this way, a sequence read or consensus sequence from a Watson strand of a duplex polynucleotide can be paired with a sequence read or consensus sequence from its complementary Crick strand. Paired sequence reads are referred to as a "Pair".

[00180] Sequence reads for which no sequence read corresponding to a complementary strand can be found among the sequence reads are termed "Singlets".

[00181] Double-stranded polynucleotides for which a sequence read for neither of the two complementary strands has been generated are referred to as "Unseen" molecules.

[00182] **I. Quantifying**

[00183] Methods disclosed herein also comprise quantifying sequence reads. For example, as shown in step (114) (**FIG. 1**), Pairs and Singlets mapping to a selected genetic locus, or to each of a plurality of selected genetic loci, are quantified, *e.g.*, counted.

[00184] The quantifying can comprise estimating number of polynucleotides in the sample (*e.g.*, Pairs polynucleotides, Singlets polynucleotides, or Unseen polynucleotides. For example, as shown in step (116) (**FIG. 1**), the number of double-stranded polynucleotides in the sample for which no sequence reads were generated ("Unseen" polynucleotides) is estimated. The probability that a double strand polynucleotide generates no sequence reads can be determined based on the relative number of Pairs and Singlets at any locus. Using this probability, the number of Unseen polynucleotide can be estimated.

[00185] In step (118) an estimate for the total number of double-stranded polynucleotides in a sample mapping to a selected locus is the sum of the number of Pairs, the number of Singlets and the number of Unseen molecules mapping to the locus.

[00186] The number of Unseen original molecules in a sample can be estimated based on the relative number of Pairs and Singlets (**FIG. 2**). Referring to **FIG. 2**, as an example, counts for a particular genomic locus, Locus A, are recorded, where 1000 molecules are paired and 1000 molecules are unpaired. Assuming a uniform probability, p, for an individual Watson or Crick strand to make it through the process subsequent to conversion, one can calculate the proportion of molecules that fail to make it through the process (Unseen) as follows: Let R = ratio of paired to unpaired molecules = 1, so $R=1=p^2/(2p(1-p))$. This implies that $p=2/3$ and that the quantity of lost molecules is equal to $(1-p)^2 = 1/9$. Thus in this example, approximately 11% of converted molecules are lost and never detected. Consider another genomic locus, Locus B, in the same sample where 1440 molecules are paired and 720 are unpaired. Using the same method, we can infer the number of molecules that are lost, is only 4%. Comparing the two areas, it may be assumed that Locus A had 2000 unique molecules as compared to 2160 molecules in Locus B – a difference of almost 8%. However, by correctly adding in the lost molecules in each region, we infer there are 2000/(8/9)=2250 molecules in Locus A and 2160/.96=2250 molecules in Locus B. Hence, the counts in both regions are actually equal. This correction and thus much higher sensitivity can be achievable by converting the original double-stranded nucleic acid

molecules and bioinformatically keeping track of all those that are paired and unpaired at the end of the process. Similarly, the same procedure can be used to infer true copy number variations in regions that appear to have similar counts of observed unique molecules. By taking the number of unseen molecules into consideration in the two or more regions, the copy number variation becomes apparent.

[00187] In addition to using binomial distribution, other methods of estimating numbers of unseen molecules include exponential, beta, gamma or empirical distributions based on the redundancy of sequence reads observed. In the latter case, the distribution of read counts for paired and unpaired molecules can be derived from such redundancy to infer the underlying distribution of original polynucleotide molecules at a particular locus. This can often lead to a better estimation of the number of unseen molecules.

[00188] **J. <u>CNV Detection</u>**

[00189] Methods disclosed herein also comprise detecting CNV. For example, as shown in step (120) (**FIG. 1**), once the total number of polynucleotides mapping to a locus is determined, this number can be used in standard methods of determining CNV at the locus. A quantitative measure can be normalized against a standard. The standard can be an amount of any polynucleotides. In one method, a quantitative measure at a test locus can be standardized against a quantitative measure of polynucleotides mapping to a control locus in the genome, such as gene of known copy number. Quantitative measures can be compared against the amount of nucleic acid in any sample disclosed herein. For example, in another method, the quantitative measure can be compared against the amount of nucleic acid in the original sample. For example, if the original sample contained 10,000 haploid gene equivalents, the quantitative measure can be compared against an expected measure for diploidy. In another method, the quantitative measure can be normalized against a measure from a control sample, and normalized measures at different loci can be compared.

[00190] In some cases, in which copy number variation analysis is desired, sequence data may be: 1) aligned with a reference genome; 2) filtered and mapped; 3) partitioned into windows or bins of sequence; 4) coverage reads counted for each window; 5) coverage reads can then be normalized using a stochastic or statistical modeling algorithm; 6) and an output file can be generated reflecting discrete copy number states at various positions in the genome. In other

cases, in which rare mutation analysis is desired, sequence data may be 1) aligned with a reference genome; 2) filtered and mapped; 3) frequency of variant bases calculated based on coverage reads for that specific base; 4) variant base frequency normalized using a stochastic, statistical or probabilistic modeling algorithm; 5) and an output file can be generated reflecting mutation states at various positions in the genome.

[00191] After the sequence read coverage ratios have been determined, a stochastic modeling algorithm can be optionally applied to convert the normalized ratios for each window region into discrete copy number states. In some cases, this algorithm may comprise a Hidden Markov Model. In other cases, the stochastic model may comprise dynamic programming, support vector machine, Bayesian modeling, probabilistic modeling, trellis decoding, Viterbi decoding, expectation maximization, Kalman filtering methodologies, or neural networks.

[00192] Methods disclosed herein can comprise detecting SNVs, CNVs, insertions, deletions, and/or rearrangements at a specific region in a genome. The specific genomic region can comprise a sequence in a gene, such as ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, or NTRK1.

[00193] In some cases, the method uses a panel which comprises exons of one or more genes. The panel can comprise introns of one or more genes as well. The panel can also comprise exons and introns of one or more genes. The one or more genes can be those disclosed above. The panel can comprise about 80,000 bases which cover a panel of genes. The panel can comprise about 1000, 2000, 3000, 4000, 5000, 10000, 15000, 20000, 25000, 30000, 35000, 40000, 45000, 50000, 55000, 60000, 65000, 70000, 75000, 80000, 85000, 90000, 95000, 100000, 105000, 110000, 115000, 120000, 125000, or more bases.

[00194] In some aspects, copy number of a gene can be reflected in the frequency of a genetic form of the gene in a sample. For example, in a healthy individual, no copy number variation is

reflected in a variant in a gene in one chromosome (*e.g.*, heterozygosity) being detected in about 50% of detected molecules in a sample. Also, in a healthy individual, duplication of a gene bearing a variant can be reflected in the variant being detected in about 66% of detected molecules in a sample. Accordingly, if the tumor burden in a DNA sample is 10%, the frequency of a somatic mutation in a gene in one chromosome of cancer cells, without CNV, can be about 5%. The converse can be true in the case of aneuploidy.

[00195] The methods disclosed herein can be used to determine whether a sequence variant is more likely present in the germ line level or resulted from a somatic cell mutation, *e.g.*, in a cancer cell. For example, a sequence variant in a gene detected at levels arguably consistent with heterozygosity in the germ line is more likely the product of a somatic mutation if CNV is also detected in that gene. In some cases, to the extent we expect that a gene duplication in the germ line bears a variant consistent with genetic dose (*e.g.*, 66% for trisomy at a locus), detection gene amplification with a sequence variant dose that deviates significantly from this expected amount indicates that the CNV is more likely present as a result of somatic cell mutation.

[00196] The methods disclosed herein can also be used to infer tumor heterogeneity in a situation in which sequence variants in two genes are detected at different frequencies. For example, tumor heterogeneity can be inferred when two genes are detected at different frequencies but their copy numbers are relatively equal. Alternatively, tumor homogeneity can be inferred when the difference in frequency between two sequence variants is consistent with difference in copy number for the two genes. Thus, for example, if an EGFR variant is detected at 11% and a KRAS variant is detected at 5%, and no CNV is detected at these genes, the difference in frequency likely reflects tumor heterogeneity (*e.g.*, all tumor cells carry an EGFR mutant and half the tumor cells also carry a KRAS mutant). Alternatively, if the EGFR gene carrying the mutant is detected at 2-times normal copy number, one interpretation is a homogenous population of tumor cells, each cell carrying a mutant in the EGFR and KRAS genes, but in which the KRAS gene is duplicated.

[00197] In response to chemotherapy, a dominant tumor form can eventually give way through Darwinian selection to cancer cells carrying mutants that render the cancer unresponsive to the therapy regimen. Appearance of these resistance mutants can be delayed through methods of this invention. In one embodiment of this method, a subject is subjected to one or more

pulsed therapy cycles, each pulsed therapy cycle comprising a first period during which a drug is administered at a first amount and a second cycle during which the drug is administered at a second, reduced amount. The first period can be characterized by a tumor burden detected above a first clinical level. The second period can be characterized by a tumor burden detected below a second clinical level. First and second clinical levels can be different in different pulsed therapy cycles. For example, the first clinical level can be lower in succeeding cycles. A plurality of cycles can include at least 2, 3, 4, 5, 6, 7, 8 or more cycles. For example, the BRAF mutant V600E may be detected in polynucleotides of a disease cell at an amount indicating a tumor burden of 5% in cfDNA. Chemotherapy can commence with dabrafenib. Subsequent testing can show that the amount of the BRAF mutant in the cfDNA falls below 0.5% or to undetectable levels. At this point, dabrafenib therapy can stop or be significantly curtailed. Further subsequent testing may find that DNA bearing the BRAF mutation has risen to 2.5% of polynucleotides in cfDNA. At this point, dabrafenib therapy can be re-started, *e.g.*, at the same level as the initial treatment. Subsequent testing may find that DNA bearing the BRAF mutation has decreased to 0.5% of polynucleotides in cfDNA. Again, dabrafenib therapy can be stopped or reduced. The cycle can be repeated a number of times.

[00198] A therapeutic intervention can also be changed upon detection of the rise of a mutant form resistant to an original drug. For example, cancers with the EGFR mutation L858R respond to therapy with erlotinib. However, cancers with the EGFR mutation T790M are resistant to erlotinib. However, they are responsive to ruxolitinib. A method of this invention involves monitoring changes in tumor profile and changing a therapeutic intervention when a genetic variant associated with drug resistance rises to a predetermined clinical level.

[00199] Methods disclosed in this invention can comprise a method of detecting disease cell heterogeneity from a sample comprising polynucleotides from somatic cells and disease cells, the method comprising: a) quantifying polynucleotides in the sample bearing a sequence variant at each of a plurality of genetic loci; b) determining CNV at each of the plurality of genetic loci; different relative amounts of disease molecules at a locus, wherein the CNV indicates a genetic dose of a locus in the disease cell polynucleotides; c) determining a relative measure of quantity of polynucleotides bearing a sequence variant at a locus per genetic dose at the locus for each of a plurality of the loci; and d) comparing the relative measures at each of the plurality of loci,

wherein different relative measures indicates tumor heterogeneity. In the methods disclosed herein, the genetic dose can be determined on a total molecule basis. For example, if there are 1X total molecules at a first locus, and 1.2X molecules mapped to a second locus, then the genetic dose is 1.2. Variants at this locus can be divided by 1.2. In some aspects, the method disclosed herein can be used to detect any disease cell heterogeneity, *e.g.*, tumor cell heterogeneity. The methods can be used to detect disease cell heterogeneity from a sample comprising any types of polynucleotides, *e.g.*, cfDNA, genomic DNA, cDNA, or ctDNA. In the methods, the quantifying can comprise, for example, determining the number or relative amount of the polynucleotides. Determining CNV can comprise mapping and normalizing different relative amounts of total molecules to a locus.

[00200] In another aspect, in response to chemotherapy, a dominant tumor form can eventually give way through Darwinian selection to cancer cells carrying mutants that render the cancer unresponsive to the therapy regimen. Appearance of these resistance mutants can be delayed through methods disclosed throughout. The methods disclosed herein can comprise a method comprising: a) subjecting a subject to one or more pulsed therapy cycles, each pulsed therapy cycle comprising (i) a first period during which a drug is administered at a first amount and (ii) a second period during which the drug is administered at a second, reduced amount; wherein (A) the first period is characterized by a tumor burden detected above a first clinical level; and (B) the second period is characterized by a tumor burden detected below a second clinical level.

[00201] **K. <u>Sequence Variant Detection</u>**

[00202] Systems and methods disclosed herein can be used to detect sequence variants, *e.g.*, SNVs. For example, a sequence variant can be detected from consensus sequences from multiple sequence reads, for example, from at least 2, at least 3, at least 4, 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 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50, at least 51, at least 52, at least 53, at least 54, at least 55, at least 56, at least 57, at

least 58, at least 59, at least 60, at least 61, at least 62, at least 63, at least 64, at least 65, at least 66, at least 67, at least 68, at least 69, at least 70, at least 71, at least 72, at least 73, at least 74, at least 75, at least 76, at least 77, at least 78, at least 79, at least 80, at least 81, at least 82, at least 83, at least 84, at least 85, at least 86, at least 87, at least 88, at least 89, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, at least 99, at least 100, at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800, at least 900, at least 1000, at least 2000, at least 3000, at least 4000, at least 5000, at least 6000, at least 7000, at least 8000, at least 9000, at least 10000 or more sequence reads. A consensus sequence can be from sequence reads of a single strand polynucleotide. A consensus sequence can also be from sequence reads of one strand of a double-stranded polynucleotide (*e.g.*, pairing reads). In an exemplary method, pairing reads allows one to identify with increased confidence the existence of a sequence variant in a molecule. For example, if both strands of a Pair include the same variant, one can be reasonably sure that the variant existed in the original molecule, as the chance that the same variant is introduced into both strands during amplification/sequencing is rare. In contrast, if only one strand of a Pair includes the sequence variant, this is more likely to be an artifact. Similarly, the confidence that a Singlet bearing a sequence variant existed in the original molecule is less than the confidence if the variant exists in a Duplex, as there is higher probability that the variant can be introduced once than twice during amplification/sequencing.

[00203] Other methods of copy number variation detection and the sequence variant detection are described in PCT/US2013/058061, which is entirely incorporated herein by reference.

[00204] Sequence reads can be collapsed to generate a consensus sequence, which can be mapped to a reference sequence to identify genetic variants, such as CNV or SNV. As an alternative, the sequence reads are mapped prior to or even without mapping. In such a case, the sequence reads can be individually mapped to the reference to identify a CNV or SNV.

[00205] **FIG. 3** shows a reference sequence encoding a genetic Locus A. The polynucleotides in FIG. 3 may be Y-shaped or have other shapes, such as hairpin.

[00206] In some cases, an SNV or multiple-nucleotide variant (MNV) can be determined across multiple sequence reads at a given locus (e.g., nucleotide base) by aligning sequence reads that correspond to that locus. Next, a plurality of sequential nucleotide bases from at least a

subset of the sequence reads are mapped to the reference to a SNV or MNV in a polynucleotide molecule or portion thereof that corresponds to the reads. The plurality of sequential nucleotide bases can span an actual, inferred or suspected location of the SNV or MNV. The plurality of sequential nucleotide bases can span at least 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide bases.

[00207] L. Detecting/Quantifying Nucleic Acids

[00208] The methods described throughout can be used to tag nucleic acids fragments, such as deoxyribonucleic acid (DNA), at extremely high efficiency. This efficient tagging allows a person to efficiently and accurately detect rare DNA in heterogenous populations of original DNA fragments (such as in cfDNA). A rare polynucleotide (e.g., rare DNA) can be a polynucleotide that comprises a genetic variant occurring in a population of polynucleotides at a frequency of less than 10%, 5%, 4%, 3%, 2%, 1%, or 0.1%. A rare DNA can be a polynucleotide with a detectable property at a concentration less than 50%, 25%, 10%, 5%, 1%, or 0.1%

[00209] Tagging can occur in a single reaction. In some cases, two or more reactions can be performed and pooled together. Tagging each original DNA fragments in a single reaction can result in tagging such that greater than 50% (*e.g.*, 60%, 70%, 80%, 90%, 95%, or 99%) of the original DNA fragments are tagged at both ends with tags that comprise molecular barcodes, thereby providing tagged DNA fragments. Tagging can also result in greater than 30%, 35%, 40%, 45%, 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%, or 99% of the original DNA fragments tagged at both ends with tags that comprise molecular barcodes. Tagging can also result in 100% of the original DNA fragments tagged at both ends with tags that comprise molecular barcodes. Tagging can also result in single end tagging.

[00210] Tagging can also occur by using an excess amount of tags as compared to the original DNA fragments. For example, the excess can be at least 5-fold excess. In other cases, the excess can be at least 1.25, 1.5, 1.75, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more fold excess. Tagging can comprise attachment to blunt ends or sticky ends. Tagging can also be

performed by hybridization PCR. Tagging can also be performed in low reaction volumes, such as 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, or 100 pico- and/or microliters.

[00211] The method can also include performing high fidelity amplification on the tagged DNA fragments. Any high fidelity DNA polymerases can be used. For example, the polymerase may be KAPA HiFi DNA polymerase or Phusion DNA polymerase.

[00212] Further, the method can comprise selectively enriching a subset of the tagged DNA fragments. For example, selective enrichment can be performed by hybridization or amplification techniques. The selective enrichment can be performed using a solid support (*e.g.*, beads). The solid support (*e.g.*, beads) can comprise probes (*e.g.*, oligonucleotides specifically hybridizing to certain sequences. For example, the probes can hybridize with certain genomic regions, *e.g.*, genes. In some cases, the genomic regions, *e.g.*, genes, can be regions associated with diseases, *e.g.*, cancer. After enrichment, the selected fragmented can be attached any sequencing adaptor disclosed in this invention. For example, a sequence adaptor can comprise a flow cell sequence, a sample barcode, or both. In another example, a sequence adaptor can be a hairpin shaped adaptor and/or comprises a sample barcode. Further, the resulting fragments can be amplified and sequenced. In some cases, the adaptor does not comprise a sequencing primer region.

[00213] The method can include sequencing one or both strands of the DNA fragments. In one case, both strands of the DNA fragment are independently sequenced. The tagged, amplified, and/or selectively enriched DNA fragments are sequenced to obtain sequence reads that comprise sequence information of the molecular barcodes and at least a portion of the original DNA fragments.

[00214] The method can include reducing or tracking redundancy (as described above) in the sequence reads to determine consensus reads that are representative of single-strands of the original DNA fragments. For example, to reduce or track redundancy, the method can include comparing sequence reads having the same or similar molecular barcodes and the same or

similar end of fragment sequences. The method can comprise performing a phylogentic analysis on the sequence reads having the same or similar molecular barcodes. The molecular barcodes can have a barcode with varying edit distances (including any edit distances as described throughout), for example, an edit distance of up to 3. The end of the fragment sequences can include fragment sequences having an edit distance with varying distances (including any edit distances as described throughout), for example, an edit distance of up to 3.

[00215] The method can comprise binning the sequence reads according to the molecular barcodes and sequence information. For example, binning the sequence reads according to the molecular barcodes and sequence information can be performed from at least one end of each of the original DNA fragments to create bins of single stranded reads. The method can further comprise in each bin, determining a sequence of a given original DNA fragment among the original DNA fragments by analyzing sequence reads.

[00216] In some cases, sequence reads in each bin can be collapsed to a consensus sequence and subsequently mapped to a genome. As an alternative, sequence reads can be mapped to a genome prior to binning and subsequently collapsed to a consensus sequence.

[00217] The method can also comprise sorting sequence reads into paired reads and unpaired reads. After sorting, the number of paired reads and unpaired reads that map to each of one or more genetic loci can be quantified.

[00218] The method can include quantifying the consensus reads to detect and/or quantify the rare DNA, which are described throughout. The method can comprise detecting and/or quantifying the rare DNA by comparing a number of times each base occurs at each position of a genome represented by the tagged, amplified, and/or enriched DNA fragments.

[00219] The method can comprise tagging the original DNA fragments in a single reaction using a library of tags. The library can include at least 2, at least 3, at least 4, 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 16, at least 17, at least 18, at least 19, at least 20, at least 50, at least 100, at least 500, at least 1000, at least 5000, at least 10000, or any number of tags as disclosed throughout. For example, the library of tags can include at least 8 tags. The library of tags can include 8 tags (which can generate 64 different possible combinations). The method can be conducted such

that a high percentage of fragments, *e.g.*, greater than 50% (or any percentages as described throughout) are tagged at both ends, wherein each of the tags comprises a molecular barcode.

[00220] M. Processing and/or Analyzing Nucleic Acids

[00221] The methods described throughout can be used for processing and/or analyzing a nucleic acid sample of a subject. The method can comprising exposing polynucleotide fragments of the nucleic acid sample to a plurality of polynucleotide molecules to yield tagged polynucleotide fragments. The plurality of polynucleotide molecules that can be used are described throughout the application.

[00222] For example, the plurality of polynucleotide molecules can be each less than or equal to 40 nucleic acid bases in length and have distinct barcode sequences with respect to at least 4 nucleic acid bases and an edit distance of at least 1, wherein each of the distinct barcode sequences is within 20 nucleic acid bases from a terminal end of a respective one of the plurality of polynucleotide molecules, and wherein the plurality of polynucleotide molecules are not sequencing adaptors.

[00223] The tagged polynucleotide fragments can be subjected to nucleic acid amplification reactions under conditions that yield amplified polynucleotide fragments as amplification products of the tagged polynucleotide fragments. After amplification, the nucleotide sequence of the amplified tagged polynucleotide fragments is determined. In some cases, the nucleotide sequences of the amplified tagged polynucleotide fragments are determined without the use of polymerase chain reaction (PCR).

[00224] The method can comprise analyzing the nucleotide sequences with a programmed computer processor to identify one or more genetic variants in the nucleotide sample of the subject. Any genetic alterations can be identified, including but not limited to, base change(s), insertion(s), repeat(s), deletion(s), copy number variation(s) , epigenetic modification(s), nucleosome binding site(s), copy number change(s) due to origin(s) of replication, and transversion(s). Other genetic alterations can include, but are not limited to, one or more tumor associated genetic alterations.

[00225] The subject of the methods can be suspected of having a disease. For example, the subject can be suspected of having cancer. The method can comprise collecting a nucleic acid sample from a subject. The nucleic acid sample can be collected from blood, plasma, serum,

urine, saliva, mucosal excretions, sputum, stool, cerebral spinal fluid, skin, hair, sweat, and/or tears. The nucleic acid sample can be a cell-free nucleic acid sample. In some cases, the nucleic acid sample is collected from no more than 100 nanograms (ng) of double-stranded polynucleotide molecules of the subject.

[00226] The polynucleotide fragments can comprise double-stranded polynucleotide molecules. In some cases, the plurality of polynucleotide molecules are coupled to the polynucleotide fragments via blunt end ligation, sticky end ligation, molecular inversion probes, polymerase chain reaction (PCR), ligation-based PCR, multiplex PCR, single strand ligation, or single strand circularization.

[00227] The method as described herein results in high efficiency tagging of nucleic acids. For example, exposing the polynucleotide fragments of the nucleic acid sample to the plurality of polynucleotide molecules yields the tagged polynucleotide fragments with a conversion efficiency of at least 30%, *e.g.*, of at least 50% (*e.g.*, 60%, 70%, 80%, 90%, 95%, or 99%). Conversion efficiency of at least 30%, 35%, 40%, 45%, 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%, or 99% can be achieved.

[00228] The method can result in a tagged polynucleotide fragment that share common polynucleotide molecules. For example, any of at least 5%, 6%, 7%, 8%, 9%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% of the tagged polynucleotide fragments share a common polynucleotide molecule. The method can comprise generating the polynucleotide fragments from the nucleic acid sample.

[00229] In some cases, the subjecting of the method comprises amplifying the tagged polynucleotide fragments in the presence primers corresponding to a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4,

CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1. Additionally, any combination of these genes can be amplified. For example, 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, or all 54 of these genes can be amplified.

[00230] The methods described herein can comprise generating a plurality of sequence reads from a plurality of polynucleotide molecules. The plurality of polynucleotide molecules can cover genomic loci of a target genome. For example, the genomic loci can correspond to a plurality of genes as listed above. Further, the genomic loci can be any combination of these genes. Any given genomic locus can comprise at least two nucleic acid bases. Any given genomic locus can also comprise a plurality of nucleic acid bases, for example, 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 nucleic acid bases.

[00231] The method can comprise grouping with a computer processor the plurality of sequence reads into families. Each of the family can comprises sequence reads from one of the template polynucleotides. Each family can comprise sequence reads from only one of the template polynucleotides. For each of the family, the sequence reads can be merged to generate a consensus sequence. The grouping can comprise classifying the plurality of sequence reads into families by identifying (i) distinct molecular barcodes coupled to the plurality of polynucleotide molecules and (ii) similarities between the plurality of sequence reads, wherein each family includes a plurality of nucleic acid sequences that are associated with a distinct combination of molecular barcodes and similar or identical sequence reads.

[00232] Once merged, a consensus sequence can be called at a given genomic locus among the genomic loci. At any given genomic loci, any of the following can be determined: i) genetic variants among the calls; ii) frequency of a genetic alteration among the calls; iii) total number of calls; and iv) total number of alterations among the calls. The calling can comprise calling at least one nucleic acid base at the given genomic locus. The calling can also comprise calling a plurality of nucleic acid bases at the given genomic locus. In some cases, the calling can comprise phylogenetic analysis, voting (*e.g.*, biased voting), weighing, assigning a probability to

each read at the locus in a family, or calling the base with the highest probability. The consensus sequence can be generated by evaluating a quantitative measure or a statistical significance level for each of the sequence reads. If a quantitative measure is performed, the method can comprise use of a binomial distribution, exponential distribution, beta distribution, or empirical distribution. However, frequency of the base at the particular location can also be used for calling, for example, if 51% or more of the reads is a "A" at the location, then the base may be called an "A" at that particular location. The method can further comprise mapping a consensus sequence to a target genome.

[00233] The method can further comprising performing consensus calling at an additional genomic locus among the genomic loci. The method can comprise determining a variation in copy number at one of the given genomic locus and additional genomic locus based on counts at the given genomic locus and additional genomic locus.

[00234] The methods described herein can comprise providing template polynucleotide molecules and a library of adaptor polynucleotide molecules in a reaction vessel. The adaptor polynucleotide molecules can have from 2 to 1,000 different barcode sequences and in some cases are not sequencing adaptors. Other variations of adaptor polynucleotide molecules are described throughout, which can also be used in the methods.

[00235] The polynucleotide molecules of the adaptors can have the same sample tag. The adaptor polynucleotide molecules can be coupled to both ends of the template polynucleotide molecules. The method can comprise coupling the adaptor polynucleotide molecules to the template polynucleotide molecules at an efficiency of at least 30%, *e.g.*, of at least 50% (*e.g.*, 60%, 70%, 80%, 90%, 95%, or 99%), thereby tagging each template polynucleotide with a tagging combination that is among 4 to 1,000,000 different tagging combinations, to produce tagged polynucleotide molecules. In some cases, the reaction can occur in a single reaction vessel. Coupling efficiency can also be at least 30%, 35%, 40%, 45%, 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%, or 99%. Tagging can be non-unique tagging.

[00236] The tagged polynucleotide molecules can then be subject to an amplification reaction under conditions that will yield amplified polynucleotide molecules as amplification products of the tagged polynucleotide molecules. The template polynucleotide molecules can be double-stranded. Further, the template polynucleotide molecules can be blunt ended. In some cases, the amplification reaction comprises non-specifically amplifying the tagged polynucleotide molecules. The amplification reaction can also comprises using a priming site to amplify each of the tagged polynucleotide molecules. The priming site can be a primer, *e.g.*, a universal primer. The priming site can also be a nick.

[00237] The method can also comprise sequencing the amplified polynucleotide molecules. The sequencing can comprise (i) subjecting the amplified polynucleotide molecules to an additional amplification reaction under conditions that yield additional amplified polynucleotide molecules as amplification products of the amplified polynucleotide molecules, and/or (ii) sequencing the additional amplified polynucleotide molecules. The additional amplification can be performed in the presence of primers comprising flow cells sequences, which will produce polynucleotide molecules that are capable of binding to a flow cell. The additional amplification can also be performed in the presence of primers comprising sequences for hairpin shaped adaptors. The hairpin shaped adaptors can be attached to both ends of a polynucleotide fragment to generate a circular molecule, which can be sequenced multiple times. The method can further comprise identifying genetic variants upon sequencing the amplified polynucleotide molecules.

[00238] The method can further comprising separating polynucleotide molecules comprising one or more given sequences from the amplified polynucleotide molecules, to produce enriched polynucleotide molecules. The method can also comprise amplifying the enriched polynucleotide molecules with primers comprising the flow cell sequences. This amplification with primers comprising flow cell sequences will produce polynucleotide molecules that are capable of binding to a flow cell. The amplification can also be performed in the presence of primers comprising sequences for hairpin shaped adaptors. The hairpin shaped adaptors can be attached to both ends of a polynucleotide fragment to generate a circular molecule, which can be sequenced multiple times.

[00239] Flow cell sequences or hairpin shaped adaptors can be added by non-amplification methods such as through ligation of such sequences. Other techniques such as hybridization methods can be used, *e.g.*, nucleotide overhangs.

[00240] The method can be performed without aliquoting the tagged polynucleotide molecules. For example, once the tagged polynucleotide molecule is made, the amplification and sequencing can occur in the same tube without any further preparation.

[00241] The methods described herein can be useful in detecting single nucleotide variations (SNV), copy number variations (CNV), insertions, deletions, and/or rearrangements. In some cases, the SNVs, CNVs, insertions, deletions, and/or rearrangements, can be associated with disease, for example, cancer.

[00242] **N. Monitoring a Patient's Status**

[00243] Methods disclosed herein can also be used to monitor a patient's disease status. The disease of a subject can be monitored over time to determine a progression of the disease (e.g., regression). Markers indicative of the disease can be monitored in a biological sample of the subject, such as a cell-free DNA sample.

[00244] For example, monitoring a subject's cancer status can comprise (a) determining an amount of one or more SNVs or copy numbers of a plurality of genes (e.g., in an exon), (b) repeating such determination at different points in time, and (c) determining if there is a difference in the number of SNVs, level of SNVs, number or level of genomic rearrangements, or copy numbers between (a) and (b). The genes can be selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1. The genes can be selected from any 5, 10, 15, 20, 30, 40, 50, or all of the genes in this group.

[00245] O. Sensitivity and Specificity

[00246] Methods disclosed herein can be used to detect cancer polynucleotides in a sample, and cancer in a subject, with high measures of agreement, *e.g.*, high sensitivity and/or specificity. For example, such methods can detect cancer polynucleotides (e.g., rare DNA) in a sample at a concentration that is less than 5%, 1%, 0.5%, 0.1%, 0.05%, or 0.01%, at a specificity of at least 99%, 99.9%, 99.99%, 99.999%, 99.9999%, or 99.99999%. Such polynucleotides may be indicative of cancer or other disease. Further, such methods can detect cancer polynucleotides in a sample with a positive predictive value of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.9%, 99.99%, 99.999%, or 99.9999%.

[00247] Subjects identified as positive in a test that are in reality positive are referred as true positives (TP). Subjects identified as positive in a test that are in reality negative are referred as false positives (FP). Subjects identified as negative in a test that are in reality negative are referred as true negatives (TN). Subjects identified as negative in a test that are in reality positive are referred as false negatives (FN). Sensitivity is the percentage of actual positives identified in a test as positive. This includes, for example, instances in which one should have found a cancer genetic variant and did. (Sensitivity =TP/(TP+FN).) Specificity is the percentage of actual negatives identified in a test as negative. This includes, for example, instances in which one should have found no cancer genetic variant and did not. Specificity can be calculated using the following equation: Specificity = TN/(TN+FP). Positive predictive value (PPV) can be measured by the percentage of subjects who test positive that are true positives. PPV can be calculated using the following equation: PPV= TP/(TP+FP). Positive predictive value can be increased by increasing sensitivity (*e.g.*, chance of an actual positive being detected) and/or specificity (*e.g.*, chance of not mistaking an actual negative for a positive).

[00248] Low conversion rates of polynucleotides into adaptor-tagged polynucleotides can compromise sensitivity as it decreases the chance of converting, and therefore detecting, rare polynucleotide targets. Noise in a test can compromise specificity as it increases the number of false positives detected in a test. Both low conversion rate and noise compromise positive predictive value as they decrease the percentage of true positives and increase the percentage of false positives.

[00249] The methods disclosed herein can achieve high levels of agreement, *e.g.*, sensitivity and specificity, leading to high positive predictive values. Methods of increasing sensitivity include high efficiency conversion of polynucleotides into adaptor-tagged polynucleotides in a sample. Methods of increasing specificity include reducing sequencing errors, for example, by molecular tracking.

[00250] Methods of the present disclosure can be used to detect genetic variation in non-uniquely tagged initial starting genetic material (e.g., rare DNA) at a concentration that is less than 5%, 1%, 0.5%, 0.1%, 0.05%, or 0.01%, at a specificity of at least 99%, 99.9%, 99.99%, 99.999%, 99.9999%, or 99.99999%. In some aspects, the methods can further comprise converting polynucleotides in the initial starting material at an efficiency of at least at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%. Sequence reads of tagged polynucleotides can be subsequently tracked to generate consensus sequences for polynucleotides with an error rate of no more than 2%, 1%, 0.1%, or 0.01%.

[00251] **2. <u>Pooling Methods</u>**

[00252] Disclosed herein are methods of detecting copy number variation and/or sequence variants at one or more genetic loci in a test sample. One embodiment is shown in **FIG. 8**. Typically, detecting copy number variation involves determining a quantitative measure (*e.g.*, an absolute or relative number) of polynucleotides mapping to a genetic locus of interest in a genome of a test sample, and comparing that number to a quantitative measure of polynucleotides mapping to that locus in a control sample. In certain methods, the quantitative measure is determined by comparing the number of molecules in the test sample that map to a locus of interest with a number of molecules in the test sample mapping to a reference sequence, *e.g.*, a sequence expected to be present at wild type ploidy number. In some examples, the reference sequence is HG19, build 37, or build 38. The comparison could involve, for example, determining a ratio. Then, this measure is compared with a similar measure determined in a control sample. So, for example, if a test sample has a ratio of 1.5:1 for locus of interest versus reference locus, and a control sample has a ratio of 1:1 for the same loci, one may conclude that the test sample exhibits polyploidy at the locus of interest.

[00253] When the test sample and the control sample are analyzed separately, the work flow can introduce distortions between final numbers in the control and test samples.

[00254] In one method disclosed herein (*e.g.*, flow chart 800), polynucleotides are provided from a test and a control sample (802). Polynucleotides in a test sample and those in a control sample are tagged with tags that identify the polynucleotides as originating from the test or control sample (a source tag). (804.) The tag can be, for example, a polynucleotide sequence or barcode that unambiguously identifies the source.

[00255] The polynucleotides in each of the control and test samples also can be tagged with identifier tags that will be carried by all amplification progeny of a polynucleotide. Information from start and end sequences of a polynucleotide and identifier tags can identify sequence reads from polynucleotides amplified from an original parent molecule. Each molecule can be uniquely tagged compared with other molecules in the sample. Alternatively, each molecule need not be uniquely tagged compared with other molecules in the sample. That is, the number of different identifier sequences can be fewer than that the number of molecules in sample. By combining identifier information with start/stop sequence information, the probability of confusing two molecules having the same start/stop sequence is significantly diminished.

[00256] Number of different identifiers used to tag a nucleic acid (*e.g.*, cfDNA) can dependent on the number of different haploid genome equivalents. Different identifiers can be used to tag at least 2, least 10, least 100, least 200, least 300, least 400, least 500, least 600, least 700, least 800, least 900, least 1,000, least 2,000, least 3,000, least 4,000, least 5,000, least 6,000, least 7,000, least 8,000, least 9,000, least 10,000 or more different haploid genome equivalents. Accordingly, the number of different identifiers used to tag a nucleic acid sample, *e.g.*, cell-free DNA from 500 to 10,000 different haploid genome equivalents and be between any of 1, 2, 3, 4 and 5 and no more than 100, 90, 80, 70, 60, 50, 40 or 30. For example, the number of different identifier used to tag a nucleic acid sample from 500 to 10,000 different haploid genome equivalents can be 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 or less.

[00257] Polynucleotides can be tagged by ligation of adaptors comprising the tags or identifiers before amplification. Ligation can be performed using an enzyme, *e.g.*, a ligase. For example, tagging can be performed using a DNA ligase. The DNA ligase can be a T4 DNA ligase, *E. coli* DNA ligase, and/or mammalian ligase. The mammalian ligase can be DNA ligase I, DNA ligase III, or DNA ligase IV. The ligase may also be a thermostable ligase. Tags can be ligated to a blunt-end of a polynucleotide (blunt-end ligation). Alternatively, tags can be ligated to a sticky end of a polynucleotide (sticky-end ligation). The polynucleotides can be tagged by blunt end ligation using adaptors (*e.g.*, adaptors having forked ends). High efficiency of ligation can be achieved using high excess of adaptors (*e.g.*, more than 1.5X, more than 2X, more than 3X, more than 4X, more than 5X, more than 6X, more than 7X, more than 8X, more than 9X, more than 10X, more than 11X, more than 12X, more than 13X, more than 14X, more than 15X, more than 20X, more than 25X, more than 30X, more than 35X, more than 40X, more than 45X, more than 50X, more than 55X, more than 60X, more than 65X, more than 70X, more than 75X, more than 80X, more than 85X, more than 90X, more than 95X, or more than 100).

[00258] Once tagged with tags that identify source of the polynucleotides, polynucleotides from different sources (*e.g.*, different samples) can be pooled. After pooling, polynucleotides from different sources (*e.g.*, different samples) can be distinguished by any measurement using the tags, including any process of quantitative measurement. For example, as shown in (806) (**FIG. 8**), polynucleotides from the control sample and the test sample can be pooled. The pooled molecules can be subject to the sequencing (808) and bioinformatic work flow. Both will be subject to the same variations in the process and, therefore, any differential bias is reduced. Because molecules originating from control and test samples are differently tagged, they can be distinguished in any process of quantitative measurement.

[00259] The relative amount of control and test sample pooled can be varied. The amount of control sample can be same as the amount of test sample. The amount of control sample can also be larger than the amount of test sample. Alternatively, the amount of control sample can be smaller than the amount of test sample. The smaller the relative amount of one sample to the total, the fewer identifying tags needed in the original tagging process. A number can be selected to reduce to acceptable levels the probability that two parent molecules having the same start/end sequences will bear the same identifying tag. This probability can be less than 10%,

less than 1%, less than 0.1% or less than 0.01%. The probability can be less than 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%.

[00260] Methods disclosed herein can also comprise grouping sequence reads. For example, bioinformatic workflow can include grouping sequence reads produced from progeny of a single parent molecule, as shown in (810) (**FIG. 8**). This can involve any of the redundancy reduction methods described herein. Molecules sourced from test and control samples can be differentiated based on source tags they carry (812). Molecules mapping to a target locus are quantified for both test-sourced and control-sourced molecules (812). This can include the normalization methods discussed herein, *e.g.*, in which numbers at a target locus are normalized against numbers at a reference locus.

[00261] Normalized (or raw) quantities at a target locus from test and control samples are compared to determine presence of copy number variation (814).

[00262] **3. <u>Computer Control Systems</u>**

[00263] The present disclosure provides computer control systems that are programmed to implement methods of the disclosure. **FIG. 6** shows a computer system 1501 that is programmed or otherwise configured to implement the methods of the present disclosure. The computer system 1501 can regulate various aspects sample preparation, sequencing and/or analysis. In some examples, the computer system 1501 is configured to perform sample preparation and sample analysis, including nucleic acid sequencing. The computer system 1501 can be an electronic device of a user or a computer system that is remotely located with respect to the electronic device. The electronic device can be a mobile electronic device.

[00264] The computer system 1501 includes a central processing unit (CPU, also "processor" and "computer processor" herein) 1505, which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system 1501 also includes memory or memory location 1510 (*e.g.*, random-access memory, read-only memory, flash memory), electronic storage unit 1515 (*e.g.*, hard disk), communication interface 1520 (*e.g.*, network adapter) for communicating with one or more other systems, and peripheral devices 1525, such as cache, other memory, data storage and/or electronic display adapters. The memory 1510, storage unit 1515, interface 1520 and peripheral devices 1525 are in

communication with the CPU 1505 through a communication bus (solid lines), such as a motherboard. The storage unit 1515 can be a data storage unit (or data repository) for storing data. The computer system 1501 can be operatively coupled to a computer network ("network") 1530 with the aid of the communication interface 1520. The network 1530 can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network 1530 in some cases is a telecommunication and/or data network. The network 1530 can include one or more computer servers, which can enable distributed computing, such as cloud computing. The network 1530, in some cases with the aid of the computer system 1501, can implement a peer-to-peer network, which may enable devices coupled to the computer system 1501 to behave as a client or a server.

[00265] The CPU 1505 can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location, such as the memory 1510. The instructions can be directed to the CPU 1505, which can subsequently program or otherwise configure the CPU 1505 to implement methods of the present disclosure. Examples of operations performed by the CPU 1505 can include fetch, decode, execute, and writeback.

[00266] The CPU 1505 can be part of a circuit, such as an integrated circuit. One or more other components of the system 1501 can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

[00267] The storage unit 1515 can store files, such as drivers, libraries and saved programs. The storage unit 1515 can store user data, *e.g.*, user preferences and user programs. The computer system 1501 in some cases can include one or more additional data storage units that are external to the computer system 1501, such as located on a remote server that is in communication with the computer system 1501 through an intranet or the Internet.

[00268] The computer system 1501 can communicate with one or more remote computer systems through the network 1530. For instance, the computer system 1501 can communicate with a remote computer system of a user (*e.g.*, an operator). Examples of remote computer systems include personal computers (*e.g.*, portable PC), slate or tablet PC's (*e.g.*, Apple® iPad, Samsung® Galaxy Tab), telephones, Smart phones (*e.g.*, Apple® iPhone, Android-enabled

device, Blackberry®), or personal digital assistants. The user can access the computer system 1501 via the network 1530.

[00269] Methods as described herein can be implemented by way of machine (*e.g.*, computer processor) executable code stored on an electronic storage location of the computer system 1501, such as, for example, on the memory 1510 or electronic storage unit 1515. The machine executable or machine readable code can be provided in the form of software. During use, the code can be executed by the processor 1505. In some cases, the code can be retrieved from the storage unit 1515 and stored on the memory 1510 for ready access by the processor 1505. In some situations, the electronic storage unit 1515 can be precluded, and machine-executable instructions are stored on memory 1510.

[00270] The code can be pre-compiled and configured for use with a machine have a processer adapted to execute the code, or can be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion.

[00271] Aspects of the systems and methods provided herein, such as the computer system 1501, 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 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 links, 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" refer to any medium that participates in providing instructions to a processor for execution.

[00272] Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, etc. shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire 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 or DVD-ROM, any other optical medium, punch cards paper tape, 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 a 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.

[00273] The computer system 1501 can include or be in communication with an electronic display 1535 that comprises a user interface (UI) 1540. The UI can allow a user to set various conditions for the methods described herein, for example, PCR or sequencing conditions. Examples of UI's include, without limitation, a graphical user interface (GUI) and web-based user interface.

[00274] Methods and systems of the present disclosure can be implemented by way of one or more algorithms. An algorithm can be implemented by way of software upon execution by the

central processing unit 1505. The algorithm can, for example, process the reads to generate a consequence sequence.

[00275] **FIG. 7** schematically illustrates another system for analyzing a sample comprising nucleic acids from a subject. The system includes a sequencer, bioinformatic software and internet connection for report analysis by, for example, a hand held device or a desktop computer

[00276] Disclosed herein is a system for analyzing a target nucleic acid molecule of a subject, comprising: a communication interface that receives nucleic acid sequence reads for a plurality of polynucleotide molecules that cover genomic loci of a target genome; computer memory that stores the nucleic acid sequence reads for the plurality of polynucleotide molecules received by the communication interface; and a computer processor operatively coupled to the communication interface and the memory and programmed to (i) group the plurality of sequence reads into families, wherein each family comprises sequence reads from one of the template polynucleotides, (ii) for each of the families, merge sequence reads to generate a consensus sequence, (iii) call the consensus sequence at a given genomic locus among the genomic loci, and (iv) detect at the given genomic locus any of genetic variants among the calls, frequency of a genetic alteration among the calls, total number of calls; and total number of alterations among the calls, wherein the genomic loci correspond to a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1. The different variations of each component of the system are described throughout the disclosure within the methods and compositions. These individual components and variations thereof, are also applicable in this system.

[00277] **4. Kits**

[00278] Kits comprising the compositions as described herein. The kits can be useful in performing the methods as described herein. Disclosed herein is a kit comprising a plurality of

oligonucleotide probes that selectively hybridize to least 5, 6, 7, 8, 9, 10, 20, 30, 40 or all genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1. The number genes to which the oligonucleotide probes can selectively hybridize can vary. For example, the number of genes 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, or 54. The kit can include a container that includes the plurality of oligonucleotide probes and instructions for performing any of the methods described herein.

[00279] The oligonucleotide probes can selectively hybridize to exon regions of the genes, *e.g.*, of the at least 5 genes. In some cases, the oligonucleotide probes can selectively hybridize to at least 30 exons of the genes, *e.g.*, of the at least 5 genes. In some cases, the multiple probes can selectively hybridize to each of the at least 30 exons. The probes that hybridize to each exon can have sequences that overlap with at least 1 other probe. In some embodiments, the oligoprobes can selectively hybridize to non-coding regions of genes disclosed herein, for example, intronic regions of the genes. The oligoprobes can also selectively hybridize to regions of genes comprising both exonic and intronic regions of the genes disclosed herein.

[00280] Any number of exons can be targeted by the oligonucleotide probes. For example, at least 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, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, , 295, 300, 400, 500, 600, 700, 800, 900, 1,000, or more, exons can be targeted.

[00281] The kit can comprise at least 4, 5, 6, 7, or 8 different library adaptors having distinct molecular barcodes and identical sample barcodes. The library adaptors may not be sequencing

adaptors. For example, the library adaptors do not include flow cell sequences or sequences that permit the formation of hairpin loops for sequencing. The different variations and combinations of molecular barcodes and sample barcodes are described throughout, and are applicable to the kit. Further, in some cases, the adaptors are not sequencing adaptors. Additionally, the adaptors provided with the kit can also comprise sequencing adaptors. A sequencing adaptor can comprise a sequence hybridizing to one or more sequencing primers. A sequencing adaptor can further comprise a sequence hybridizing to a solid support, *e.g.*, a flow cell sequence. For example, a sequencing adaptor can be a flow cell adaptor. The sequencing adaptors can be attached to one or both ends of a polynucleotide fragment. In some cases, the kit can comprise at least 8 different library adaptors having distinct molecular barcodes and identical sample barcodes. The library adaptors may not be sequencing adaptors. The kit can further include a sequencing adaptor having a first sequence that selectively hybridizes to the library adaptors and a second sequence that selectively hybridizes to a flow cell sequence. In another example, a sequencing adaptor can be hairpin shaped. For example, the hairpin shaped adaptor can comprise a complementary double stranded portion and a loop portion, where the double stranded portion can be attached (*e.g.*, ligated) to a double-stranded polynucleotide. Hairpin shaped sequencing adaptors can be attached to both ends of a polynucleotide fragment to generate a circular molecule, which can be sequenced multiple times. A sequencing adaptor can be up to 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, or more bases from end to end. The sequencing adaptor can comprise 20-30, 20-40, 30-50, 30-60, 40-60, 40-70, 50-60, 50-70, bases from end to end. In a particular example, the sequencing adaptor can comprise 20-30 bases from end to end. In another example, the sequencing adaptor can comprise 50-60 bases from end to end. A sequencing adaptor can comprise one or more barcodes. For example, a sequencing adaptor can comprise a sample barcode. The sample barcode can comprise a pre-determined sequence. The sample barcodes can be used to identify the source of the polynucleotides. The sample barcode can be at least 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, or more (or any length as described throughout) nucleic acid bases, *e.g.*, at least 8 bases. The barcode can be contiguous or non-contiguous sequences, as described above.

[00282] The library adaptors can be blunt ended and Y-shaped and can be less than or equal to 40 nucleic acid bases in length. Other variations of the can be found throughout and are applicable to the kit.

EXAMPLES

[00283] **Example 1. Methods for copy number variation detection.**

[00284] Blood collection

[00285] 10-30 mL Blood samples are collected at room temperature. The samples are centrifuged to remove cells. Plasma is collected after centrifugation.

[00286] cfDNA extraction

[00287] The sample is subjected to proteinase K digestion. DNA is precipitated with isopropanol. DNA is captured on a DNA purification column (*e.g.*, a QIAamp DNA Blood Mini Kit) and eluted in 100 µl solution. DNAs below 500 bp are selected with Ampure SPRI magnetic bead capture (PEG/salt). The resulting production is suspended in 30 µl H_2O. Size distribution is checked (major peak = 166 nucleotides; minor peak = 330 nucleotides) and quantified. 5 ng of extracted DNA contain approximately 1700 haploid genome equivalents ("HGE"). The general correlation between the amount of DNA and HGE is as follow: 3 pg DNA = 1 HGE; 3 ng DNA = 1K HGE; 3 µg DNA = 1M HGE; 10 pg DNA = 3 HE; 10 ng DNA = 3K HGE; 10 µg DNA = 3M HGE.

[00288] "Single Molecule" library prep

[00289] High-efficiency DNA tagging (>80%) is performed by blunt-end repair and ligation with 8 different octomers (*i.e.*, 64 combinations) with overloaded hairpin adaptors. 2.5 ng DNA (*i.e.* approximately 800 HGE) is used as the starting material. Each hairpin adaptor comprises a random sequence on its non-complementary portion. Both ends of each DNA fragment are attached with hairpin adaptors. Each tagged fragment can be identified by the random sequence on the hairpin adaptors and a 10 p endogenous sequence on the fragment.

[00290] Tagged DNA is amplified by 10 cycles of PCR to produce about 1-7 µg DNAs that contain approximately 500 copies of each of the 800 HGE in the starting material.

[00291] Buffer optimization, polymerase optimization and cycle reduction may be performed to optimize the PCR reactions. Amplification bias, *e.g.*, non-specific bias, GC bias, and/or size bias are also reduced by optimization. Noise(s) (*e.g.*, polymerase-introduced errors) are reduced by using high-fidelity polymerases.

[00292] The Library may be prepared using Verniata or Sequenom methods.

[00293] Sequences may be enriched as follow: DNAs with regions of interest (ROI) are captured using biotin-labeled bead with probe to ROIs. The ROIs are amplified with 12 cycles of PCR to generate a 2000 times amplification. The resulting DNA is then denatured and diluted to 8 pM and loaded into an Illumina sequencer.

[00294] Massively parallel sequencing

[00295] 0.1 to 1% of the sample (approximately 100pg) are used for sequencing.

[00296] Digital bioinformatics

[00297] Sequence reads are grouped into families, with about 10 sequence reads in each family. Families are collapsed into consensus sequences by voting (*e.g.*, biased voting) each position in a family. A base is called for consensus sequence if 8 or 9 members agree. A base is not called for consensus sequence if no more than 60% of the members agree.

[00298] The resulting consensus sequences are mapped to a reference genome. Each base in a consensus sequence is covered by about 3000 different families. A quality score for each sequence is calculated and sequences are filtered based on the their quality scores.

[00299] Sequence variation is detected by counting distribution of bases at each locus. If 98% of the reads have the same base (homozygous) and 2% have a different base, the locus is likely to have a sequence variant, presumably from cancer DNA.

[00300] CNV is detected by counting the total number of sequences (bases) mapping to a locus and comparing with a control locus. To increase CNV detection, CNV analysis is performed specific regions, including regions on ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET,SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A,

BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, or NTRK1 genes.

[00301] Example 2. Method for Correcting Base Calling by Determining the Total Number Unseen Molecules in a Sample

[00302] After fragments are amplified and the sequences of amplified fragments are read and aligned, the fragments are subjected to base calling. Variations in the number of amplified fragments and unseen amplified fragments can introduce errors in base calling. These variations are corrected by calculating the number of unseen amplified fragments.

[00303] When base calling for locus A (an arbitrary locus), it is first assumed that there are N amplified fragments. The sequence readouts can come from two types of fragments: double-strand fragments and single-strand fragments. The following is a theoretical example of calculating the total number of unseen molecules in a sample.

[00304] N is the total number of molecules in the sample.

Assuming 1000 is the number of duplexes detected.

Assuming 500 is the number of single-stranded molecule detected.

P is the probability of seeing a strand.

Q is the probability of not detecting a strand.

[00305] Since Q = 1 -P.

1000 = NP(2).

500 = N2PQ.

1000 / P(2) = N.

500÷2 PQ = N.

1000 / P(2) = 500÷2PQ.

1000 * 2 PQ = 500 P(2).

2000 PQ = 500 P(2).

2000 Q = 500 P.

2000 (1-P) = 500P

2000-2000 P = 500P.

2000 = 500P +2000 P.

2000 = 2500 P.

2000÷2500 = P.

0.8 = P.

1000/ P(2) = N.

1000÷0.64 = N.

1562 = N.

Number of unseen fragments = 62.

[00306] Example 3. Identification of genetic variants in cancer-associated somatic variants in a patient.

[00307] An assay is used to analyze a panel of genes to identify genetic variants in cancer-associated somatic variants with high sensitivity.

[00308] Cell-free DNA is extracted from plasma of a patient and amplified by PCR. Genetic variants are analyzed by massively parallel sequencing of the amplified target genes. For one set of genes, all exons are sequenced as such sequencing coverage had shown to have clinically utility (Table 1). For another set of genes, sequencing coverage included those exons with a previously reported somatic mutation (Table 2). The minimum detectable mutant allele (limit of detection) is dependent on the patient's sample cell-free DNA concentration, which varied from less than 10 to over 1,000 genomic equivalents per mL of peripheral blood. Amplification may not be detected in samples with lower amounts of cell-free DNA and/or low-level gene copy amplification. Certain sample or variant characteristics resulted in reduced analytic sensitivity, such as low sample quality or improper collection.

[00309] The percentage of genetic variants found in cell-free DNA circulating in blood is related to the unique tumor biology of this patient. Factors that affected the amount/percentages of detected genetic variants in circulating cell-free DNA in blood include tumor growth, turn-over, size, heterogeneity, vascularization, disease progression or treatment. Table 3 annotates the percentage, or allele frequency, of altered circulating cell-free DNA (% cfDNA) detected in this patient. Some of the detected genetic variants are listed in descending order by % cfDNA.

[00310] Genetic variants are detected in the circulating cell-free DNA isolated from this patient's blood specimen. These genetic variants are cancer-associated somatic variants, some of

which have been associated with either increased or reduced clinical response to specific treatment. "Minor Alterations" are defined as those alterations detected at less than 10% the allele frequency of "Major Alterations". The detected allele frequencies of these alterations (Table 3) and associated treatments for this patient are annotated.

[00311] All genes listed in Tables 1 and 2 are analyzed as part of the Guardant360™ test. Amplification is not detected for *ERBB2*, *EGFR*, or *MET* in the circulating cell-free DNA isolated from this patient's blood specimen.

[00312] Patient test results comprising the genetic variants are listed in Table 4.

Table 1. Genes in which all exons are sequenced

GENES IN WHICH ALL EXONS ARE SEQUENCED			
ALK	< 0.1%	APC	< 0.1%
AR	< 0.1%	BRAF	< 0.1%
CDKN2A	< 0.1%	EGFR	< 0.1%
ERBB2	< 0.1%	FBXW7	< 0.1%
KRAS	< 0.1%	MET	< 0.1%
MYC	< 0.1%	NOTCH1	< 0.1%
NRAS	< 0.1%	PIK3CA	< 0.1%
PTEN	< 0.1%	PROC	< 0.1%
RB1	< 0.1%	TP53	< 0.1%

LOD: Limit of Detection. The minimum detectable mutant allele frequency for this specimen in which 80% of somatic variants is detected.

Table 2. Genes in which exons with a previously reported somatic mutation are sequenced

GENES IN WHICH EXONS WITH A PREVIOUSLY REPORTED SOMATIC MUTATION ARE SEQUENCED			
ABL1	< 0.1%	AKT1	< 0.1%
ATM	< 0.1%	CDH1	< 0.1%
CSF1R	< 0.1%	CTNNB1	< 0.1%
ERBB4	< 0.1%	EZH2	< 0.1%
FGFR1	< 0.1%	FGFR2	< 0.1%
FGFR3	< 0.1%	FLT3	< 0.1%
GNA11	< 0.1%	GNAQ	< 0.1%
GNAS	< 0.1%	HNF1A	< 0.1%
HRAS	< 0.1%	IDH1	< 0.1%

IDH2	< 0.1%	JAK2	< 0.1%
JAK3	< 0.1%	KDR	< 0.1%
KIT	< 0.1%	MLH1	< 0.1%
MPL	< 0.1%	NPM1	< 0.1%
PDGFRA	< 0.1%	PTPN11	< 0.1%
RET	< 0.1%	SMAD4	< 0.1%
SMARCB1	< 0.1%	SMO	< 0.1%
SRC	< 0.1%	STK11	< 0.1%
TERT	< 0.1%	VHL	< 0.1%

LOD: Limit of Detection. The minimum detectable mutant allele frequency for this specimen in which 80% of somatic variants is detected.

Table 3. Allele frequency of altered circulating cell-free DNA detected in this patient

Gene	cfDNA with alterations (%)	cfDNA without alterations (%)
BRAF V600E	8.9	91.1
NRAS Q61K	6.2	93.8
JAK V617F	1.5	98.6

Table 4. Genomic alterations detected in selected genes

Detected: 51 Genomic Alterations							
Gene	**Chromo-some**	**Position**	**Mutation (nt)**	**Mutation (AA)**	**Percentage**	**Cosmic ID**	**DBSNP ID**
KRAS	12	25368462	C>T		100.0%		rs4362222
ALK	2	29416572	T>C	I1461V	100.0%		rs1670283
ALK	2	29444095	C>T		100.0%		rs1569156
ALK	2	29543663	T>C	Q500Q	100.0%		rs2293564
ALK	2	29940529	A>T	P234P	100.0%		rs2246745
APC	5	112176756	T>A	V1822D	100.0%		rs459552
CDKN2A	9	21968199	C>G		100.0%	COSM14251	rs11515
FGFR3	4	1807894	G>A	T651T	100.0%		rs7688609
NOTCH1	9	139410424	A>G		100.0%		rs3125006
PDGFRA	4	55141055	A>G	P567P	100.0%		rs1873778
HRAS	11	534242	A>G	H27H	100.0%	COSM249860	rs12628
EGFR	7	55214348	C>T	N158N	99.9%	COSM42978	rs2072454
TP53	17	7579472	G>C	P72R	99.8%		rs1042522
APC	5	112162854	T>C	Y486Y	55.0%		rs2229992
APC	5	112177171	G>A	P1960P	53.8%		rs465899
EGFR	7	55266417	T>C	T903T	53.6%		rs1140475
APC	5	112176325	G>A	G1678G	53.2%		rs42427
APC	5	112176559	T>G	S1756S	53.0%		rs866006
EGFR	7	55229255	G>A	R521K	53.0%		
MET	7	116397572	A>G	Q648Q	52.7%		
APC	5	112175770	G>A	T1493T	52.7%		rs41115

EGFR	7	55249063	G>A	Q787Q	52.6%		rs1050171
NOTCH1	9	139411714	T>C		52.4%		rs11145767
EGFR	7	55238874	T>A	T629T	52.0%		rs2227984
ERBB2	17	37879588	A>G	I655V	51.6%		rs1136201
NOTCH1	9	139397707	G>A	D1698D	51.3%	COSM33747	rs10521
ALK	2	30143499	G>C	L9L	51.0%		rs4358080
APC	5	112164561	G>A	A545A	51.0%		rs351771
FLT3	13	28610183	A>G		50.8%		rs2491231
NOTCH1	9	139418260	A>G	N104N	50.5%		rs4489420
ALK	2	29444076	G>T		50.4%		rs1534545
PIK3CA	3	178917005	A>G		50.3%		rs3729674
NOTCH1	9	139412197	G>A		50.2%		rs9411208
ALK	2	29455267	A>G	G845G	50.0%	COSM148825	rs2256740
KIT	4	55593464	A>C	M541L	49.9%	COSM28026	
NOTCH1	9	139391636	G>A	D2185D	48.9%		rs2229974
PDGFRA	4	55152040	C>T	V824V	48.9%	COSM22413	rs2228230
ALK	2	29416481	T>C	K1491R	48.9%	COSM1130802	rs1881420
ALK	2	29445458	G>T	G1125G	48.6%		rs3795850
NOTCH1	9	139410177	T>C		48.5%		rs3124603
RET	10	43613843	G>T	L769L	48.2%		rs1800861
EGFR	7	55214443	G>A		48.0%		rs7801956
ALK	2	29416366	G>C	D1529E	47.2%		rs1881421
EGFR	7	55238087	C>T		45.5%		rs10258429
RET	10	43615633	C>G	S904S	44.8%		rs1800863
BRAF	7	140453136	A>T	V600E	8.9%	COSM476	
NRAS	1	115256530	G>T	Q61K	6.2%	COSM580	rs121913254
JAK2	9	5073770	G>T	V617F	1.5%	COSM12600	rs77375493

[00313] Example 4. Determining patient-specific limits of detection for genes analyzed by Guardant360™ assays.

[00314] Using the method of Example 3, Genetic alterations in cell-free DNA of a patient are detected. The sequence reads of these genes include exon and/or intron sequences.

[00315] Limits of detection of the test are shown in Table 5. The limits of detection values are dependent on cell-free DNA concentration and sequencing coverage for each gene.

Table 5. Limits of Detection of selected genes in a patient using Guardant

Complete Exon and Partial Intron Coverage					
APC	0.1%	AR *	0.2%	ARID1A	
BRAF *	0.1%	BRCA1		BRCA2	
CCND1 *		CCND2 *		CCNE1 *	
CDK4 *		CDK6 *		CDKN2A	0.1%
CDKN2B		EGFR *	< 0.1%	ERBB2 *	0.1%
FGFR1 *	< 0.1%	FGFR2 *	0.1%	HRAS	0.1%
KIT *	0.1%	KRAS *	0.1%	MET *	0.1%

MYC *	0.1%	NF1		NRAS	0.1%
PDGFRA *	0.1%	PIK3CA*	0.1%	PTEN	0.1%
RAF1 *		TP53	0.1%		
Exons Covered with Reported Somatic Mutations					
AKT1	0.1%	ALK	< 0.1%	ARAF	
ATM	0.1%	CDH1	0.1%	CTNNB1	0.1%
ESR1		EZH2	0.1%	FBXW7	0.1%
FGFR3	0.1%	GATA3		GNA11	0.1%
GNAQ	0.1%	GNAS	0.1%	HNF1A	0.1%
IDH1	0.1%	IDH2	0.1%	JAK2	0.1%
JAK3	0.1%	MAP2K1		MAP2K2	
MLH1	0.1%	MPL	0.2%	NFE2L2	
NOTCH1	0.1%	NPM1	0.1%	PTPN11	0.1%
RET	0.1%	RHEB		RHOA	
RIT1		ROS1		SMAD4	0.1%
SMO	0.1%	SRC	< 0.1%	STK11	0.2%
TERT	0.1%	VHL	0.2%		
Fusions					
ALK	< 0.1%	RET	0.1%	ROS1	
NTRK1					

LOD: Limit of Detection. The minimum detectable mutant allele frequency for this specimen in which 80% of somatic variants is detected. * indicates CNV genes.

[00316] Example 5. Correcting Sequence Errors Comparing Watson and Crick Sequences

[00317] Double-stranded cell-free DNA is isolated from the plasma of a patient. The cell-free DNA fragments are tagged using 16 different bubble-containing adaptors, each of which comprises a distinctive barcode. The bubble-containing adaptors are attached to both ends of each cell-free DNA fragment by ligation. After ligation, each of the cell-free DNA fragment can be distinctly identified by the sequence of the distinct barcodes and two 20 bp endogenous sequences at each end of the cell-free DNA fragment.

[00318] The tagged cell-free DNA fragments are amplified by PCR. The amplified fragments are enriched using beads comprising oligonucleotide probes that specifically bind to a group of

cancer-associated genes. Therefore, cell-free DNA fragments from the group of cancer-associated genes are selectively enriched.

[00319] Sequencing adaptors, each of which comprises a sequencing primer binding site, a sample barcode, and a cell-flow sequence, are attached to the enriched DNA molecules. The resulting molecules are amplified by PCR.

[00320] Both strands of the amplified fragments are sequenced. Because each bubble-containing adaptor comprises a non-complementary portion (*e.g.*, the bubble), the sequence of the one strand of the bubble-containing adaptor is different from the sequence of the other strand (complement). Therefore, the sequence reads of amplicons derived from the Watson strand of an original cell-free DNA can be distinguished from amplicons from the Crick strand of the original cell-free DNA by the attached bubble-containing adaptor sequences.

[00321] The sequence reads from a strand of an original cell-free DNA fragment are compared to the sequence reads from the other strand of the original cell-free DNA fragment. If a variant occurs in only the sequence reads from one strand, but not other strand, of the original cell-free DNA fragment, this variant will be identified as an error (*e.g.*, resulted from PCR and/or amplification), rather than a true genetic variant.

[00322] The sequence reads are grouped into families. Errors in the sequence reads are corrected. The consensus sequence of each family is generated by collapsing.

[00323] 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. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations

or equivalents. 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.

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

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

First Named Inventor:	AmirAli TALASAZ	Nonprovisional Application Number (if known):	
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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 /Timothy A. Hott/	Date 2020-07-31
Name (Print/Typed) Timothy A. Hott	Practitioner Registration Number 67740

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.**

☑ *Total of 1 forms are submitted.

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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 inspection 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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	AmirAli TALASAZ et al.	Confirmation No.:	To be assigned
Serial No.:	To be assigned	Art Unit:	To be assigned
Filing Date:	July 31, 2020	Examiner:	To be assigned
Title:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

A. ☒ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☒ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☐ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☐ a fee of $240.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $240.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure

statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☐ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☐ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☐ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☒ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☒ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: 16/601,168

Information Disclosure Statement(s) filed on: 01/14/2020, 05/06/2020 and 06/03/2020

AND

☒ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 60-2231(Docket No. GH0004US-CON5).

Respectfully submitted,

Dated: July 31, 2020

By: /Timothy A. Hott/
Timothy A. Hott, Reg. No. 67740

Customer No. 115823
GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063

PTO/AIA/15 (10-17)
Approved for use through 11/30/2020. 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.

UTILITY PATENT APPLICATION TRANSMITTAL

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

Attorney Docket No.	42534-708.306
First Named Inventor	AmirAli TALASAZ
Title	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
Priority Mail Express® 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* 92] 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* 11]
6. **Inventor's Oath or Declaration** [*Total Pages* 3] *(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** (*when there is an assignee*) [✔] **Power of Attorney**
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:** Track One Request

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: 115823 **OR** [ ] Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Timothy A. Hott/	Date	2020-07-31
Name (Print/Type)	Timothy A. Hott	Registration No. (Attorney/Agent)	67740

This collection of information is required by 37 CFR 1.53(b). 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 12 minutes 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.

Privacy Act Statement

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 disclosure of these records is required by the Freedom of Information Act.
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 inspection 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 Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Filer:	Timothy A Hott/Michelle Chan
Attorney Docket Number:	GH0004US-C5/42534-708.306

Filed as Large Entity

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4000	4000
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1202	10	100	1000
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 ($)	**6860**

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 NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/945,124	07/31/2020	1636	2720	42534-708.306	30	2

CONFIRMATION NO. 2506

115823
Wilson Sonsini Goodrich & Rosati / Guardant Health
650 Page Mill Road
Palo Alto, CA 94304

FILING RECEIPT

OC000000119087741

Date Mailed: 08/10/2020

Receipt is acknowledged of this non-provisional utility 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 FIRST INVENTOR, 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 corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction 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 provided that the request is grantable.

Inventor(s)

AmirAli TALASAZ, Atherton, CA;
Helmy ELTOUKHY, Atherton, CA;
Stefanie Ann Ward MORTIMER, Morgan Hill, CA;

Applicant(s)

GUARDANT HEALTH, INC., Redwood City, CA;

Power of Attorney: The patent practitioners associated with Customer Number 115823

Domestic Priority data as claimed by applicant

This application is a CON of 16/601,168 10/14/2019
which is a CON of 15/892,178 02/08/2018
which is a CON of 14/861,989 09/22/2015 PAT 9920366
which is a CON of PCT/US2014/072383 12/24/2014
which claims benefit of 61/948,509 03/05/2014
and claims benefit of 61/921,456 12/28/2013

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 Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/07/2020
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/945,124**
Projected Publication Date: 11/19/2020
Non-Publication Request: No
Early Publication Request: No
Title

METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

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).

SelectUSA

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.

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

115823 7590 08/10/2020

Wilson Sonsini Goodrich & Rosati / Guardant Health
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
08/10/2020	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):

Patents@guardanthealth.com
patentdocket@wsgr.com

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 16/945,124	**Applicant(s)** TALASAZ et al.	
	Examiner BRIAN W BROWN	**Art Unit** OPET	**AIA (FITF) Status** Yes

1. THE REQUEST FILED 31 July 2020 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 BRIAN BROWN at (571)272-5338. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/BRIAN W BROWN/ Petitions Examiner, OPET	

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/945,124

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	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	30 minus 20 =	* 10			OR	x 100 =	1000
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*				x 460 =	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	2720

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.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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					

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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
	1					

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16945124
	Filing Date	2020-07-31
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	1	EPO Preliminary Opinion, dated August 25, 2020, in EP2893040
	2	Guardant Health, Inc. Response to Notices of Opposition in EP2893040 filed May 29, 2020.
	3	IPR2019-00634, Final Written Decision of U.S. Patent 9,840,743, dated August 18, 2020
	4	IPR2019-00652, Final Written Decision of U.S. Patent 9,834,822, dated August 18, 2020
	5	LO, DENNIS Interview with Professor Dennis Lo, Qiagen News (2002)
	6	Opponents Reply dated August 20, 2020 to Proprietor's Observation in EP2893040.
	7	Opposition Form and Statement to EP3470533 filed August 6, 2020 by Foundation Medicine Inc.
	8	US Provisional Appl. No. 61/625,623 filed 04/17/2012.

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-08-28
Name/Print	Timothy A. Hott	Registration Number	67740

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.**

Privacy Act Statement

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:	40418014
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	28-AUG-2020
Filing Date:	31-JUL-2020
Time Stamp:	17:43:36
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	Transmittal Letter	2020-08-28_GH0004US-CON5_IDSTrans.pdf	144958 e59be8e0b2c491ecfc1f4003c0f23e82ee67bb62	no	4

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	2020-08-28_GH0004US-CON5_SB08.pdf	1053506 57e74a771ac84395dc522b3b676da11f7b025857	no	4

Warnings:

Information:

A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.

Total Files Size (in bytes):	1198464

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.

Attorney Docket No. 42534-708.306/GH0004US-CON5
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	AmirAli TALASAZ et al.	Confirmation No.:	2506
Serial No.:	To be assigned	Art Unit:	1636
Filing Date:	July 31, 2020	Examiner:	To be assigned
Title:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

A. ☒ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☒ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☐ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☐ a fee of $240.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $240.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure

statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☐ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☐ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☐ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☒ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☒ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: 15/892,178

Information Disclosure Statement(s) filed on: 8/19/20; 8/26/20

AND

☒ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 60-2231(Docket No. GH0004US-CON5).

Respectfully submitted,

Dated: August 28, 2020

By: /Timothy A. Hott/
Timothy A. Hott, Reg. No. 67740

Customer No. 115823
GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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
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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
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

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]

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	1	Guardant Health vs. FMI Invalidity Exhibit C-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	2	Guardant Health vs. FMI Invalidity Exhibit C-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	3	Guardant Health vs. FMI Invalidity Exhibit C-4, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	4	Guardant Health vs. FMI Invalidity Exhibit C-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	5	Guardant Health vs. FMI Invalidity Exhibit D-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	6	Guardant Health vs. FMI Invalidity Exhibit D-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	7	Guardant Health vs. FMI Invalidity Exhibit D-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	8	Guardant Health vs. FMI Invalidity Exhibit D-5, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	9	Guardant Health vs. FMI Second Amended Complaint dated March 6, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	10	Guardant Health vs. FMI Suppl Invalidity References dated February 15, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	
	11	Guardant Health vs. FMI Supplemental Invalidity Contentions dated March 29, 2019 (C.A. No. 17-cv-1616-LPS-CJB)	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

	12	Guardant Health vs. PGDx Amended Answer to Second Amended Complaint dated April 30, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	13	Guardant Health vs. PGDx Invalidity Claim Chart A, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	14	Guardant Health vs. PGDx Invalidity Claim Chart B, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	15	Guardant Health vs. PGDx Invalidity Claim Chart C, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	16	Guardant Health vs. PGDx Invalidity Claim Chart D, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	17	Guardant Health vs. PGDx Invalidity Claim Chart E, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	18	Guardant Health vs. PGDx Invalidity Claim Chart F, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	19	Guardant Health vs. PGDx Invalidity Claim Chart G, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
	20	Guardant Health vs. PGDx Invalidity Claim Chart H, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
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	22	Guardant Health vs. PGDx Invalidity Claim Chart J, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	

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	23	Guardant Health vs. PGDx Invalidity Claim Chart K, dated May 13, 2019 (C.A. No. 17-cv-1623-LPS-CJB)	
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CERTIFICATION STATEMENT

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

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

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

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Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

/K.R.H/	1	EPO Preliminary Opinion, dated August 25, 2020, in EP2893040	
/K.R.H/	2	Guardant Health, Inc. Response to Notices of Opposition in EP2893040 filed May 29, 2020.	
/K.R.H/	3	IPR2019-00634, Final Written Decision of U.S. Patent 9,840,743, dated August 18, 2020	
/K.R.H/	4	IPR2019-00652, Final Written Decision of U.S. Patent 9,834,822, dated August 18, 2020	
/K.R.H/	5	LO, DENNIS Interview with Professor Dennis Lo, Qiagen News (2002)	
/K.R.H/	6	Opponents Reply dated August 20, 2020 to Proprietor's Observation in EP2893040.	
/K.R.H/	7	Opposition Form and Statement to EP3470533 filed August 6, 2020 by Foundation Medicine Inc.	
/K.R.H/	8	US Provisional Appl. No. 61/625,623 filed 04/17/2012.	

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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.

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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-08-28
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Application Number	
Filing Date	
First Named Inventor	AmirAli TALASAZ
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Examiner Name	
Attorney Docket Number	42534-708.306

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	1	Illumina "Preparing Samples for Paired-End Sequencing (2008)	
	2	INGOLIA, et al. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science. 2009 Apr 10;324(5924):218-23. Epub 2009 Feb 12.	
	3	Instructions for Norit Rapid DNA Ligation Kit (November 6, 2004)	
	4	International search report and written opinion dated 04/03/2015 for PCT/US2014/072383.	
	5	International search report and written opinion dated 05/07/2012 for PCT/IB2011/003160.	
	6	International search report and written opinion dated 06/06/2012 for PCT/US2011/065291.	
	7	International search report and written opinion dated 07/15/2015 for PCT/US2015/030639.	
	8	International search report and written opinion dated 09/05/2014 for PCT/US2014/000048.	
	9	International search report and written opinion dated 09/06/2017 for PCT Application No. US-201727809	
	10	International search report and written opinion dated 11/18/2013 for PCT/US2013/058061.	
	11	Invitrogen Instructions for T4 DNA Ligase (May 5, 2002)	

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	12	IPR2018-00130, Petition for Inter Partes Review of U.S. Patent 9,598,731, dated November 7, 2018	
	13	IPR2019-00130, Decision Denying Inter Partes Review U.S. 9,598,731, issued June 5, 2019	
	14	IPR2019-00130, Preliminary Response to Petition for Inter Partes Review U.S. 9,598,731, filed March 6, 2019	
	15	IPR2019-00634, Decision Instituting Inter Partes Review U.S.9,840,743, issued August 19, 2019	
	16	IPR2019-00634, Petition for Inter Partes Review of U.S. Patent 9,840,743, dated February 1, 2019	
	17	IPR2019-00636 & 637, Exhibit 3001 Memo for Inter Partes Review of U.S. Patent 9,902,992, dated July 1, 2019	
	18	IPR2019-00636 & 637, Order - Conduct of the Proceedings for Inter Partes Review of U.S. Patent 9,902,992, dated July 22, 2019	
	19	IPR2019-00636 and IPR2019-00637, Decision Denying Inter Partes Review U.S.9,902,992, issued August 20, 2019	
	20	IPR2019-00636, Petition for Inter Partes Review of U.S. Patent 9,902,992, dated February 1, 2019	
	21	IPR2019-00637, Petition for Inter Partes Review of U.S. Patent 9,902,992, dated February 1, 2019	
	22	IPR2019-00652, Decision Granting Inter Partes Review U.S.9,834,822, issued August 19, 2019	

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	25	IPR2019-00653, Petition for Inter Partes Review of U.S. Patent 9,834,822, dated February 1, 2019	
	26	JABARA, C. Capturing the cloud: High throughput sequencing of multiple individual genomes from a retroviral population. Biology Lunch Bunch Series, Training Initiatives in Biomedical & Biological Sciences of the University of North Carolina at Chapel Hill, April 23, 2010	
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	29	JAHR, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001 Feb 15;61(4):1659-65.	
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	31	JIANG, H. et al., "SeqMap: mapping massive amount of oligonucleotides to the genome," Bioinformatics 2008, 24 (20):2395-2396	
	32	KAMPS-HUGHES, N. et al. "ERASE-Seq: Leveraging replicate measurements to enhance ultralow frequency variant detection in NGS data"PLOS One (2018)	
	33	KANAGAWA. Bias and artifacts in multitemplate polymerase chain reactions (PCR), 2003, Journal of Bioscience and Bioengineering, vol. 96, no. 4, p. 317-323.	

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	34	KAO,W-C. et al., "BayesCall: A model-based base-calling algorithm for high-throughput short-read sequencing," Genome Res. 2009 19(10), 1884-1895	
	35	KAROW, J. "Hopkins Team Develops Method to Improve Rare Mutation Detection for Early Cancer Dx" Genome Web (2011) 3 pages	
	36	KENNEDY, S.R. et al., "Detecting ultralow-frequency mutations by Duplex Sequencing" Nature Protocols (2014) 9 (11):2586-2606	
	37	KENNEDY, S.R. et al., "Ultra-Sensitive Sequencing Reveals an Age-Related Increase in Somatic Mitochondrial Mutations That Are Inconsistent with Oxidative Damage" PLOS Genetics (2013) 9:e1003794	
	38	KENNEDY, S.R. et al., "Detecting ultralow-frequency mutations by Duplex Sequencing," Nat. Protoc. 2014, 9(11), 2586-2606	
	39	KIMURA, et al. EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib. Br J Cancer. 2006 Nov 20;95(10):1390-5. Epub 2006 Oct 24.	
	40	KINDE, et al. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011 Jun 7;108(23):9530-5. doi: 10.1073/pnas.1105422108. Epub 2011 May 17.	
	41	KINDE, et al. Supplemental Information, Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011 Jun 7;108(23):1-10.	
	42	KINDE, I. et al., "Evaluation of DNA from the Papanicolaou Test to Detect Ovarian and Endometrial Cancers," Sci. Transl. Med. 2013, 5(167):167ra4	
	43	KINDE, I. et al., "FAST-SeqS: a simple and efficient method for the detection of aneuploidy by massively parallel sequencing," PLoS One 2012, 7(7), e41162	
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	45	KIRSCH, S. et al. "Sequence error storms and the landscape of mutations in cancer," Proc. Natl. Acad. Sci. USA 2012, 109(36), 14289-14290	
	46	KIVIOJA, T., et al. Counting absolute numbers of molecules using unique molecular identifiers. Nat Methods. Nov. 20, 2011;9(1):72-4. doi: 10.1038/nmeth.1778.	
	47	KOBOLDT, D.C.et al., "The next-generation sequencing revolution and its impact on genomics," Cell 2013, 155(1), 27-38	
	48	KOBOLDT, et al. Massively parallel sequencing approaches for characterization of structural variation. 08/12/2011. Methods Mol Biol. 2012;838:369-84. doi: 10.1007/978-1-61779-507-7_18.	
	49	KOLBLE, et al. Microsatellite alterations in serum DNA of patients with colorectal cancer. Lab Invest. 1999 Sep;79 (9):1145-50	
	50	KONIG, et al. iCLIP reveals the function of hnRNAP particles in splicing at individual nucleotide resolution, 2010 Jul, Nature Structural & Molecular Biology, 17(7):909-916.	

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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.

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

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	1	MOHAN, et al.,Changes in colorectal carcinoma genomes under anti-EGFR therapy identified by whole-genome plasma DNA sequencing.,PLoS Genet,doi: 10.1371/journal.pgen.1004271. eCollection 2014.,2014 Mar 27,10(3), e1004271.	
	2	MORI, et al. Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. J Clin Oncol. 2005 Dec 20;23(36):9351-8.	
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	6	NAWROZ, et al. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med. 1996 Sep;2 (9):1035-7.	
	7	NEWMAN, A. et al. "Integrated digital error suppression for improved detection of circulating tumor DNA" Nature Biotech (2016) 34(5):547-555	
	8	NEWMAN, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014 May;20(5):548-54. doi: 10.1038/nm.3519. Epub 2014 Apr 6.	
	9	NG, S.B., et al., "Targeted capture and massively parallel sequencing of 12 human exomes," Nature 2009, 461(7261), 272-276	
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	12	Notice of Allowance dated 01/31/2017 for U.S. Patent Application No. 14/712,754.	
	13	Notice of allowance dated 03/21/2014 for US Application No. 12/969,581.	
	14	Notice of allowance dated 06/15/2017 for US Application No.15/076,565.	
	15	Notice of allowance dated 06/19/2014 for US Application No. 12/969,581.	
	16	Notice of allowance dated 08/01/2017 for US Application No. 15/492,659	
	17	Notice of allowance dated 08/04/2017 for US Application No.15/467,570	
	18	Notice of allowance dated 08/22/2014 for US Application No. 12/969,581.	
	19	Notice of allowance dated 08/29/2017 for US Application No. 15/492,659	
	20	Notice of allowance dated 09/11/2017 for US Application No. 15/076,565	
	21	Notice of allowance dated 10/03/2017 for US Application No. 15/076,565.	
	22	Notice of allowance dated 10/25/2017 for US Application No. 14/861,989	

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	23	Notice of allowance dated 12/28/2017 for US Application No. 14/861,989.	
	24	ODEGAARD, J.I. et al. "Validation of a Plasma-Based Comprehensive Cancer Genotyping Assay Utilizing Orthogonal Tissue- and Plasma-Based Methodologies" Clin Canc Res (2018) 24(15):3539-3549	
	25	Office Action dated 02/09/2017 for U.S. Patent Application No. 15/076,565.	
	26	Office action dated 05/13/2019 for US Application No. 15/669,779.	
	27	Office action dated 05/20/2016 for US Application No. 14/855,301.	
	28	Office action dated 05/31/2016 for US Application No. 14/712,754.	
	29	Office action dated 06/01/2017 for US Application No. 15/467,570	
	30	Office action dated 06/03/2016 for US Application No. 14/861,989.	
	31	Office action dated 06/12/2017 for US Application No. 15/492,659.	
	32	Office action dated 07/18/2017 for US Application No. 14/861,989	
	33	Office Action dated 07/30/2019 for U.S. Application No. 16/283,635.	

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	34	Office action dated 08/06/2019 for US Application No. 14/855,301.	
	35	Office action dated 10/03/2013 for US Application No. 12/969,581.	
	36	Office Action dated 10/12/2016 for U.S. Patent Application No. 14/712,754.	
	37	Office action dated 10/20/2017 for US Application No. 14/425,189.	
	38	Office Action dated 11/21/2016 for U.S. Patent Application No. 14/855,301.	
	39	Office action dated 12/04/2015 for US Application No. 14/712,754.	
	40	Office action dated 12/07/2017 for US Application No. 14/855,301	
	41	Office Action dated 12/09/2016 for U.S. Patent Application No. 14/861,989.	
	42	Office action dated 12/21/2018 for US Application No. 15/978,848.	
	43	OGINO, et al. Quantification of PCR bias caused by a single nucleotide polymorphism in SMN gene dosage analysis. J Mol Diagn. 2002 Nov;4(4):185-90.	
	44	Opposition Brief dated August 7, 2018 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	X

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	45	Opposition Decision dated January 15, 2019 for Japanese Opposition No. 2018-700659 to JP Patent 6275145	☒
	46	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Foundation Medicine Inc.	☐
	47	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Grunecker Patent-und Rechtsanwalte PartGmbB	☐
	48	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Personal Genome Diagnostics, Inc.	☐
	49	Opposition Form and Statement to EP2893040 filed October 2, 2019 by Strawman Limited	☐
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	Examiner Name	
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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

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Name/Print	Timothy A. Hott	Registration Number	67740

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Attorney Docket Number	42534-708.306

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	3	2013181170	WO	A1	2013-12-05	OTWINOWSKI et al.	Entire Document	
	4	2013188471	WO	A2	2013-12-19	Faham et al.		
	5	2013190441	WO	A2	2013-12-27	CHIU et al.	Entire Document	
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	9	2014039556	WO	A1	2014-03-13	TALASAZ et al.	Entire Document	
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	14	2014152990	WO	A1	2014-09-25	SPENCE et al.		
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	16	2016015058	WO	A2	2016-01-28	Shendure et al.	Entire Document	
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	1	D'ANTONI, et al. Rapid quantitative analysis using a single molecule counting approach. Anal Biochem. 2006 May 1;352(1):97-109. Epub 2006 Feb 10.	
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	10	EHRICH, et al. Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: a study in a clinical setting. Am J Obstet Gynecol. 2011 Mar;204(3):205.e1-11. doi: 10.1016/j.ajog.2010.12.060. Epub 2011 Feb 18.	
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	41	Guardant Health vs. FMI Invalidity Exhibit A-1, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
	42	Guardant Health vs. FMI Invalidity Exhibit A-2, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	

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	43	Guardant Health vs. FMI Invalidity Exhibit A-3, dated June 25, 2018 (C.A. No. 17-cv-1616-LPS-CJB)	
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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

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	1	OU, SHI et al. "Liquid Biopsy to Identify Actionable Genomic Alterations" Am Soc Clin Onc (2018) 978	
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	45	SORENSON, et al. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev. 1994 Jan-Feb;3(1):67-71.	
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	First Named Inventor	AmirAli TALASAZ
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	Examiner Name	
	Attorney Docket Number	42534-708.306

CERTIFICATION STATEMENT

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

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	2	10370713	B2	2019-08-06	Salk et al.	Entire Document
	3	10385393	B2	2019-08-20	Salk et al.	Entire Document
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	6	4725536	A	1988-02-16	Fritsch et al.	
	7	4942124	A	1990-07-17	Church	
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	9	5149625	A	1992-09-22	Church et al.	
	10	5200314	A	1993-04-06	Urdea	
	11	5424186	A	1995-06-13	Fodor et al.	
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	31	5952170	A	1999-09-14	Stroun et al.	Entire Document
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See attached certification statement.

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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.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.R.H/

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	AmirAli TALASAZ
Art Unit	
Examiner Name	
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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
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	1							

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NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

/K.R.H/	1	ZHANG et al. "Comprehensive One-Step Molecular Analysis of Mitochondrial Genome by Massively Parallel Sequencing" Clinical Chem (2012) 58(9):1322-1331	
/K.R.H/	2	ZHANG, et al. The impact of next-generation sequencing on genomics. J Genet Genomics. 2011 Mar 20;38(3):95-109. doi: 10.1016/j.jgg.2011.02.003. Epub 2011 Mar 15.	
/K.R.H/	3	ZHAO, et al. Homozygous Deletions and Chromosome Amplifications in Human Lung Carcinomas Revealed by Single Nucleotide Polymorphism Array Analysis. Cancer Research, 65: 5561-5570 (2005).	
/K.R.H/	4	ZHOU, et al. Counting alleles reveals a connection between chromosome 18q loss and vascularinvasion. Nature Biotechnology, 19: 78-81 (2001).	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/KENNETH R HORLICK/	Date Considered	09/02/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**	Application Number	
	Filing Date	
	First Named Inventor	AmirAli TALASAZ
	Art Unit	
	Examiner Name	
	Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-07-31
Name/Print	Timothy A. Hott	Registration Number	67740

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.**

Privacy Act Statement

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.

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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.

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BIB DATA SHEET

CONFIRMATION NO. 2506

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
16/945,124	07/31/2020	435	1637	42534-708.306

APPLICANTS

GUARDANT HEALTH, INC., Redwood City, CA;

INVENTORS

AmirAli TALASAZ, Atherton, CA;
Helmy ELTOUKHY, Atherton, CA;
Stefanie Ann Ward MORTIMER, Morgan Hill, CA;

**** CONTINUING DATA ***************************

This application is a CON of 16/601,168 10/14/2019
which is a CON of 15/892,178 02/08/2018
which is a CON of 14/861,989 09/22/2015 PAT 9920366
which is a CON of PCT/US2014/072383 12/24/2014
which claims benefit of 61/948,509 03/05/2014
and claims benefit of 61/921,456 12/28/2013

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

08/07/2020

Foreign Priority claimed ❑ Yes ☑ No 35 USC 119(a-d) conditions met ❑ Yes ☑ No Verified and Acknowledged /KENNETH R HORLICK/ Examiner's Signature	❑ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	11	30	2

ADDRESS

Wilson Sonsini Goodrich & Rosati / Guardant Health
650 Page Mill Road
Palo Alto, CA 94304
UNITED STATES

TITLE

METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

FILING FEE RECEIVED		
2720	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	❑ All Fees ❑ 1.16 Fees (Filing) ❑ 1.17 Fees (Processing Ext. of time) ❑ 1.18 Fees (Issue) ❑ Other _____ ❑ Credit

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

115823 7590 09/09/2020

Wilson Sonsini Goodrich & Rosati / Guardant Health
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER
HORLICK, KENNETH R

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
09/09/2020	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):

Patents@guardanthealth.com
patentdocket@wsgr.com

Office Action Summary	Application No. 16/945,124	Applicant(s) TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	AIA (FITF) 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 3 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) 1-30 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) 1-30 is/are rejected.

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

9) ☐ Claim(s) _____ 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 7/31/20 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 See Continuation Sheet.

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

4) ☐ Other: _____.

Continuation of Attachment(s) 2) Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date: 8/28/20; 7/31/20; 7/31/20; 7/31/20; 7/31/20; 7/31/20; 7/31/20; 7/31/20; 7/31/20; 7/31/20.

Notice of Pre-AIA or AIA Status

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

OBJECTION

2. Claim 22 is objected to because of the following informality: it lacks a period at the end. Correction is required.

NON-PRIOR ART REJECTIONS

3. The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 20 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention. This claim is indefinite because 'the plurality of double-stranded cfDNA molecules' lacks proper antecedent basis. Correction is required.

4. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., In re Berg, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); In re Goodman, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); In re Longi, 759 F.2d 887, 225 USPQ645 (Fed. Cir. 1985); In re Van Ornum, 686 F.2d 937, 214 USPQ761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ619 (CCPA 1970); In re Thorington, 418 F.2d 528, 163 USPQ644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(l) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

5. Claims 1-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 2 of U.S. Patent No. 9,920,366. Although the claims at issue are not identical, they are not patentably distinct from each other because the patented claim and the instant claims are related as species-genus. That is, the steps of the instant claims, including analysis of paired reads and unpaired reads, are included in the steps of the patented claim.

6. Claims 1-30 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 31, 34-42, 44-49, 52-56, and 61-68 of copending Application No. 16/714,579 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the copending claims and the instant claims are related as

species-genus. That is, the steps of the instant claims, including analysis of paired reads and unpaired reads, are included in the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

7. Claims 1-30 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 61-90 of copending Application No. 16/672,267 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the copending claims and the instant claims are related as species-genus. That is, the steps of the instant claims, including analysis of paired reads and unpaired reads, are included in the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

8. Claims 1-30 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 31, 33-41, 47-49, 51, 57-59, and 61-71 of copending Application No. 16/601,168 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the copending claims and the instant claims are related as species-genus. That is, the steps of the instant claims, including analysis of paired reads and unpaired reads, are included in the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

9. Claims 1-30 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 54-74 of copending Application No. 15/892,178 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the copending claims and the instant claims are related as species-genus. That is, the steps of the instant claims, including analysis of paired reads and unpaired reads, are included in the steps of the copending claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

CONCLUSION

10. Claims 1-30 are free of the prior art, but are rejected for another reason. No claims are allowable.

11. Any inquiry concerning this communication or earlier communications from the examiner should be directed to KENNETH R HORLICK whose telephone number is (571)272-0784. The examiner can normally be reached on Mon. - Thurs. 8:30 - 6:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

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 https://ppair-my.uspto.gov/pair/PrivatePair. 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.

09/02/20

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/945,124	TALASAZ et al.
	Examiner	Art Unit
	KENNETH R HORLICK	1637

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
inventor name search	09/02/2020	KH
reviewed parent applications and references therein	09/02/2020	KH
updated parent searches in USPAT and PGPUB	09/02/2020	KH

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637	

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306

CONFIRMATION NO. 2506

115823
Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

PUBLICATION NOTICE

OC000000121456095

Title:METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

Publication No.US-2020-0362405-A1
Publication Date:11/19/2020

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): TALASAZ et al.	Confirmation No.: 2506
Serial Number: 16/945,124	Customer No.: 115823
Filing Date: July 31, 2020	Group Art Unit: 1637
Title: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	Examiner: Kenneth R. HORLICK

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

RESPONSE TO NON-FINAL OFFICE ACTION

Sir:

This communication is in response to the Office Action mailed on September 9, 2020. The shortened statutory period for reply expires December 9, 2020; therefore, this response is timely filed. Applicant respectfully requests reconsideration of the above-referenced application in view of the following remarks:

Amendments to the Specification begin on page 2 of this paper

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 8 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings in the above-referenced patent application. The foregoing amendments are without prejudice and do not constitute an admission regarding the patentability of the amended subject matter and should not so be construed. Applicant reserves the right to pursue the subject matter of the canceled claims in this or any other appropriate patent application.

Listing of Claims:

1. (Currently amended) A method, comprising:

(a) providing a population of cell-free deoxyribonucleic acid (cfDNA) molecules having first and second complementary strands;

(b) tagging a plurality of the cfDNA molecules in the population with duplex tags comprising molecular barcodes from a set of molecular barcodes to produce tagged parent polynucleotides, wherein the duplex tags are attached [[at]] to both ends of a molecule of the plurality of cfDNA molecules;

(c) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(d) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(e) reducing and/or tracking redundancy in a plurality of sequence reads from the set of sequence reads to determine distinct cfDNA molecules from among the tagged parent polynucleotides, wherein the distinct cfDNA molecules are determined based on (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, and/or (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides.

2. (Currently amended) The method of claim 1, wherein the ~~sample~~ population of cfDNA molecules is obtained or derived from a subject having cancer.

3. (Currently amended) The method of claim 1, wherein the ~~plurality~~ population of cfDNA molecules comprises 1 nanogram (ng) to 100 ng of cfDNA molecules.

4. (Currently amended) The method of claim 1, wherein the molecular barcodes are ligated to the plurality of the cfDNA molecules using more than a 10X molar excess of duplex tags as compared to the population of cfDNA molecules.

5. (Currently amended) The method of claim 1, wherein the molecular barcodes are ligated to the plurality of the cfDNA molecules using more than an 80X molar excess of duplex tags as compared to the population of cfDNA molecules.

6. (Original) The method of claim 5, wherein at least 40% of the cfDNA molecules from the population are tagged with the duplex tags.

7. (Currently amended) The method of claim 1, wherein the tagging comprises non-uniquely tagging the plurality of the cfDNA molecules, wherein the cfDNA molecules that map to a mappable base position of a reference sequence are tagged with a number of different molecular barcodes ranging from at least 2 to fewer than a number of the cfDNA molecules that map to the mappable base position.

8. (Currently amended) The method of claim 1, wherein ~~the molecular barcodes in~~ the set of molecular barcodes [[have]] comprises 5 to 10,000 different molecular barcode sequences, and [[are]] wherein the molecular barcodes in the set of molecular barcodes have a length of 5 to 20 base pairs ~~in length~~.

9. (Currently amended) The method of claim 1, further comprising, prior to the sequencing, enriching at least a subset of the amplified progeny polynucleotides for target regions of interest to produce enriched progeny polynucleotides ~~prior to sequencing~~.

10. (Original) The method of claim 9, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

11. (Currently amended) The method of claim 10, further comprising, prior to the sequencing, amplifying a plurality of the enriched progeny polynucleotides ~~prior to sequencing~~.

12. (Original) The method of claim 1, wherein the duplex tags are part of sequencing adapters.

13. (Original) The method of claim 12, wherein the sequencing adapters are Y-shaped adapters.

14. (Currently amended) The method of claim 1, wherein the reducing and/or tracking the redundancy in the [[set]] plurality of sequence reads comprises mapping at least a subset of the plurality of [[the]] sequence reads to a reference sequence.

15. (Currently amended) The method of claim 14, further comprising:

(f) determining quantitative measures of (i) paired reads and (ii) unpaired reads that map to a locus of [[a]] the reference sequence.

16. (Original) The method of claim 15, further comprising:

(g) estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides that map to the locus based on the quantitative measures of the paired reads and the unpaired reads.

17. (Currently amended) The method of claim 16, further comprising:

(h) detecting copy number variation in the ~~sample~~ population of cfDNA molecules by determining a normalized quantitative measure determined in [[step]] (g) at each of one or more loci.

18. (Original) A method, comprising:

(a) tagging a population of double-stranded cell-free deoxyribonucleic acid (cfDNA) molecules from a sample of a subject with a set of tags comprising molecular barcodes to generate tagged parent polynucleotides;

(b) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(c) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(d) sorting a plurality of sequence reads from the set of sequence reads into (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged

parent polynucleotides, and (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides.

19. (Original) The method of claim 18, wherein the sample is blood, plasma, or serum.

20. (Currently amended) The method of claim 18, wherein the ~~plurality~~ population of double-stranded cfDNA molecules comprises 1 nanogram (ng) to 100 ng of double-stranded cfDNA molecules.

21. (Original) The method of claim 18, wherein the tagging is performed by ligating the molecular barcodes to double-stranded cfDNA molecules.

22. (Currently amended) The method of claim 18, wherein the ~~molecular barcodes in the~~ set of tags comprises [[have]] 2 to 10,000 different molecular barcode sequences. ~~from a set of molecular barcodes having a plurality of different molecular barcode sequences~~

23. (Currently amended) The method of claim 18, further comprising, prior to the sequencing, enriching at least a subset of the amplified progeny polynucleotides for target regions of interest to produce enriched progeny polynucleotides ~~prior to sequencing~~.

24. (Original) The method of claim 23, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

25. (Currently amended) The method of claim 18, further comprising, prior to (d), reducing and/or tracking redundancy [[for]] in a plurality of sequence reads from the set of sequencing reads ~~that map to the reference sequence~~.

26. (Currently amended) The method of claim 25, wherein the reducing and/or tracking the redundancy in the plurality of sequence reads comprises mapping at least a subset of the plurality of [[the]] sequence reads to a reference sequence.

27. (Currently amended) The method of claim 26, wherein the reducing and/or tracking the redundancy in the plurality of sequence reads comprises ~~making~~ determining a base call at one or more genomic loci for a plurality of sequence reads that map to the one or more loci on the reference sequence.

28. (Currently amended) The method of claim 26, wherein the reducing and/or tracking the redundancy in the plurality of sequence reads comprises collapsing the plurality of [[the]] sequence reads to generate consensus sequences representative of original double-stranded cfDNA molecules from among the tagged parent polynucleotides.

29. (Currently amended) The method of claim 26 [[18]], further comprising determining quantitative measures of at least two of (i) paired reads, (ii) unpaired reads, (iii) a read depth of the paired reads, and (iv) a read depth of the unpaired reads.

30. (Currently amended) The method of claim 29, further comprising estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides ~~mapping~~ that map to a locus of [[a]] the reference sequence based on the quantitative measures of at least two of (i) paired reads, (ii) unpaired reads, (iii) a read depth of the paired reads, and (iv) a read depth of the unpaired reads at the locus.

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☒ APPROVED

This patent is subject to a terminal disclaimer

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Electronic Petition Request	**TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION AND TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT**
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Filing Date	31-Jul-2020
First Named Inventor	AmirAli TALASAZ
Attorney Docket Number	42534-708.306
Title of Invention	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action

☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

Owner	Percent Interest
GUARDANT HEALTH, INC.	100 %

The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Number(s)

16714579 filed on 12/13/2019

15892178 filed on 02/08/2018

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Registration Number 67740

( ) A sole inventor

( ) A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application

( ) A joint inventor; all of whom are signing this request

Signature	/Timothy A. Hott/
Name	Timothy A. Hott

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Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

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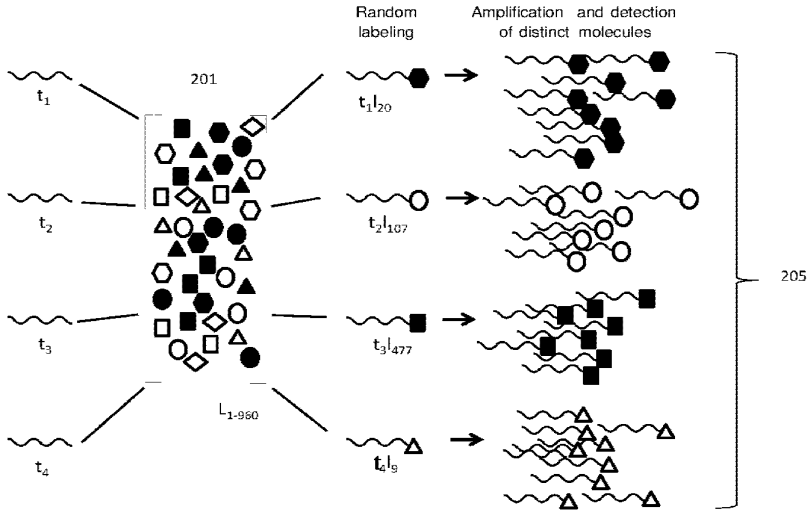
[Continued on next page]

(54) **Title:** DIGITAL COUNTING OF INDIVIDUAL MOLECULES BY STOCHASTIC ATTACHMENT OF DIVERSE LA-BEL-TAGS

FIG. 1



(57) **Abstract:** Compositions, methods and kits are disclosed for high-sensitivity counting of individual molecules by stochastic labeling of a identical molecules in mixtures of molecules by attachment of a unique label-tags from a diverse pool of label tags to confer uniqueness to otherwise identical or indistinguishable events. Individual occurrences of target molecules randomly choose from a non- depleting reservoir of diverse label-tags. Labeled molecules may be detected by hybridization or sequencing based methods. Molecules that would otherwise be identical in information content are labeled to create a separately detectable product that can be distinctly detected. The disclosed stochastic transformation methods reduce the problem of counting molecules from one of locating and identifying identical molecules to a series of binary digital questions detecting whether preprogrammed label-tags are present. The methods may be used, for example, to count a given species of molecule within a sample.

WO 2012/148477 A1

— *with sequence listing part of description (Rule 5.2(a))*

DIGITAL COUNTING OF INDIVIDUAL MOLECULES BY STOCHASTIC ATTACHMENT OF DIVERSE LABEL-TAGS

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/286,768 filed December 15, 2009, and is a continuation-in-part of U.S. Application No. 12/969,581, filed December 15, 2010, the contents of which are incorporated herein in their entireties.

FIELD OF THE INVENTION

[0002] Methods, compositions and products for counting individual molecules by stochastic attachment of diverse label-tags from a set of label-tags, followed by amplification and detection are disclosed.

INCORPORATION OF SEQUENCE LISTING

[0003] The content of the Sequence Listing filed electronically concurrently with the filing of this application is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0004] Many processes are characterized or regulated by the absolute or relative amounts of a plurality of items. For example, in biology, the level of expression of particular genes or groups of genes or the number of copies of chromosomal regions can be used to characterize the status of a cell or tissue. Analog methods such as microarray hybridization methods and real-time PCR are alternatives, but digital readout methods such as those disclosed herein have advantages over analog methods. Methods for estimating the abundance or relative abundance of genetic material having increased accuracy of counting would be beneficial.

[0005] The availability of convenient and efficient methods for the accurate identification of genetic variation and expression patterns among large sets of genes may be applied to understanding the relationship between an organism's genetic make-up and the state of its health or disease, Collins et al, Science, 282: 682-689 (1998). In this regard, techniques have been developed for the analysis of large populations of polynucleotides based either on specific hybridization of probes to microarrays, e.g. Lockhart et al. Hacia *et al, Nature Genetics,* 21: 4247 (1999), or on the

counting of tags or signatures of DTSIA fragments, e.g. Velculescu *et al, Science,* 270: 484-487 (1995); Brenner *et al, Nature Biotechnology,* 18: 630-634 (2000). These techniques have been used in discovery research to identify subsets of genes that have coordinated patterns of expression under a variety of circumstances or that are correlated with, and predictive of events, of interest, such as toxicity, drug responsiveness, risk of relapse, and the like, e.g. Golub *et al, Science,* 286: 531-537 (1999); Alizadeh *et al, Nature,* 403: 503-5 l 1 (2000); Perou *et al, Nature,* 406: 747-752 (2000); Shipp *et al, Nature Medicine,* 8: 68-74 (2002); Hakak *et al, Proc. Natl. Acad. ScL,* 98: 47454751 (2001); Thomas *et al, Mol. Pharmacol,* 60: l 189-1 194 (2001); De Primo *et al, BMC Cancer* 2003, 3:3. Not infrequently the subset of genes found to be relevant has a size in the range of from ten or under to a few hundred.

[0006] In addition to gene expression, techniques have also been developed to measure genome-wide variation in gene copy number. For example, in the field of oncology, there is interest in measuring genome-wide copy number variation of local regions that characterize many cancers and that may have diagnostic or prognostic implications. For a review see Zhang *et al. Annu. Rev. Genomics Hum. Genet.* 2009. 10:45 1-81 .

[0007] While such hybridization-based techniques offer the advantages of scale and the capability of detecting a wide range of gene expression or copy number levels, such measurements may be subject to variability relating to probe hybridization differences and cross-reactivity, eiement-to-element differences within microarrays, and microarray-to-microarray differences, Audic and Claverie, *Genomic Res.,* 7: 986-995 (1997); Wittes and Friedman, *J Natl. Cancer Inst.* 91: 400-401 (1999).

[0008] On the other hand, techniques that provide digital representations of abundance, such as SAGE (Velculescu et al. Science 270, 484-487 (1995) and Velculescu *et al. Cell* 88 (1997) or MPSS (Brenner *et al,* cited above), are statistically more robust; they do not require repetition or standardization of counting experiments as counting statistics are well-modeled by the Poisson distribution, and the precision and accuracy of relative abundance measurements may be increased by increasing the size of the sample of tags or signatures counted, e.g. Audic and Claverie (cited above). SAGE relies on short sequence tags (10-14bp) within transcripts as an indicator of the presence of a given transcript. The tags are separated from the rest of the RNA and can be linked together to form long serial molecules that can be cloned and sequenced. Quantitation of the number of times a particular tag is observed provides an estimate of the relative expression level of

the corresponding transcript, relative to other tagged transcripts, but not an actual count of the number of times that transcript appears. Other methods based on estimating relative abundance have also been described. See, for example, Wang *et al. Nat. Rev. Genet.* 10, 57-63 (2009).

[0009] Both digital and non-digital hybridization-based assays have been implemented using oligonucleotide tags that are hybridized to their complements, typically as part of a detection or signal generation schemes that may include solid phase supports, such as microarrays, microbeads, or the like, e.g. Brenner et al, Proc. Natl. Acad. Sci., 97: 1665-1670 (2000); Church et al, Science, 240: 185-188 (1988); Chee, *Nucleic Acids Research,* 19: 3301-3305 (1991); Shoemaker *et al, Nature Genetics,* 14: 450456 (1996); Wallace, U.S. Pat. No. 5,981,179; Gerry *et al, J. Mol. Biol,* 292: 251-262 (1999); Fan *et al, Genome Research,* 10: 853-860 (2000); Ye *et al, Human Mutation,* 17: 305-316 (2001); and the like. Bacterial transcript imaging by hybridization of total RNA to nucleic acid arrays may be conducted as described in Saizieu *et al, Nature Biotechnology,* 16:45-48 (1998). Accessing genetic information using high density DNA arrays is further described in Chee et al., *Science* 274:610-614 (1996). Additional methods for digital profiling are disclosed, for example, in U.S. Patent Pub. 20050250147 and US 7,537,897. Tagging approaches have also been used in combination with next-generation sequencing methods, see for example, Smith et al. NAR (May 11, 2010), 1-7.

[0010] A common feature among all of these approaches is a one-to-one correspondence between probe sequences and oligonucleotide tag sequences. That is, the oligonucleotide tags have been employed as probe surrogates for their favorable hybridizations properties, particularly under multiplex assay conditions.

[0011] Determining small numbers of biological molecules and their changes is essential when unraveling mechanisms of cellular response, differentiation or signal transduction, and in performing a wide variety of clinical measurements. Although many analytical methods have been developed to measure the *relative* abundance of different molecules through sampling (e.g., microarrays and sequencing), few techniques are available to determine the *absolute* number of molecules in a sample. This can be an important goal, for example in single cell measurements of copy number or stochastic gene expression, and is especially challenging when the number of molecules of interest is low in a background of many other species. As an example, measuring the relative copy number or expression level of a gene across a wide number of genes can currently be performed using PGR, hybridization to a microarray or by direct sequence counting. PGR and

microarray analysis rely on the specificity of hybridization to identify the target of interest for amplification or capture respectively, then yield an analog signal proportional to the original number of molecules. A major advantage of these approaches is in the use of hybridization to isolate the specific molecules of interest within the background of many other molecules, generating specificity for the readout or detection step. The disadvantage is that the readout signal to noise is proportional to all molecules (specific and non-specific) specified by selective amplification or hybridization. The situation is reversed for sequence counting. No intended sequence specificity is imposed in the sequence capture step, and all molecules are sequenced. The major advantage is that the detection step simply yields a digital list of those sequences found, and since there is no specificity in the isolation step, all sequences must be analyzed at a sufficient statistical depth in order to learn about a specific sequence. Although significant technical advances in sequencing speed and throughput have occurred, the statistical requirements imposed to accurately measure small changes in concentration of a specific gene (or target) or a few targets within the background of many other sequences requires measuring many sequences that are not of interest to find the ones that are of interest. Each of these techniques, PCR, array hybridization and sequence counting is a comparative technique in that they primarily measure relative abundance, and do not typically yield an absolute number of molecules in a solution. Digital PCR is one method that may be used for absolute counting of nucleic acids (B. Vogelstein, K. W. Kinzler, *Proc Natl Acad Sci USA 96,* 9236 (Aug 3, 1999). In this application of PCR solutions are progressively diluted into individual compartments until there is an average probability of one molecule per two wells, then detected by PCR. Although digital PCR can be used as a measure of absolute abundance, the dilutions must be customized for each type of molecule, and thus in practice the method is generally limited to the analysis of a small number of different molecules.

SUMMARY OF THE INVENTION

[0012] High-sensitivity single molecule digital counting by the stochastic labeling of a collection of identical molecules is disclosed. Each copy of a molecule randomly chooses from a non-depleting reservoir of diverse label-tags. The uniqueness of each labeled molecule is determined by the statistics of random choice, and depends on the number of copies of identical molecules in the collection compared to the diversity of label-tags. The size of the resulting set of labeled molecules is determined by the stochastic nature of the labeling process, and analysis reveals the original

number of molecules. When the number of copies of a molecule to the diversity of label-tags is low, the labeled molecules are highly unique, and the digital counting efficiency is high. This stochastic transformation relaxes the problem of counting molecules from one of locating and identifying identical molecules to a series of yes/no digital questions detecting whether preprogrammed label-tags are present. The conceptual framework for stochastic mapping of a variety of molecule types is developed and the utility of the methods are demonstrated by stochastically labeling 360,000 different fragments of the human genome. The labeled fragments for a target molecule of choice are detected with high specificity using a microarray readout system, and with DNA sequencing. The results are consistent with a stochastic process, and yield highly precise relative and absolute counting statistics of selected molecules within a vast background of other molecules.

[0013] The methods disclosed herein for digital counting of individual molecules may be applied to a single target or to a plurality of different targets simultaneously. In preferred embodiments the targets are nucleic acids, but may be a variety of biological or non-biological elements. Targets are labeled so that individual occurrences of the same target are marked by attachment of a different label-tag to difference occurrences. The attachment of the label-tag confers a separate, determinable identity to each occurrence of targets that may otherwise be indistinguishable. Preferably the label-tags are different sequences that tag or mark each target occurrence uniquely. The resulting modified target comprises the target sequence and the unique label-tag identifier (which also may be referred to herein as label-tag, counter, or identifier). The junction of the target and identifier forms a uniquely detectable mechanism for counting the occurrence of that copy of the target. The attachment of the identifier to each occurrence of the target is a random sampling event. Each occurrence of target could choose any of the identifiers from a pool of identifiers. Each identifier is present in multiple copies so selection of one copy does not remove that identifier sequence from the pool of identifiers so it is possible that the same identifier will be selected twice. The probability of that depends on the number of target occurrences relative to the number of different identifier sequences.

[0014] Each stochastic attachment event, where a target occurrence is attached to a unique identifier, results in the creation of a novel sequence formed at the junction of the identifier and the target. For a given target, the resulting products will contain the same target portion, but each will contain a different identifier sequence (T1L1, T1L2, ...T1LN where N is the number of different occurrences of target 1, "T1" and L is the identifier, L1, L2...LN). In preferred aspects the

occurrences are detected by hybridization. In some aspects the methods and systems include a probe array comprising features, wherein each feature has a different combination of target sequence with identifiers, 1 to N wherein N is the number of unique identifiers in the pool of identifiers. The array has N features for each target, so if, for example, there are 8 targets to be analyzed there are 8 times N features on the array to interrogate the 8 targets.

[0015] In some aspects methods and kits for counting the number of occurrences of a two or more different species of target nucleic in a complex mixture of target and non-target species are disclosed. The steps may include covalently or non-covalently attaching a label-tag from a pool of label-tags to individual occurrences of the each of the target nucleic acid species of interest to form sets of targets that are distinctly labeled within species so that instead of many undistinguishable molecules of each species there are many distinguishable molecules of each species. The species are distinguishable from one another on the basis of differences between species. The label-tag are selected from a pool for attaching in an approximately random manner and preferably at least 90% of the labeled targets within a species have a label-tag that is different from all other labeled targets in that species. The number of different molecules of a given species is then approximately (within the counting error of the system) equal to the number of different label-tags that are counted. In many aspects the label-tag-targets can be amplified prior to detection.

[0016] Methods of detection include hybridization based methods and sequencing based methods. Other methods such as size separation, mass spec or optical scanning methods may also be used. They nature of the label-tags will guide the detection mechanism.

[0017] Attachment of the label-tags may be by any means available. In preferred aspects where the targets are biological molecules such as nucleic acids or proteins the labels are preferably oligonucleotides or peptides. Methods for attaching oligonucleotide or nucleic acid sequences to nucleic acids may include ligation, polymerization and primer mediated extension reactions, for example.

[0018] Kits for labeling targets within mixtures and for amplification and detection of the label-tag-targets are also disclosed. Kits may include materials or reagents for carrying out a method of the invention and may include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents

and/or supporting materials for assays of the invention. In one aspect, kits of the invention comprises pools of label-tags, pools of label-tag containing primers or pools of label-tag containing adaptors. Enzymes that may be included in the disclosed kits include a ligase, a terminal transferase, RNaseH, reverse transcriptase, flap endonuclease, DNA polymerase, and restriction enzymes.

[0019] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several exemplary embodiments of the disclosure and together with the description, serve to explain certain teachings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] The skilled artisan will understand that the drawings described below are for illustration purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0021] FIG. 1 shows a schematic of labeling target molecules with a pool of label-tags.

[0022] FIG. 2A shows a schematic of detection of labeled targets on an array having features that are label-tag specific and target specific.

[0023] FIG. 2B shows a schematic of detection of several different targets by hybridization to an array having features containing support bound probes that are specific for junctions between label-tags and each of three different target species to facilitate counting of targets.

[0024] FIG. 2C shows a schematic for detection of individual label-target combinations by label-specific and target directed PCR.

[0025] FIG. 3 shows a schematic of a method for circularizing targets and amplifying with gene specific primers followed by detection of the target-label-tag junction.

[0026] FIG. 4 provides an example of a genomic target ligated at either end to a label-tag adaptor.

[0027] FIG. 5 shows a schematic of a method for target preparation wherein the label-tags are ligated to the targets as part of adaptors that have common priming sequences to facilitate amplification.

[0028] FIG. 6 shows a method for stochastic counting by fragmentation where the unique end of the fragment is the label-tag used for counting.

[0029] FIG. 7 shows a schematic of a method for detection of ligation products by hybridization to array probes that are complementary to the sequence resulting from the ligation.

[0030] FIG. 8 shows a schematic of the arrangement and position of the adaptors, PCR primers, and the biotinylated array-ligation probe in one exemplary sample prep method.

[0031] FIG. 9 is a schematic of a method for using ligation based read out on arrays to detect labeled targets and minimize partial target hybridization.

[0032] FIG. 10 shows the arrangement of the adaptors, label-tags and primers used to convert the labeled sample into sequencing template.

[0033] FIG. 11 is a plot of the number of label-tags from a non-depleting reservoir of 960 label-tags that are predicted to be captured at least once, exactly once or exactly twice.

[0034] FIG. 12 is a plot of counting results for 4 different DNA copy number titrations using microarray hybridization (on left) or DNA sequencing (on right).

[0035] FIG. 13 shows relative copy ratios of three tested gene targets representing copy number 1, 2 or 3 at different dilutions as analyzed using the disclosed methods.

[0036] FIG. 14 shows a comparison between experimentally observed label-tag usage and predicted label-tag usage from stochastic modeling.

[0037] FIG. 15 shows a plot of the expected label-tag usage (y-axis) when ligating to a given number of target molecules (x-axis).

[0038] FIG. 16 shows a plot of number of target molecules (x-axis) compared to counting efficiency (y-axis). The nset is a blow-up of the upper left region of the graph.

[0039] FIG. 17 shows a schematic of a method for attaching label-tags to targets using a splint having a variable region.

[0040] FIG. 18 shows a schematic of a method for enriching for molecules that contain label-tags, target or both.

[0041] FIG. 19 is a plot of the intensity observed compared to the number of fragments when fragments are binned according to size.

[0042] FIG. 20 shows label-tags observed by microarray hybridization plotted against intensity (y-axis) for each of 960 label-tags for the chromosome 4 gene target.

[0043] FIG. 21 shows frequency plots (y-axis, log-scale) of intensity distributions of the 960 label-tags in the microarray experiments with the counting threshold applied indicated by the dashed line.

[0044] FIG. 22 shows a simulated PCR run showing the replication outcome for 500 molecules of a target fragment ligated to a library of 960 label-tag counters.

[0045] FIG. 23 shows intensities of 1,152 array probes associated with a single gene target on chromosome 4 in the upper panel and a histogram of the intensity data corresponding to 960 label-tags in the lower panel.

[0046] FIG. 24 shows a plot of the number of times each of the 960 label-tags was observed in ligations with low DNA target amounts.

[0047] FIG. 25 shows plots of label-tags observed in the mapped reads from the first sequencing run for chromosome 4 with the horizontal dashed line indicating the counting threshold applied and the vertical dashed line indicating the break separating the 192 negative controls from the expected label-tags (controls to the right of the line).

[0048] FIG. 26 shows a method for adding counting label-tags to polyadenylated RNA.

[0049] FIG. 27 shows another method for adding counting label-tags to polyadenylated RNA.

[0050] FIG. 28A shows a method for adding label-tags to targets using a primer that has a stem loop between two poly (dT) regions.

[0051] FIG. 28B shows amplification of the products of the method followed by fragmentation and adaptor ligation to generate a product to be sequenced.

DETAILED DESCRIPTION OF THE INVENTION

[0052] Reference will now be made in detail to exemplary embodiments of the invention. While the invention will be described in conjunction with the exemplary embodiments, it will be understood that they are not intended to limit the invention to these embodiments. On the contrary, the invention is intended to cover alternatives, modifications and equivalents, which may be included within the spirit and scope of the invention.

[0053] The invention has many preferred embodiments and relies on many patents, applications and other references for details known to those of the art. Therefore, when a patent, application, or other reference, such as a printed publication, is cited or repeated below, it should be understood that it is incorporated by reference in its entirety for all purposes and particularly for the proposition that is recited.

[0054] As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "an agent" includes a plurality of agents, including mixtures thereof.

[0055] An individual is not limited to a human being, but may also be other organisms including, but not limited to, mammals, plants, bacteria, or cells derived from any of the above.

[0056] Throughout this disclosure, various aspects of this invention can be 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.

[0057] Suitable samples for analysis may be derived from a variety of sources. Biological samples may be of any biological tissue or fluid or cells from any organism. Frequently the sample will be a "clinical sample" which is a sample derived from a patient. Clinical samples provide a rich source of information regarding the various states of gene expression and copy number. Typical clinical samples include, but are not limited to, sputum, blood, tissue or fine needle biopsy samples, urine, peritoneal fluid, and pleural fluid, or cells therefrom. Biological samples may also include sections of tissues, such as frozen sections or formalin-fixed sections taken for histological purposes, which may include formalin-fixed, paraffin embedded (FFPE) samples and samples derived therefrom. FFPE samples are a particularly important source for study of archived tissue as they can nucleic acids can be recovered from these samples even after long term storage of the samples at room temperature. See, for example, Specht *et al. Am J. Path.* (2001), 158(2):419-429. Nucleic acids isolated from fresh-frozen samples may also be analyzed using the disclosed methods.

[0058] The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series (Vols.*

I-IV), Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular *Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), *Gait, "Oligonucleotide Synthesis: A Practical Approach " 1984, IRL Press, London,* Nelson and Cox (2000), Lehninger *et ah,* (2008) *Principles of Biochemistry* 5th Ed., W.H. Freeman Pub., New York, NY and Berg *et ah* (2006) *Biochemistry,* 6th Ed., W.H. Freeman Pub., New York, NY, all of which are herein incorporated in their entirety by reference for all purposes.

[0059] The present invention can employ solid substrates, including arrays in some preferred embodiments. Methods and techniques applicable to polymer (including protein) array synthesis have been described in U.S. Patent Pub. No. 20050074787, WO 00/58516, U.S. Patent Nos. 5,143,854, 5,242,974, 5,252,743, 5,324,633, 5,384,261, 5,405,783, 5,424,186, 5,451,683, 5,482,867, 5,491,074, 5,527,681, 5,550,215, 5,571,639, 5,578,832, 5,593,839, 5,599,695, 5,624,71 1, 5,631,734, 5,795,716, 5,831,070, 5,837,832, 5,856,101, 5,858,659, 5,936,324, 5,968,740, 5,974,164, 5,981,185, 5,981,956, 6,025,601, 6,033,860, 6,040,193, 6,090,555, 6,136,269, 6,269,846 and 6,428,752, in PCT Publication No. WO 99/36760 and WO 01/58593, which are all incorporated herein by reference in their entirety for all purposes. Patents that describe synthesis techniques in specific embodiments include U.S. Patent Nos. 5,412,087, 6,147,205, 6,262,216, 6,310,189, 5,889,165, and 5,959,098. Nucleic acid arrays are described in many of the above patents, but many of the same techniques may be applied to polypeptide arrays.

[0060] The present invention also contemplates many uses for polymers attached to solid substrates. These uses include gene expression monitoring, transcript profiling, library screening, genotyping, epigenetic analysis, methylation pattern analysis, tumor typing, pharmacogenomics, agrigenetics, pathogen profiling and detection and diagnostics. Gene expression monitoring and profiling methods have been shown in U.S. Patent Nos. 5,800,992, 6,013,449, 6,020,135, 6,033,860, 6,040,138, 6,177,248 and 6,309,822. Genotyping and uses therefore are shown in U.S. Patent Publication Nos. 20030036069 and 20070065816 and U.S. Patent Nos. 5,856,092, 6,300,063, 5,858,659, 6,284,460, 6,361,947, 6,368,799 and 6,333,179. Other uses are embodied in U.S. Patent Nos. 5,871,928, 5,902,723, 6,045,996, 5,541,061, and 6,197,506.

[0061] The present invention also contemplates sample preparation methods in certain embodiments. Prior to or concurrent with analysis, the sample may be amplified by a variety of mechanisms. In some aspects nucleic acid amplification methods such as PCR may be combined with the disclosed methods and systems. *See,* for example, *PCR Technology: Principles and*

Applications for DNA Amplification (Ed. H.A. Erlich, Freeman Press, NY, NY, 1992); *PCR Protocols: A Guide to Methods and Applications* (Eds. Innis, et al., Academic Press, San Diego, CA, 1990); Mattila et al., *Nucleic Acids Res.* 19, 4967 (1991); Eckert et al., *PCR Methods and Applications* 1, 17 (1991); *PCR* (Eds. McPherson et al., IRL Press, Oxford); and U.S. Patent Nos. 4,683,202, 4,683,195, 4,800,159, 4,965,188, and 5,333,675, each of which is incorporated herein by reference in their entireties for all purposes. Additional methods of sample preparation and techniques for reducing the complexity of a nucleic sample are described in Dong et al., *Genome Research* 11, 1418 (2001), in U.S. Patent Nos. 6,300,070 (amplification on an array), 6,361,947, 6,391,592, 6,872,529 and 6,458,530 and U.S. Patent Pub. Nos. 20030096235, 20030082543, 20030039069, 20050079536, 20040072217, 20050142577, 20050233354, 20050227244, 20050208555, 20050074799, 20050042654, and 20040067493, which are each incorporated herein by reference in their entireties..

[0062] Many of the methods and systems disclosed herein utilize enzyme activities. Enzymes and related methods of use in molecular biology that may be used in combination with the disclosed methods and systems are reviewed, for example, in Rittie and Perbal, *J. Cell Commun. Signal.* (2008) 2:25-45, incorporated herein by reference in its entirety. A variety of enzymes are well known, have been characterized and many are commercially available from one or more supplier. Exemplary enzymes include DNA dependent DNA polymerases (such as those shown in Table 1 of Rittie and Perbal), RNA dependent DNA polymerase (see Table 2 of Rittie and Perbal), RNA polymerases (such as T7 and SP6), ligases (see Table 3 of Rittie and Perbal), enzymes for phosphate transfer and removal (see Table 4 of Rittie and Perbal), nucleases (see Table 5 of Rittie and Perbal), and methylases.

[0063] Other methods of genome analysis and complexity reduction include, for example, AFLP, *see* US Patent 6,045,994, which is incorporated herein by reference, and arbitrarily primed-PCR (AP-PCR) *see* McClelland and Welsh, in *PCR Primer: A laboratory Manual,* (1995) eds. C. Dieffenbach and G. Dveksler, Cold Spring Harbor Lab Press, for example, at p 203, which is incorporated herein by reference in its entirety.

[0064] Other suitable amplification methods include the ligase chain reaction (LCR) *(for example,* Wu and Wallace, *Genomics* 4, 560 (1989), Landegren et al., ***Science*** 241, 1077 (1988) and Barringer et al. *Gene* 89:1 17 (1990)), transcription amplification (Kwoh et al, *Proc. Natl. Acad. Sci. USA* 86, 1173 (1989) and WO88/10315), self-sustained sequence replication (Guatelli et al.,

Proc. Nat. Acad. Set USA, 87, 1874 (1990) and WO90/06995), selective amplification of target polynucleotide sequences (U.S. Patent No. 6,410,276), consensus sequence primed polymerase chain reaction **(CP-PCR)** (U.S. Patent No. 4,437,975), arbitrarily primed polymerase chain reaction **(AP-PCPv)** (U.S. PatentNos. 5, 413,909, 5,861,245), rolling circle amplification **(RCA)** (for example, Fire and Xu, PNAS 92:4641 (1995) and Liu et al., J. Am. Chem. Soc. 118:1587 (1996)) and U.S. Pat. No. 5,648,245, strand displacement amplification (see Lasken and Egholm, *Trends Biotechnol.* 2003 2 1(12):53 1-5; Barker et al. *Genome Res.* 2004 May;14(5):901-7; Dean *et al. Proc Natl Acad Sci* U S A. 2002; 99(8):526 1-6; Walker et al. 1992, Nucleic Acids Res. 20(7): 1691 -6. 1992 and Paez, J.G., *et al. Nucleic Acids Res.* 2004; 32(9):e71), Qbeta Replicase, described in PCX Patent Application No. PCT/US87/00880 and nucleic acid based sequence amplification (NABSA). *(See,* U.S. PatentNos. 5,409,818, 5,554,517, and 6,063,603, each of which is incorporated herein by reference). Other amplification methods that may be used are described in, U.S. Patent Nos. 6,582,938, 5,242,794, 5,494,810, 4,988,617, and US Pub. No. 20030143599 each of which is incorporated herein by reference. DNA may also be amplified by multiplex locus-specific PCR or using adaptor-ligation and single primer PCR (See Kinzler and Vogelstein, NAR (1989) 17:3645-53. Other available methods of amplification, such as balanced PCR (Makrigiorgos, et al. (2002), *Nat Biotechnol,* Vol. 20, pp.936-9), may also be used.

[0065] Molecular inversion probes("MIPs") may also be used for amplification of selected targets. MIPs may be generated so that the ends of the pre-circle probe are complementary to regions that flank the region to be amplified. The gap can be closed by extension of the end of the probe so that the complement of the target is incorporated into the MIP prior to ligation of the ends to form a closed circle. The closed circle can be amplified and detected by sequencing or hybridization as previously disclosed in Hardenbol *et al, Genome Res.* 15:269-275 (2005) and in U.S. Patent No. 6,858,412.

[0066] Methods of ligation will be known to those of skill in the art and are described, for example in Sambrook et at. (2001) and the New England BioLabs catalog both of which are incorporated herein by reference for all purposes. Methods include using T4 DNA Ligase which catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA with blunt and sticky ends; Taq DNA Ligase which catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini of two adjacent oligonucleotides which are hybridized to a complementary target DNA; E. coli DNA ligase which

catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA containing cohesive ends; and T4 RNA ligase which catalyzes ligation of a 5' phosphoryl-terminated nucleic acid donor to a 3' hydroxyl-terminated nucleic acid acceptor through the formation of a 3'->5' phosphodiester bond, substrates include single-stranded RNA and DNA as well as dinucleoside pyrophosphates; or any other methods described in the art. Fragmented DNA may be treated with one or more enzymes, for example, an endonuclease, prior to ligation of adaptors to one or both ends to facilitate ligation by generating ends that are compatible with ligation.

[0067] Methods for ligating adaptors to fragments of nucleic acid are well known. Adaptors may be double-stranded, single-stranded or partially single-stranded. In preferred aspects adaptors are formed from two oligonucleotides that have a region of complementarity, for example, about 10 to 30, or about 15 to 40 bases of perfect complementarity, so that when the two oligonucleotides are hybridized together they form a double stranded region. Optionally, either or both of the oligonucleotides may have a region that is not complementary to the other oligonucleotide and forms a single stranded overhang at one or both ends of the adaptor. Single-stranded overhangs may preferably by about 1 to about 8 bases, and most preferably about 2 to about 4. The overhang may be complementary to the overhang created by cleavage with a restriction enzyme to facilitate "sticky-end" ligation. Adaptors may include other features, such as primer binding sites and restriction sites. In some aspects the restriction site may be for a Type IIS restriction enzyme or another enzyme that cuts outside of its recognition sequence, such as EcoP15I (see, Mucke *et al. J Mol Biol* 2001, 312(4):687-698 and US 5,710,000 which is incorporated herein by reference in its entirety).

[0068] Methods for using mapping arrays see, for example, Applications of microarrays for SNP genotyping have been described in e.g., U.S. Patent Nos 6,300,063, 6,361,947, 6,368,799 and US Patent Publication Nos. 20040067493, 20030232353, 20030186279, 20050260628, 20070065816 and 20030186280, and Kennedy *et al, Nat. Biotech.* 21:1233-1237 (2003), Matsuzaki *et al, Genome Res.* 14:414-425 (2004), Matsuzaki *et al, Nat. Meth.* 1:109-111 (2004) and U.S. Patent Pub. Nos. 20040146890 and 20050042654, each incorporated herein by reference. Fixed content mapping arrays are available from Affymetrix, for example, the SNP 6.0 array and the AXIOM® array system. Selected panels of SNPs and markers (e.g. copy number markers) can also be interrogated using a panel of locus specific probes in combination with a universal array as

described in Hardenbol *et al, Genome Res.* 15:269-275 (2005) and in U.S. Patent No. 6,858,412. Universal tag arrays and reagent kits for performing such locus specific genotyping using panels of custom molecular inversion probes (MIPs) are available from Affymetrix.

[0069] Methods for analyzing chromosomal copy number using mapping arrays are disclosed, for example, in Bignell et al., *Genome Res.* 14:287-95 (2004), Lieberfarb, *et al, Cancer Res.* 63:4781-4785 (2003), Zhao *et al, Cancer Res.* 64:3060-71 (2004), Huang *et al, Hum Genomics* 1:287-299 (2004), Nannya *et al, Cancer Res.* 65:6071-6079 (2005), Slater *et al, Am. J. Hum. Genet.* 77:709-726 (2005) and Ishikawa *et al, Biochem. and Biophys. Res. Comm.,* 333:1309-1314 (2005). Computer implemented methods for estimation of copy number based on hybridization intensity are disclosed in U.S. Patent Pub. Nos. 20040157243, 20050064476, 20050130217, 20060035258, 20060134674 and 20060194243.

[0070] Methods for conducting polynucleotide hybridization assays have been well developed in the art. Hybridization assay procedures and conditions will vary depending on the application and are selected in accordance with known general binding methods, including those referred to in: Maniatis et al. *Molecular Cloning: A Laboratory Manual* (2nd Ed. Cold Spring Harbor, N.Y, 1989); Berger and Kimmel *Methods in Enzymology,* Vol. 152, *Guide to Molecular Cloning Techniques* (Academic Press, Inc., San Diego, CA, 1987); Young and Davis, *P.N.A.S,* 80: 1194 (1983). Methods and apparatus for carrying out repeated and controlled hybridization reactions have been described in U.S. Patent Nos. 5,871,928, 5,874,219, 6,045,996 and 6,386,749, 6,391,623 each of which are incorporated herein by reference.

[0071] The present invention also contemplates signal detection of hybridization between ligands in certain preferred embodiments. See U.S. Patent Nos. 5,143,854, 5,578,832, 5,63 1,734, 5,834,758, 5,936,324, 5,981,956, 6,025,601, 6,141,096, 6,185,030, 6,201,639, 6,218,803, and 6,225,625 in U.S. Patent Pub. No. 20040012676 and in PCT Application PCT/US99/06097 (published as W099/47964), each of which also is hereby incorporated by reference in its entirety for all purposes.

[0072] Methods and apparatus for signal detection and processing of intensity data are disclosed in, for example, U.S. Patents Nos. 5,143,854, 5,547,839, 5,578,832, 5,631,734, 5,800,992, 5,834,758, 5,856,092, 5,902,723, 5,936,324, 5,981,956, 6,025,601, 6,090,555, 6,141,096, 6,185,030, 6,201,639; 6,218,803; and 6,225,625, in U.S. Patent Pub. Nos. 20040012676 and 20050059062 and

in PCT Application PCT/US99/06097 (published as W099/47964), each of which also is hereby incorporated by reference in its entirety for all purposes.

[0073] The practice of the present invention may also employ conventional biology methods, software and systems. Computer software products of the invention typically include computer readable medium having computer-executable instructions for performing the logic steps of the method of the invention. Suitable computer readable medium include floppy disk, CD-ROM/DVD/DVD-ROM, hard-disk drive, flash memory, ROM/RAM, magnetic tapes, etc. The computer-executable instructions may be written in a suitable computer language or combination of several languages. Basic computational biology methods are described in, for example, Setubal and Meidanis et al., *Introduction to Computational Biology Methods* (PWS Publishing Company, Boston, 1997); Salzberg, Searles, Kasif, (Ed.), *Computational Methods in Molecular Biology,* (Elsevier, Amsterdam, 1998); Rashidi and Buehler, *Bioinformatics Basics: Application in Biological Science and Medicine* (CRC Press, London, 2000) and Ouelette and Bzevanis *Bioinformatics: A Practical Guidefor Analysis of Gene and Proteins* (Wiley & Sons, Inc., 2nd ed., 2001). See also US 6,420,108.

[0074] The present invention may also make use of various computer program products and software for a variety of purposes, such as probe design, management of data, analysis, and instrument operation. See, U.S. Patent Nos. 5,593,839, 5,795,716, 5,733,729, 5,974,164, 6,066,454, 6,090,555, 6,185,561, 6,188,783, 6,223,127, 6,229,91 1 and 6,308,170. Computer methods related to genotyping using high density microarray analysis may also be used in the present methods, see, for example, US Patent Pub. Nos. 20050250151, 20050244883, 20050108197, 20050079536 and 20050042654. Additionally, the present disclosure may have preferred embodiments that include methods for providing genetic information over networks such as the Internet as shown in U.S. Patent Pub. Nos. 20030097222, 20020183936, 20030100995, 20030120432, 20040002818, 20040126840, and 20040049354.

[0075] An allele refers to one specific form of a genetic sequence (such as a gene) within a cell, an individual or within a population, the specific form differing from other forms of the same gene in the sequence of at least one, and frequently more than one, variant sites within the sequence of the gene. The sequences at these variant sites that differ between different alleles are termed "variances", "polymorphisms", or "mutations". At each autosomal specific chromosomal location or "locus" an individual possesses two alleles, one inherited from one parent and one from the other

parent, for example one from the mother and one from the father. An individual is "heterozygous" at a locus if it has two different alleles at that locus. An individual is "homozygous" at a locus if it has two identical alleles at that locus.

[0076] The term "polymorphism" as used herein refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker or site is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%, and more preferably greater than 10% or 20% in a selected population. A polymorphism may comprise one or more base changes, an insertion, a repeat, or a deletion of one or more bases. Copy number variants (CNVs), transversions and other rearrangements are also forms of genetic variation. Polymorphic markers include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. The allelic form occurring most frequently in a selected population is sometimes referred to as the wildtype form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms. Single nucleotide polymorphisms (SNPs) are a form of polymorphisms. SNPs are a common type of human genetic variation and are useful in the performance of genome wide association studies (GWAS). GWAS may be used, for example for the analysis of biological pathways, see Wang and Hakonarson, Nat. Rev. Genet. 2010, 1 1:843-854.

[0077] The term genotyping refers to the determination of the genetic information an individual carries at one or more positions in the genome. For example, genotyping may comprise the determination of which allele or alleles an individual carries for a single SNP or the determination of which allele or alleles an individual carries for a plurality of SNPs or CNVs. A diploid individual may be homozygous for each of the two possible alleles (for example, AA or BB) or heterozygous (for example, AB). For additional information regarding genotyping and genome structure see, *Color Atlas of Genetics,* Ed. Passarge, Thieme, New York, NY (2001), which is incorporated by reference.

[0078] Normal cells that are heterozygous at one or more loci may give rise to tumor cells that are homozygous at those loci. This loss of heterozygosity (LOH) may result from structural deletion of normal genes or loss of the chromosome carrying the normal gene, mitotic recombination between normal and mutant genes, followed by formation of daughter cells homozygous for deleted or

inactivated (mutant) genes; or loss of the chromosome with the normal gene and duplication of the chromosome with the deleted or inactivated (mutant) gene. LOH may be copy neutral or may result from a deletion or amplification.

[0079] The term "array" as used herein refers to an intentionally created collection of molecules which can be prepared either synthetically or biosynthetically. The molecules in the array can be identical or different from each other. The array can assume a variety of formats,*for example,* libraries of soluble molecules; libraries of compounds tethered to resin beads, silica chips, microparticles, nanoparticles or other solid supports.

[0080] The term "complementary" as used herein refers to the hybridization or base pairing between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid to be sequenced or amplified. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

[0081] The term "copy number variation" or "CNV" refers to differences in the copy number of genetic information. In many aspects it refers to differences in the per genome copy number of a genomic region. For example, in a diploid organism the expected copy number for autosomal genomic regions is 2 copies per genome. Such genomic regions should be present at 2 copies per cell. For a recent review see Zhang *et al. Annu. Rev. Genomics Hum. Genet.* 2009. 10:451-81. CNV is a source of genetic diversity in humans and can be associated with complex disorders and disease, for example, by altering gene dosage, gene disruption, or gene fusion. They can also represent benign polymorphic variants. CNVs can be large, for example, larger than 1 Mb, but many are smaller, for example between 100 bp and 1 Mb. More than 38,000 CNVs greater than 100 bp (and less than 3 Mb) have been reported in humans. Along with SNPs these CNVs account for a significant amount of phenotypic variation between individuals. In addition to having deleterious impacts, e.g. causing disease, they may also result in advantageous variation.

[0082] Digital PGR is a technique where a limiting dilution of the sample is made across a large number of separate PCR reactions so that most of the reactions have no template molecules and give a negative amplification result. Those reactions that are positive at the reaction endpoint are counted as individual template molecules present in the original sample in a 1 to 1 relationship. See Kalina et al. NAR 25:1999-2004 (1997) and Vogelstein and Kinzler, PNAS 96:9236-9241 (1999). This method is an absolute counting method where solutions are partitioned into containers until

there is an average probability of one molecule per two containers or when, $P_0 = (1\text{-}e^{-n/c}) = ½$; where *n* is the number of molecules and *c* is the number of containers, or *n/c* is 0.693. Quantitative partitioning is assumed, and the dynamic range is governed by the number of containers available for stochastic separation. The molecules are then detected by PCR and the number of positive containers is counted. Each successful amplification is counted as one molecule, independent of the actual amount of product. PCR-based techniques have the additional advantage of only counting molecules that can be amplified, *e.g.* that are relevant to the massively parallel PCR step in the sequencing workflow. Because digital PCR has single molecule sensitivity, only a few hundred library molecules are required for accurate quantification. Elimination of the quantification bottleneck reduces the sample input requirement from micrograms to nanograms or less, opening the way for minute and/or precious samples onto the next-generation sequencing platforms without the distorting effects of pre-amplification. Digital PCR has been used to quantify sequencing libraries to eliminate uncertainty associated with the construction and application of standard curves to PCR-based quantification and enable direct sequencing without titration runs. See White *et al. BMC Genomics* 10: 116 (2009). To vary dynamic range, micro-fabrication can be used to substantially increase the number of containers. See, Fan et al. *Am J Obstet Gynecol* 200, 543 e1 (May, 2009).

[0083] Similarly, in stochastic labeling as disclosed herein, the same statistical conditions are met when $P_0 = (1\text{-}e^{-n/m}) = ½$; where *m* is the number of label-tags, and one half of the label-tags will be used at least once when $n/m = 0.693$. The dynamic range is governed by the number of label-tags used, and the number of label-tags can be easily increased to extend the dynamic range. The number of containers in digital PCR plays the same role as the number of label-tags in stochastic labeling and by substituting containers for label-tags identical statistical equations may be applied. Using the principles of physical separation, digital PCR stochastically expands identical molecules into *physical space,* whereas the principle governing stochastic labeling is identity based and expands identical molecules into *identity space.*

[0084] The term "hybridization" as used herein refers to the process in which two single-stranded polynucleotides bind non-covalently to form a stable double-stranded polynucleotide; triple-stranded hybridization is also theoretically possible. The resulting (usually) double-stranded polynucleotide is a "hybrid." The proportion of the population of polynucleotides that forms stable hybrids is referred to herein as the "degree of hybridization." Hybridizations may be performed

under stringent conditions, for example, at a salt concentration of no more than 1 M and a temperature of at least 25°C. For example, conditions of 5X SSPE (750 hiM NaCl, 50 mM NaPhosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C are suitable for allele-specific probe hybridizations. For stringent conditions, see, for example, Sambrook, Fritsche and Maniatis. "Molecular Cloning A laboratory Manual" 2nd Ed. Cold Spring Harbor Press (1989) which is hereby incorporated by reference in its entirety for all purposes above. In some aspects salt concentrations for hybridization are preferably between about 200 mM and about 1M or between about 200 mM and about 500 mM. Hybridization temperatures can be as low as 5 °C , but are typically greater than 22 °C, more typically greater than about 30°C, and preferably in excess of about 37°C. Longer fragments may require higher hybridization temperatures for specific hybridization. As other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one alone.

[0085] The term "mRNA" or sometimes refer by "mRNA transcripts" as used herein, include, but not limited to pre-mRNA transcript(s), transcript processing intermediates, mature mRNA(s) ready for translation and transcripts of the gene or genes, or nucleic acids derived from the mRNA transcript(s). Transcript processing may include splicing, editing and degradation. As used herein, a nucleic acid derived from an mRNA transcript refers to a nucleic acid for whose synthesis the mRNA transcript or a subsequence thereof has ultimately served as a template. Thus, a cDNA reverse transcribed from an mRNA, an RNA transcribed from that cDNA, a DNA amplified from the cDNA, an RNA transcribed from the amplified DNA, *etc., are,* all derived from the mRNA transcript and detection of such derived products is indicative of the presence and/or abundance of the original transcript in a sample. Thus, mRNA derived samples include, but are not limited to, mRNA transcripts of the gene or genes, cDNA reverse transcribed from the mRNA, cRNA transcribed from the cDNA, DNA amplified from the genes, RNA transcribed from amplified DNA, and the like.

[0086] Other classes or RNAs are also expressed, including, for example, ribosomal RNA, snRNA, miRNA and siRNA. Recent evidence suggests that the human transcriptome contains many functional RNA transcripts which are not translated into proteins. These non-coding RNAs have been recognized as important to a more complete understanding of biology. Mature miRNAs are relatively small (21-23 nucleotides) RNA duplexes that act as translational repressors of protein

expression. The guide strand of a miRNA interacts with proteins to form RNA-Induced Silencing Complexes (RISC) in the cell. These sequence-specific ribonucleoprotein complexes bind target mRNAs typically in the 3'UTR and can subsequently silence gene expression either through directed mRNA degradation or by simply sequestering the target mRNA in an ineffectual form (Lee et al., Cell (1993), 75: 843-854; Bartel, Cell (2009), 136: 215-233). It has been demonstrated that miRNA based regulation plays a significant role in routine cellular processes including metabolism (Esau et al, Cell Met. 2006, v.3, p 87-98), development (Carthew et al., Cell 2009, v.137, p. 273-282), and even apoptosis (Cheng et al, Nucl. Acids Res. 2005, v.33, pi290- 1297). Further research has revealed that miRNAs play critical roles in diverse disease processes such as hepatitis C (Jopling et al, Science 2005, v.309, p. 1577-1581), diabetes (Poy et al., Nature 2004, v.432, p. 226-230), and most notably multiple cancer types (Hammond, Can. Chemo. Pharma. 2006 v.58, s63-s68; Calin et al, Cancer Res. 2006, v.66, p. 7390-7394) including leukemia (Calin et al., PNAS 2002, v.lOl, p. 2999-3004) and glioma (Corsten et al., Cancer Res. 2007, v.67, p. 8994-9000). Over one thousand miRNAs have now been identified in animals, but only a few individual miRNAs have been linked to specific functions. Methods of the invention disclosed herein can be used for inactivation of relatively short regulatory non-coding RNAs, such as micro RNAs (miRNAs), Piwi-interacting RNAs (piRNAs), snoRNAs, snRNAs, moRNAs, PARs, sdRNAs, tel-sRNAs, crasiRNAs, and small interfering RNAs (siRNAs). Methods of the invention can also be used for long non-coding RNAs (long ncRNAs), traditional non-coding tRNAs and ribosomal RNA (rRNA).

[0087] The term "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides, deoxyribonucleotides or peptide nucleic acids (PNAs), that comprise purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups, as may typically be found in RNA or DNA, or modified or substituted sugar or phosphate groups. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. The sequence of nucleotides may be interrupted by non-nucleotide components. Thus the terms nucleoside, nucleotide, deoxynucleoside and deoxynucleotide generally include analogs such as those described herein. These analogs are those molecules having some structural features in common with a naturally occurring nucleoside or nucleotide such that when incorporated into a nucleic acid or oligonucleoside sequence, they allow hybridization with a naturally occurring nucleic acid sequence in solution. Typically, these analogs

are derived from naturally occurring nucleosides and nucleotides by replacing and/or modifying the base, the ribose or the phosphodiester moiety. The changes can be tailor made to stabilize or destabilize hybrid formation or enhance the specificity of hybridization with a complementary nucleic acid sequence as desired.

[0088] The term "oligonucleotide" or sometimes refer by "polynucleotide" as used herein refers to a nucleic acid ranging from at least 2, preferable at least 8, and more preferably at least 20 nucleotides in length or a compound that specifically hybridizes to a polynucleotide. Polynucleotides of the present invention include sequences of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) which may be isolated from natural sources, recombinantly produced or artificially synthesized and mimetics thereof. A further example of a polynucleotide of the present invention may include non natural analogs that may increase specificity of hybridization, for example, peptide nucleic acid (PNA) linkages and Locked Nucleic Acid (LNA) linkages. The LNA linkages are conformationally restricted nucleotide analogs that bind to complementary target with a higher melting temperature and greater mismatch discrimination. Other modifications that may be included in probes include: 2'OMe, 2'OAllyl, 2'0-propargyl, 2'O-alkyl, 2' fluoro, *2'* arabino, 2' xylo, 2' fluoro arabino, phosphorothioate, phosphorodithioate, phosphoroamidates, 2Amino, 5-alkyl-substituted pyrimidine, 5-halo-substituted pyrimidine, alkyl-substituted purine, halo-substituted purine, bicyclic nucleotides, 2'MOE, LNA-like molecules and derivatives thereof. The invention also encompasses situations in which there is a nontraditional base pairing such as Hoogsteen base pairing which has been identified in certain tRNA molecules and postulated to exist in a triple helix. "Polynucleotide" and "oligonucleotide" are used interchangeably in this application.

[0089] The term "primer" as used herein refers to a single-stranded oligonucleotide capable of acting as a point of initiation for template-directed DNA synthesis under suitable conditions for example, buffer and temperature, in the presence of four different nucleoside triphosphates and an agent for polymerization, such as, for example, DNA or RNA polymerase or reverse transcriptase. The length of the primer, in any given case, depends on, for example, the intended use of the primer, and generally ranges from 15 to 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with such template. The primer site is the area of the template to which a primer hybridizes. The primer pair is a set of primers including a 5' upstream primer that hybridizes with the 5' end of the

sequence to be amplified and a 3' downstream primer that hybridizes with the complement of the 3' end of the sequence to be amplified.

[0090] The term "probe" as used herein refers to a surface-immobilized molecule that can be recognized by a particular target. See U.S. Patent No. 6,582,908 for an example of arrays having all possible combinations of probes with 10, 12, and more bases. Examples of probes that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (for example, opioid peptides, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

[0091] The term "solid support", "support", and "substrate" as used herein are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations. See U.S. Patent No. 5,744,305 and US Patent Pub. Nos. 20090149340 and 20080038559 for exemplary substrates.

[0092] The terms "label-tag", "identifier" and "counter" as used herein refer to the information that is added to individual occurrences of species of molecules to be counted using the methods disclosed herein. Libraries of label-tags having a diversity of unique label-tags, for example about 1,000, about 5,000, about 10,000, about 100,000 or more than 100,000 may be used to uniquely identify occurrences of target species thereby marking each species with an identifier that can be used to distinguish between two otherwise identical or nearly identical targets. For example, each label-tag may be a short string of nucleotides that can be attached to different copies of an mRNA, for example, a first label-tag may be 5OCATCTTC3' and a second may be 5'CAAGTAAC3'. Each has a unique identity that can be determined by determining the identity and order of the bases in the label-tag. Although nucleic acids are used throughout as a preferred embodiment of label-tag, one of skill in the art will appreciate that a number of types of molecules or items that can be generated with the diversity needed may be used as label-tags. Label-tags should be compounds, structures or elements that are amenable to at least one method of detection that allows for discrimination between different label-tags and should be associable in some means with the

elements to be counted. For example, a pool of label-tags may be comprises of a collection of different semiconductor nanocrystals, metal compounds, peptides, antibodies, small molecules, isotopes, particles or structures having different shapes, colors, barcodes or diffraction patterns associated therewith or embedded therein, strings of numbers, random fragments of proteins or nucleic acids, or different isotopes (see, Abdelrahman, A.I. et al. *Journal of Analytical Atomic Spectrometry* 25 (3):260-268, 2010 for use of metal containing polystyrene beads as standards for mass cytometry, incorporated herein by reference). Pools of label-tags may be partitioned into distinct sets that can be attached to separate sample mixtures and then combined for later analysis. For example, a set of 1,000,000 different label-tags could be physically divided into 10 sets of 100,000 different label-tags and each could be used to label-tag a different mixture. The identity of the label-tags in each set can be used as an indication of the original source. Counting of multiple samples in parallel can be facilitated.

[0093] The term "detectable label" as used herein refers to any chemical moiety attached to a nucleotide, nucleotide polymer, or nucleic acid binding factor, wherein the attachment may be covalent or non-covalent. Preferably, the label is detectable and renders the nucleotide or nucleotide polymer detectable to the practitioner of the invention. Detectable labels that may be used in combination with the methods disclosed herein include, for example, a fluorescent label, a chemiluminescent label, a quencher, a radioactive label, biotin and gold, or combinations thereof. Detectable labels include luminescent molecules, fluorochromes, fluorescent quenching agents, colored molecules, radioisotopes or scintillants. Detectable labels also include any useful linker molecule (such as biotin, avidin, streptavidin, HRP, protein A, protein G, antibodies or fragments thereof, Grb2, polyhistidine, Ni^2+, FLAG tags, myc tags), heavy metals, enzymes (examples include alkaline phosphatase, peroxidase and luciferase), electron donors/acceptors, acridinium esters, dyes and calorimetric substrates. It is also envisioned that a change in mass may be considered a detectable label, as is the case of surface plasmon resonance detection. The skilled artisan would readily recognize useful detectable labels that are not mentioned above, which may be employed in the operation of the present invention.

[0094] A stochastic process is the counterpart to a deterministic process. Instead of dealing with only one possible "reality" of how the process might evolve under time, in a stochastic or random process there is some indeterminacy in its future evolution described by probability distributions. This means that even if the initial condition (or starting point) is known, there are many possibilities

the process might go to, but some paths are more probable and others less. When all of the molecules in a uniform mixture of fixed volume collide and react randomly, the chemical events follow a stochastic process governed in part by the molecule concentration of each species (D. T. Gillespie, *The Journal of Physical Chemistry* 81, 2340 (1977)).

[0095] In the simplest possible case, a stochastic process amounts to a sequence of random variables known as a time series. Another basic type of a stochastic process is a random field, whose domain is a region of space, in other words, a random function whose arguments are drawn from a range of continuously changing values. One approach to stochastic processes treats them as functions of one or several deterministic arguments ("inputs" or "time") whose values ("outputs") are random variables: non-deterministic (single) quantities which have certain probability distributions. Random variables corresponding to various times (or points, in the case of random fields) may be completely different. The main requirement is that these different random quantities all have the same "type". Although the random values of a stochastic process at different times may be independent random variables, they often exhibit complicated statistical correlations. Familiar examples of processes modeled as stochastic time series include stock market and exchange rate fluctuations, signals such as speech, audio and video, medical data such as a patient's EKG, EEG, blood pressure or temperature, and random movement such as Brownian motion or random walks.

STOCHASTIC LABELING OF INDIVIDUAL MOLECULES

[0096] Methods are disclosed herein that may be applied to determining small numbers of biological molecules and their changes in response to, for example, cellular response, differentiation or signal transduction. The methods may also be used in performing a wide variety of clinical measurements. Although many analytical methods have been developed to measure the *relative* abundance of different molecules through sampling (e.g., microarrays and sequencing), the methods disclosed herein are able to determine the *absolute* number of molecules in a sample.

[0097] The stochastic labeling methods may also be generalized as follows. Consider a given target sequence defined as $T = \{t_1, t_2 \ldots t_n\}$; where n is the number of copies of T. A set of label-tags is defined as $L = \{l_1, l_2 \ldots l_m\}$; where m is the number of different label-tags. l^i reacts stochastically with L, such that each t becomes attached to one l. If the l's are in non-depleting excess, each t will choose one l randomly, and will take on a new identity $l_i t_j$; where l_i is chosen from L and j is the jth copy from the set of n molecules. We identify each new molecule $l_i t_j$ by its label-tag subscript and

drop the subscript for the copies of T, because they are identical. The new collection of molecules becomes $T^* = Iit + l_2t + ... lit;$ where l_i is the ith choice from the set of m label-tags. It is important to emphasize that the subscripts of / at this point refer only to the ith choice and provide no information about the identity of each /. In fact, $/_1$ and l_2 will have some probability of being identical, depending upon the diversity m of the set of label-tags. Overall, T^* will contain a set of k unique label-tags resulting from n targets choosing from the non-depleting reservoir of m label-tags. Or, $T^*(m,n) = \{//_i\}$; where k represents the number of unique label-tags that have been captured. In all cases, k will be smaller than m, approaching m only when n becomes very large. We can define the stochastic attachment of the set of label-tags on a target using a stochastic operator S with m members, acting upon a target population of n, such that $S(m)T(n) = T^*(m,n)$ generating the set $\{ht\}$.

[0098] Furthermore, because 5' operates on all molecules independently, it can act on many different target sequences. Hence, the method can simultaneously count copies of multiple target sequences by combining the information of target sequence and label. The probability of the number of label-tags generated by the number of trials n, from a diversity of m, can be approximated by the Poisson equation, $P_X = [(n/m)^x/xl]e^{-(n/m)}$. Then P_0 is the probability that a label-tag will not be chosen in n trials, and therefore, $1 - P_0$ is the probability that a label-tag will occur at least once. It follows that the number of unique label-tags captured is given by: $k = m(1-Po) = m[1- e^{-(n/m)}]$. Given k, we can calculate n. In addition to using the Poisson approximation, the relationship for k, n and m can be described analytically using the binomial distribution, or simulated using a random number generator, each yielding similar results.

[0099] The stochastic labeling process employed herein can be generalized as follows for illustrative purposes. Consider n copies of a given target molecule T, where $T = \{t_j, j = 1,2,\cdots,«\}$, and a non-depleting reservoir of m diverse label-tags L, where $L = \{l_i, i = 1,2,\cdots,m\}$. T reacts with L stochastically, such that each t_j will choose exactly one $l_{i(j)}$, $I \leq i\{j) \leq_m$ to take on a new identity $i_{i(j)}t_j$, and may be identified by its label-tag subscript (the subscript j for the copy of target molecult $/_j$ may be dropped). Therefore, the new collection of molecules T^* may be denoted as $T^* = \{l_{i(j)}t, 7 = 1,2,- ./!, \setminus \leq i(j) \leq m\}$.

[00100] When different copies of the target molecules react with the same label-tag, $i(j)$ for those molecules will assume the same value, therefore, the number of uniquely labeled target

molecules k cannot be greater than m. The stochastic reaction of the set of label-tags on a target may be described by a stochastic operator S with m members, acting upon a target population of n, such that $S(m)T(n) = ST = T*(m,n)$ generating the set $\tau* = \{l_{i(j)}t,\ j = \backslash,2,\cdots,n,\ \backslash \leq i(j) \leq m\}$. For simplicity, we may writer* $= \{l_k t\}$. Furthermore, since S operates on all molecules independently, it will act on many different target sequences and the method can be expanded to count copies of multiple target sequences, w,

simultaneously: $ST^w = ST, + ST_2 + \cdots + ST_W = T_1* + T_2* + \cdots + T_W* = \{l_k t\}_1 + \{l_k t\}_2 + \cdots + \{l_k t\}_w$,

where each $T_i*,\ i = 1,2,\cdots,w$ consists of a set $\{l_k t\}_i$. The net result of S operating on a specific target population is to map the number of molecules, n, of that target, to the number of distinct label-tags captured, k, which is a random variable.

[0100] Because target molecules randomly react with a label-tag with probability $\frac{1}{m}$, the probability of a label-tag being captured by exactly x out of n copies of a target molecule can be modeled as a Binomial distribution, $P(x) = \frac{n!}{x\backslash(n - x)!}(\frac{1}{m})^x(1 - \frac{1}{m})^{n-x}$, where xl denotes the factorial of x. The probability that a label-tag will not be captured by any copy of the target molecule is $P(\mathbf{0}) = (1 - 1/m)^n$, and the probability that a label-tag will be captured at least once is **1- P(0)**. When $n \to \infty$ and $1/m \to \mathbf{0}$ in the way $thatn/m \to \lambda$, $P\{x\}$ converges to the Poisson distribution with mean λ, i.e., $P(x) = \frac{\lambda^x}{x!}e^{-\lambda}$.

[0101] To compute the number of unique label-tags captured by n copies of a target molecule, an index random variable, X_i may be introduced, which is **1** if a label-tag has been captured at least once, and **0** otherwise. The number of unique label-tags captured is thus $k = \sum_{i=1}^{m} X_i$. The mean and variance of k can be derived,

$$E[k] = rn[l - (1 - \frac{1}{m})^n] \qquad (1)$$

$$Var[k] = m[\backslash - (1 - \frac{1}{m})^n](i - \frac{1}{m})^{n} + m(m - 1)[(1 - \frac{2}{m})^n - (1 - \frac{1}{m})^{2n}] \qquad (2)$$

[0102] Similarly, to compute the number of label-tags captured by exactly x copies of a target molecule, we introduce another index random variable, Y_j, which is **1** if a label-tag has been

captured exactly x times, and 0 otherwise. The number of label-tags captured x times is thus $t = \sum_{i=1}^{m} Y_i$. The mean and variance of t are,

$$E[t] = \frac{m \cdot n!}{x!(n-x)!}(\frac{1}{m})^x(1-\frac{1}{m})^{n-x} \qquad (3)$$

$$Var[t] = A(1-A) + (m-1)m \cdot \binom{n}{2x} \cdot (\frac{2}{m})^{2x}(1-\frac{2}{m})^{n-2x}\binom{2x}{x}(\frac{1}{2})^{2x} \qquad (4)$$

where $A = m \cdot \binom{n}{x}(\frac{1}{m})^x(1-\frac{1}{m})^{n-x}$, and the combination $\binom{n}{x} = \frac{n!}{x!(n-x)!}$.

The equations were experimentally validated by performing numerical simulations with 5000 independent runs for each simulated case. Complete agreement with the analytical solutions was observed.

[0103] The theoretical outcome of stochastic labeling is illustrated by examining the graph of k verses n (curve **1101** in FIG. 11) calculated using a label-tag diversity (m) of 960 and assuming a non-depleting reservoir. As expected, the number of unique label-tags captured depends on the ratio of molecules to label-tags, n/m. When n is much smaller than m, each molecule almost always captures a unique label-tag, and counting k is equivalent to counting n. As n increases, k increases more slowly as given by equation 1, and yet remains a very precise estimate of n. For example, when n/m is -0.01, the ratio of unique label-tags to molecules k/n -0.99, and we expect an increase of 10 molecules will generate 10 +/- X new label-tags. As n/m approaches 0.5 (i.e., -480 molecules reacted with 960 label-tags), k/n -0.79 and - 6 +/- X new label-tags are expected with an increase of 10 molecules. At high n/m, k increases more slowly as label-tags in the library are more likely to be captured more than once. Curve 1101 shows the number of label-tags captured at least once, curve **1102** is the number of label-tags chosen exactly once, and curve **1103** shows the number of label-tags chosen exactly twice as n increases. Curve **1101** shows the number of label-tags captured at least once. Equation (1) above was used to calculate the at least once curve, equation (2) the exactly once curve and equation (3) the exactly twice curve. The error bars indicate one standard deviation from the corresponding mean value.

[0104] A more complete description of the number of times a label-tag is chosen as a function of n is shown in FIGS. 15 and 16 (which correspond to Figs. S1 and S2 in Fu et al. PNAS, 108(22):9026-9031 (2011), which is incorporated herein in its entirety by reference). FIG. 15, plots

the expected label-tag usage (y axis) when ligating to a given number of target molecules (x axis). Each copy of a target molecule randomly ligates to only a single copy of one of 1000 distinct label-tags equally represented in a library pool in non-depleting quantities. The number of occurrences that each label-tag is used is plotted as indicated. The expected label-tag usage was obtained from Eqs. (1) and (3). Ratios can also be expressed as *m* to *n* (or *m/n*) which may be, for example, greater than 50, 100, 500 or 1,000. FIG. 16 shows the predicted target molecule counting efficiency (Y-axis) based on libraries of 1000, 2000, 4000, 8000 or 10,000 label-tags (X-axis). Counting efficiency is expressed as the ratio of label-tags observed at least once over the number of target molecules present. At lower target numbers (inset plot) a near-linear relationship is expected, but counting efficiency decreases nonlinearly upon increased repetitive usage of the same label-tags. Larger numbers of label-tags have higher counting efficiencies.

[0105] Methods for performing single molecule digital counting by the stochastic labeling of a collection of identical molecules are disclosed. As illustrated in FIGS. 1, 2A and 2B, each copy of a molecule (from a collection of identical target molecules (target types 203a, 203b and 203c, in a mixture 203) randomly captures a label-tag by choosing from a large, non-depleting reservoir of diverse label-tags 201. The uniqueness of each labeled molecule is governed by the statistics of random choice, and depends on the number of copies of identical molecules in the collection compared to the diversity of label-tags. Once the molecules are labeled each has been given a unique identity and can now be separately detected. In some aspects, it is preferable to first amplify the labeled targets prior to detection so that simple present/absent threshold detection methods can be used. Counting the number of label-tags is used to determine the original number of molecules in solution. In further aspects, the molecules to be counted are each members of a class that shares some common feature, for example, they may each be a single copy of a particular gene sequence or nucleic acid sequence. Counting may be applied, for example, to mRNA targets, including splicing products and alternatively spliced products and pre-mRNA. The methods may be used for counting of short regulatory non-coding RNAs, such as micro RNAs (miRNAs), Piwi-interacting RNAs (piRNAs), snoRNAs, snRNAs, moRNAs, PARs, sdRNAs, tel-sRNAs, crasiRNAs, and small interfering RNAs (siRNAs). Methods of the invention can also be used for long non-coding RNAs (long ncRNAs), traditional non-coding tRNAs, and ribosomal RNA (rRNA).

[0106] Similarly, counting may be applied to DNA, for example, gene copy number, chromosome number, mitochondrial DNA, bacterial genomes, pathogen nucleic acid, viral nucleic acids and the

like. Counting may be applied in research of disease in humans or other mammals or agricultural organisms, e.g. cattle, chicken, wheat, rice, fish, etc. Counting may also be applied to counting aspects of microbes, such as environmental measurements, e.g. water quality testing. The methods may be particularly useful where small numbers of items are to be counted and an accurate count is desirable rather than a relative estimate.

[0107] FIG. 1 illustrates the labeling process schematically. The different label-tags or identifiers (represented by different shapes) from the pool of label-tags 201, $\{l_1, l_2—l_m\}$ are attached to each of 4 different copies of the same target "t". Label-tag 20, l_{20} is attached to ti, label-tag 107 (l_{107}) to t_2, label-tag 477 (l_{477}) to t_3 and label-tag 9 (l_9) to t_4. The target-label-tag products, t_1l_{20}, t_2li_{07}, t_3l477 and t_{419}, are then amplified to generate four unique populations in a pool of amplified targets 205, each population representing a single occurrence of the target in the starting sample. Each target molecule randomly captures and joins with a label-tag by choosing from a large, nondepleting reservoir of m label-tags. Each resulting labeled target molecule takes on a new identity. The subsequent diversity of the labeled molecules is governed by the statistics of random choice, and depends on the number of copies of identical molecules in the collection compared to the number of kinds of label-tags. Once the molecules are labeled, they can be amplified so that simple present/absent threshold detection methods can be used for each. Counting the number of distinctly labeled targets reveals the original number of molecules of each species.

[0108] FIG. 2A illustrates a method for comparing two samples, identified as sample 1 and 2. The target 203 Gene A is present in 2 copies in sample 1 and 8 copies in sample 2. Both samples have non-target molecules 204. The label-tags 201 are combined with the samples and target molecules are attached to individual label-tag molecules in a stochastic manner. The targets with attached label-tags are hybridized to an array 211 having many features (illustrated schematically as squares), there is a feature for each possible target-label-tag combination. Some of the features are labeled, for example, 209 and others are not, for example, 207. The labeled features indicate the presence of a specific target-label-tag combination and each corresponds to a count. As shown for gene A in sample 1 there are two labeled features so the count is 2. For Gene A in sample 2 there are 8 labeled features so the count is 8.

[0109] FIG. 2C shows a further schematic illustration of one embodiment. A library of different label-tag sequences 201 (here shown as identical lines) is combined with a sample that includes an unknown number of each of several targets of interest in a mixture of targets 203. Three different

species of target are shown, **203a, 203b** and **203c,** present at **4, 6** and **3** copies respectively (non-targets may be present but are not shown). The individual label-tag oligonucleotides from library **201** are covalently attached to the different targets to form target-label-tag molecules **205.** Each target has a collection of different label-tag molecules **205a, 205b** and **205c** (again present at **4, 6** and **3** copies respectively) and within each target-specific collection the members differ in the label-tag oligo that is attached, so that each different target occurrence is uniquely marked with a different label-tag from the pool **201.** On the array **207,** each target is tiled in combination with all possible label-tag combinations represented with each different combination being present at a different known or determinable location on the array. In the figure each different possible combination of target and label-tag is represented by a single probe for illustration purposes, but on the array each different probe is preferably present in a feature having multiple copies of the same probe sequence. The array is divided into subarrays **207a, 207b** and **207c** for illustrative purposes, each corresponding to a different target species (**203a, 203b** or **203c** respectively). The upper portion **209** of the probes (shown as vertical lines) varies at each feature according to the different label-tag. The lower portion **213** is the same for all features of each subarray and is complementary to the target. Preferably there is a different probe for each possible combination of label-tag and target. For example, if there are three targets being interrogated and **1000** different label-tags there would be **3,000** different probe features (**3** x **1000**). After hybridization individual features of the array are labeled through hybridization of the complementary target-label-tag molecule to the feature. The figure shows a detectable label **211,** for example a biotin labeled nucleotide, may be used to detect features where a target-label-tag is hybridized.

[0110] Although the array illustrated in FIG. 2C is shown as a single planar support, any type of array could be used. In many aspects the methods do not require that the identity of the probe at a given feature be known or determinable and this allows for many different types of arrays to be used. Because the readout can be simply binary (i.e. yes, the feature is labeled or no, the feature is not labeled) it is not necessary to determine which probe sequence is present at a given feature. If a single target is being analyzed such that all features of the array correspond to the same target, the number of labeled features corresponds to the count and it is not necessary to determine which label-tags are being counted. The array can be one or more solid supports with different probes arranged in features at known or determinable locations or the probes may be arranged in a random manner where the region of the probe that is complementary to the label-tags is not known. This

allows for a variety of synthesis approaches in addition to methods where the sequence to be synthesized or deposited at any given feature is known, for example, synthesis may include a pooled synthesis approach.

[0111] The array may also be beads or microparticles in solution. Different beads may be labeled with different probes and detection may be by arranging the beads on a solid support and imaging the beads on the support, (see for example, Oliphant *et. al, Biotechniques* 2002, 56-8, 60-1 and Kuhn et al. Genome Res. 2004 14(1 1):2347-56) or the beads may be imaged in solution as they flow through a detection chamber, see for example, Dunbar, SA, *Clin Chim Acta.* (2006), 363(1-2):7 1-82. A differentially identifiable bead type can be used for each target and within that bead type different individual beads can be attached to different target-label-tag probes. Each bead that is labeled can be counted as an occurrence of that target. Methods for making and using microparticles are also disclosed in US 7,745,091 and 7,745,092 and PCT Publication No. WO 2007/081410, each of which is incorporated herein by reference in their entireties. Probes may be attached to a microparticle carrying a distinguishable code that may be characteristic of a target being counted. Such microparticles are preferably encoded such that the identity or type of a probe borne by a microparticle can be determined from a distinguishable code. The code can be in the form of a tag, which may itself be a probe, such as an oligonucleotide, a detectable label, such as a fluorophore, or embedded in the microparticle, for example, as a bar code. Microparticles bearing different probes may have different codes or the code may indicate members of a class of probes, for example, all probes for a given target, the probes varying in the label-tag region. Microparticles are typically distributed on a support by a sorting process in which a collection of microparticles are placed on the support and the microparticles distributed on the support. The location of the microparticles after distribution on the support can be defined by indentations such as wells or by association to adhesive regions on the support, among other methods. The microparticles may be touching or they may be separated so that individual microparticles are not touching. In many aspects, populations of microparticles for detection of a single target may be encoded with a single code indicative of that target while other populations may be labeled with a code for another target, even though each microparticle in the population may be specific for a different label-tag.

[0112] FIG. 2C illustrates another method for detection and counting of the number of label-tags and thus the number of individual targets. As above the label-tags, LI, L2, L3 and L4 are attached to the copies of a target 203 to form target-label-tag molecules 205. The target-label-tag molecules

are then subjected to PGR amplification in individual reactions, each having a single primer pair that includes a first target specific primer "IS" and a label-tag specific primer, "LI", "L2", "L3" or "L4" in the figure. The "TS" primer is the same for each reaction because a single target is being interrogated. The LI-L4 primers are different for each reaction. Each target-label-tag combination that is present after ligation will result in an amplification product in the reaction that includes the corresponding label-tag specific primer (indicated by "YES" in the figure). Each label-tag that isn't selected (L3) will result in no amplification (indicated by "NO" in the figure). The amplification products can be detected by any method available, including real time PGR or end point PGR where products are separated according to size.

[0113] The label-tags may be attached to the targets through any of a variety of mechanisms. Examples for methods for attachment of label-tags to targets are provided herein. Attachment may be, for example, by extension of a primer that has a label-tag portion and a target specific portion. The target specific portion may be specific for a single target, for example, a locus specific primer, or it may be specific for a class of targets, for example, an oligo dT region. Extension may be polymerase mediated, e.g. DNA or RNA polymerase, and may be template mediated (where the target serves as template for extension of the primer) or template independent. For template mediated extension the template may be RNA or DNA or an RNA:DNA chimera. The primer may be RNA or DNA or an RNA:DNA chimera (see. for example, US 6,251,639 which is incorporated herein by reference). The primer may also include modified bases such as locked nucleic acids (LNAs), peptide nucleic acids (PNAs), inosines, isoC, isoG, or the like. In some aspects label-tags may be attached by ligation using RNA or DNA ligase that may be heat labile or heat stabile.

[0114] In one aspect, the targets are small RNAs that are not polyadenylated. To attach identifiers to these small RNAs a first pool of identifier primers may be used to generate labeled cDNA product. The identifier primers may have (in the 5' to 3' direction) a common priming sequence, an identifier region and a target specific region that is complementary to a first region in the small RNA. The priming sequence and the target specific region are common in all identifier primers in the pool and the identifier region varies. The primers are hybridized to the targets and extended using a reverse transcriptase. This generates an identifiable cDNA for each small RNA target occurrence. The pool of cDNAs can be amplified using a primer specific for the small RNA target and a common primer complementary to the common priming sequence. The amplification products can be analyzed to determine how many different identifiers are included in the

amplification product. The pool of cDNAs can also be analyzed by performing separate PCR amplifications for each identifier in the pool as illustrated in FIG. 2C. The reactions may include a primer for one of the identifiers and a primer for the target. A separate reaction is used for each identifier so if the pool has 1,000 different identifiers the analysis may include 1,000 different amplification reactions, although a subset may be analyzed, for example, reactions may be performed for more than 90% of the identifiers, more than 80% or more than 50%. Each reaction may be analyzed for the presence or absence of an amplification product where the number of "present" calls indicates the number of occurrences of the target in the sample. Methods for performing many individual amplification reactions by PCR are disclosed, for example, in Heyries *et al. Nature Methods* 8, 649-65 1 (201 1), which is incorporated herein by reference. Methods for Digital PCR may be used but the partitioning step may be omitted since each reaction is specific for a single identifier. The reactions may be run in micro well plates, capillaries, arrays or miniaturized chambers, or on localized features of an array, e.g. arrays or beads. In some aspects each feature of an array has a different identifier primer attached to the array and an amplification reaction is performed using that primer and a common primer that may be complementary to a specific target to be counted. The common primer may be in solution or it may be attached to all of the features of an array for detection of a specific target.

[0115] FIG. 3 shows a schematic of one method for attachment of label-tags to fragments of DNA. The nucleic acid target 301, which may be genomic DNA, has fragmentation sites, for example, restriction sites, indicated as vertical lines 303. The target 301 is fragmented to generate fragments 305. Fragmentation may be site specific, e.g. restriction sites, or non-specific, e.g. by shearing or sonication. Adaptors that preferably contain label-tags 306 are ligated to the fragments to generate adaptor-ligated fragments 307. The adaptors may ligate to all fragments. The adaptor-ligated fragments can be circularized to generate circles 309 that include the two label-tag containing adaptors ligated together 311 and the fragment 305. The circularized fragments can be treated with exonuclease to remove uniigated fragments. Target specific primer pair 313 can be used to amplify fragment 314. In some aspects the circles are linearized prior to amplification (after exonuclease treatment if it is used) by cleavage within fragment 305, preferably at a position between the sites of binding of the primers of the primer pair 313 to facilitate amplification. During amplification non-target circles will be amplified poorly or not at all because the primers in primer pair 313 are not complementary to the non-targets. The PCR product has the label-tag region 311 flanked by target

specific regions. Counting of the targets is by detection of the junction sequences 315 and/or 317. Detection may be, for example, by sequencing through the junction or by hybridization to a probe complementary to the junction. If an array is used, the array probe is preferably complementary to the junction between the target specific region and the label. The two such junctions 315 and 317 in the PGR product could each be targeted on either strand (since the product is double stranded). The products may be fragmented, labeled and hybridized to an array of probes to the junctions. The label-target combination can be hybridized to an array for counting.

[0116] The methods disclosed herein and the examples below demonstrate that a population of indistinguishable molecules can be stochastically expanded to a population of uniquely identifiable and countable molecules. High-sensitivity threshold detection of single molecules is demonstrated, and the process can be used to count both the absolute and relative number of molecules in a sample. The method should be well suited for determining the absolute number of multiple target molecules in a specified container, for example in high-sensitivity clinical assays, or for determining the number of transcripts in single cells. The approach should also be compatible with other molecular assay systems. For example, antibodies could be stochastically labeled with DNA fragments and those that bind antigen harvested. After amplification, the number of label-tags detected will reflect the original number of antigens in solutions. In the examples shown here, DNA is used because of the great diversity of sequences available, and because it is easily detectable. In principle, any molecular label-tag could be used, for example fluorescent groups or mass spectroscopy tags, as long as they are easily detected and they have sufficient diversity for the desired application. Although many of the examples refer to populations

[0117] It is instructive to contrast the attributes of stochastic labeling with other quantitative methods. Microarray and sequencing technologies are commonly used to obtain relative abundance of multiple targets in a sample. In the case of microarray analysis, intensity values reflect the relative amount of hybridization bound target and can be used to compare to the intensity of other targets in the sample. In the case of sequencing, the relative number of times a sequence is found is compared to the number of times other sequences are found. Although the techniques differ by using intensity in one case and a digital count in the other, they both provide *relative* comparisons of the number of molecules in solution. In order to obtain *absolute* numbers, *quantitative* capture of all sequences would need to be assured; however in practice the efficiency of capture with microarray and sequencing technologies is unknown.

[0118] Unlike the stochastic labeling methods disclosed herein, digital PCR confers uniqueness by partitioning into unique containers. Digital PCR is an *absolute* counting method where solutions are stochastically partitioned into multi-well containers until there is an average probability of one molecule per two containers, then detected by PCR. This condition is satisfied when, $P_0 = (1\text{-}e^{-n/c}) = ½$; where n is the number of molecules and c is the number of containers, or n/c is 0.693. Quantitative partitioning is assumed, and the dynamic range is governed by the number of containers available for stochastic separation. Once the molecules are partitioned, high efficiency PCR detection gives the yes/no answer and absolute counting enabled. To vary dynamic range, micro-fabrication can be used to substantially increase the number of containers. Similarly, in stochastic labeling, the same statistical conditions are met when $Po = (1\text{-}e^{-n/m}) = ½$; where m is the number of label-tags, and one half of the label-tags will be used at least once when $n/m = 0.693$. The dynamic range is governed by the number of label-tags used, and the number of label-tags can be easily increased to extend the dynamic range. The number of containers in digital PCR plays the same role as the number of label-tags in stochastic labeling and by substituting containers for label-tags we can write identical statistical equations. Using the principles of physical separation, digital PCR stochastically expands identical molecules into *physical space,* whereas the principle governing stochastic labeling is identity based and expands identical molecules into *identity space.*

[0119] In preferred aspects, of the presently disclosed methods, a diverse set of label-tags is randomly attached to a population of identical molecules and the population of identical molecules is thereby converted into a population of distinct molecules suitable for threshold detection. Random attachment as used herein refers to a process whereby any label-tag can be attached to a given molecule with the same probability. To demonstrate stochastic labeling methods experimentally the absolute and relative number of selected genes were determined after stochastically labeling 360,000 different fragments of the human genome. The approach does not require the physical separation of molecules and may take advantage of highly parallel methods such as microarray and sequencing technologies to simultaneously count absolute numbers of multiple targets. In some embodiments, stochastic labeling may be used for determining the absolute number of RNA or DNA molecules within single cells.

[0120] The methods disclosed herein may be used to take quantitative measurements of copies of identical molecules in a solution by transformation of the information to a digital process for detecting the presence of different label-tags. The stochastic properties of the method have been

measured, and the relative and absolute digital counting of nucleic acid molecules is demonstrated. The method is extremely sensitive, quantitative, and can be multiplexed to high levels. In some aspects a microarray-based detection method is used, but the method is extendable to many other detection formats.

[0121] Methods for analyzing genomic information often utilize a correlation between a measurement of the amount of material associated with a location. The location can be, for example, a feature of an array that contains a specific sequence that is known or can be determined or any type of solid support such as a bead, particle, membrane, etc. A common aspect to these methods is often hybridization of a target to be measured to a complementary probe attached to the solid support. The probe may be, for example, an oligonucleotide of known or determinable sequence, but may also be BACs, PACs, or PGR amplicons.

[0122] Methods that use signal intensity to estimate relative concentrations of targets typically label the targets with a detectable label, often after an amplification step, and through hybridization of the detectably labeled target to the probe, the probe and thus the feature is also labeled. The amount of label-tag is detected and correlated with a measurement of the amount of target in the sample. The estimate of amount of a given target in a sample is typically relative to other targets in the sample or to previously obtained measurements and may be based on comparison to targets present in the sample at known or expected levels or to controls within the sample. This type of analysis can and has been used successfully, for example, to estimate genomic copy number to detect copy number variation in individuals or in cell populations (see, for example, Pinkel and Albertson, *Annu. Rev. Genomics Hum. Genet.* 6, 331-354 (2005), Lucito *et al. Genome Res.* 13, 229102305 (2004), Sebat *et al. Science* 305, 525-528 (2004), Zhou *et al, Nat. Biotechnol.* 19, 78-81 (2001) and Zhao *et al. Cancer Res.* 65, 5561-5570 (2005) and US Patent Pub. Nos. 200401 57243 and 20060035258) or to estimate gene expression levels (see, for example, Lockhart *et al, Nat. Biotechnol.* 14:1675-1680 (1996), and Wodicka *et al, Nat. Biotechnol.* 15:1359-1367 (1997)).

[0123] Correlating intensity of hybridization signal or signal intensity with concentration of target molecules has limitations and can typically provide only an estimate of the absolute amount of a target, and may not be an accurate count of the actual amount of target present. The estimate may be an under or over estimate, particularly when comparing different targets or different samples. This is the result of many different factors, including but not limited to, differences between probes, feature specific effects, sample specific effects, feature size (as it decreases the ability to correlate

accurately decreases) and experimental variation. Much of this variation can be addressed by data analysis methods, but the methods do not provide counting of individual molecules or events and are therefore subject to estimation errors.

[0124] Because of the density of different features that can be obtained using synthesis methods such as photolithography, microarrays can be applied to high density applications. For example, at feature sizes of 1 micron square an array can have about 10^8 features per cm^2. Array densities that are currently commercially available include feature sizes of between 4 x 4 microns to 10 x 10 microns or feature spacing (distance between the centers of neighboring features of about 4 to about 10 microns). Within a feature, depending on the chemistry used for synthesis, the probes may be spaced typically at about 10 nm spacing resulting in about 10^4 molecules in a 1 x 1 micron feature. At approximately full saturation about 10% of those probes are hybridized with target (with some photolithographic synthesis methods some portion of the probes are not full length and may not participate in specific hybridization to the target). Assuming that only about 80% of the size of the feature is being analyzed (to mitigate for overlap between features at the edge) there are only about -800 nm^2 functional area for the probe so about 640 functional molecules in an array having 1 $micron^2$ spacing between features. This relatively small number of functional molecules in a feature limits the dynamic range for estimating relative concentration from hybridization signal intensity. The methods disclosed herein do not require estimation of relative concentration; rather it is sufficient to have a binary readout that is not quantitative with respect to feature intensity. The dynamic range limitations observed with small feature sizes and small numbers of molecules on the array surface, are mitigated by the disclosed methods by using a counting or digital readout as a substitute for the typical analog signal resulting from array hybridization.

[0125] In further aspects a different label-tag sequence is attached to each molecule of a particular target sequence or more preferably a collection of target sequences of interest. To achieve unique or nearly unique labeling of targets the number of different label-tags to be attached to the targets should exceed the number of targets. For example, a sample having 100 molecules of target type 1 may be mixed with an excess of about 1000 different-identity label-tag sequences, forming a library of label-tag sequences. Multiple copies of the library of label-tag sequences are used so there are many copies of each different-identity label-tag. Different label-tag sequences from the library are appended to each of the 100 target molecules so that each of the 100 molecules of the first target sequence has a unique label-tag sequence appended thereto. This results in 100 different target-

label-tag combinations that are each unique. The target-label-tag molecules may then be amplified to enrich the target-label-tag products relative to other non-targets. Amplification after labeling alters the absolute amount of the target, but because each occurrence in the original sample has been uniquely labeled this will not alter the count. The amplified target-label-tag products, whether amplified or not, can then be labeled with a detectable label like biotin, and hybridized to an array of probes or detected by other means.

[0126] If an array is used for detection, the features of the array that have target-label -tag hybridized thereto can be detected, for example, by labeling the hybridization complex with a fluorescent label-tag and detecting the presence of signal at the features. In this example, because there are 1000 different label-tags possible and a single target being analyzed, there are 1000 different possible label-target sequences that might be generated so an array having a different feature for each of the 1000 different possibilities can be used. Assuming each target is labeled and no label-tag is used twice, 100 of the 1000 different features should be detectable, indicating the corresponding label-tag has been used. A simple detector having m elements for each target sequence can be constructed. An array having 10^8 features or elements could assay 10^5 different targets using 10^3 different label-tags, for example. Other detection methods do not require individual elements for each counter, for example, sequencing.

[0127] The number of different-identity label-tags that are needed depends upon the maximum number of expected targets and the requirement for accuracy in the count. Consider 1 copy of a target molecule in solution identified as ti reacted against a set of 10 label-tags, $L_m = \{l_1, l_2, \ldots l_{10}\}$. Each label-tag has a 0.1 probability of being chosen. Next consider multiple copies of the target, t_n, reacted against the set of L_m (assume a non-depleting reservoir of label-tags). For simplicity, consider 3 copies of t: ti, t_2 and t_3. Target t_1 will choose a label-tag, t_2 has a 0.9 probability of choosing a different label-tag, t_3 has a predictable probability of choosing the same label-tag as ti or t_2. For n copies choosing from m label-tags, outcomes can be modeled by the binomial distribution as discussed above. For 3 targets and 10 label-tags, the probability of a label-tag not being chosen, Po is $(1-(1/10))^3 = 0.729$. The probability Pi of being chosen exactly once is $(3/10)(1-(1/10))^2 = 0.243$. The probability of being chosen twice, P_2 is 0.027 and the probability P_3 of being chosen 3 times is 0.001 . Since P_0 is the probability of not being chosen, $1-P_0$ is the probability of being chosen at least once. We define $k = m(1-Po)$ as the number of label-tags we expect to see in an experiment. Conversely, if we know m, and observe k we can solve for the number of molecules.

In the previous example where n=3 and *m=l0* we expect to see $10(1-P_0)$ or 2.71 label-tags as our most probable outcome. Increasing m dramatically increases the counting efficiency, accuracy and dynamic range, e.g. for m = 1,000, ^(number of label-tags expected for «=10, $k = 9.96$, for $n = 20$, k = 19.8. However, it may be desirable to use a lower number of label-tags to reduce cost and complexity of the analysis with the understanding that there is an increased probability of undercounting because label-tags are used multiple times for the same target.

[0128] In some aspects the pool of label-tags has approximately the same numbers of copies of each label-tag in the library. The label-tag sequences are not target specific, but may be similar to the tags that have been used for other tagging applications, for example, the Affymetrix GENFLEX tag array. The label-tags in a pool may be a collection of random sequences, such as a pool of $\mathbf{N_6}$, $\mathbf{N_7}$, N_8, N_9 or N_{10}, or mixtures of these lengths, where N can be any of the four bases. Preferably all label-tags in a set of label-tags will have similar hybridization characteristics so that the label-tags of the set can be detected under similar conditions. In some aspects the pool of label-tags has similar amounts of each tag, for example, within 2 fold or within 10 fold. In other aspects some label-tags may be present at high amounts relative to other label-tags. For example, some label-tags may be at high concentration in the pool, and others low, for example at a ratio of 100 to 1 or 1000 to 1.

[0129] In some aspects, the label-tag is attached without requirement for it to hybridize to any portion of the target so there is no or minimal bias as to which label-tag sequence is attached. In one embodiment, the label-tag is attached by ligation of the label-tag to one of the ends of the target. When array hybridization is used for detection the probes of the array may be complementary to a predicted junction between target and label-tag so it is preferable that the label-tags are attached to all occurrences of a target at the same position. This is facilitated if the termini of each occurrence of a selected target are the same and are known. This may be how the target exists in the unprocessed sample, for example for small RNAs that have defined and known ends such as miPvNAs, siRNAs and other non-coding and structural RNAs or known ends can be generated in a target by cleavage at a specific site of known sequence. Such cleavage can be enzymatic, for example site specific restriction digestion or at a site that is targeted by hybridization of an oligo to facilitate cleavage by RNaseH or by an enzyme that has flap endonucleoase activity such as FEN 1 or Taq DNA polymerase.

[0130] In another aspect the label-tag may be attached as a partially double stranded adaptor. One strand of the adaptor may serve as a splint to bring the target and the label-tag sequence together for ligation. The splint may include a first region that is complementary to one end of the target and a second region that is complementary to at least a portion of the oligonucleotide that carries the label-tag. The splint hybridizes to both target and label-tag oligo and brings the ends together for ligation by a ligase enzyme. In some aspects if there is a gap between the two it can be filled by extension of one or the other or by ligation of a gap filling sequence into the gap.

[0131] To facilitate amplification, adaptors may contain a universal or common priming sequence that is 5' of the label-tag sequence. Attachment of universal or common primers to either end of a target can be followed by PCR amplification. The universal primers may be added along with the label-tag or at a subsequent ligation step or primer extension step.

[0132] For RNA targets an RNA ligase, such as T4 RNA ligase may be used. T4 RNA ligase 1 catalyses the ligation of a 5' phosphryl-terminated nucleic acid donor to a 3' hydroxyl-terminated nucleic acid acceptor. Substrates include single-stranded RNA and DNA. See, for example, Romaniuk, P. and Uhlenbeck, O. (1983) R. Wu, L. Grossman and K. Moldave (Eds.), *Methods Enzymol,* 100, pp. 52-56. New York: Academic Press and Moore, M.J. and Sharp, P.A. (1992) *Science,* 256, 992-997. RNA targets may also be circularized and used as template for rolling circle amplification using an enzyme having reverse transcriptase activity. T4 RNA ligase 1 may be used for circularization of RNA by ligating the ends of the molecule together. T4 RNA ligase 1 can also be used to ligate RNA to DNA.

[0133] Full-length mRNA can be selected by treating total or poly(A) RNA with calf intestinal phosphatase (CIP) to remove the 5' phosphate from all molecules which contain free 5' phosphates (e.g. ribosomal RNA, fragmented mRNA, tRNA and genomic DNA). Full-length mRNAs are not affected. The RNA can then be treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from the full-length mRNA leaving a 5'-monophosphate. A synthetic RNA adapter can be ligated to the RNA population. Only molecules containing a 5'-phosphate, (i.e. the uncapped, full-length mRNAs) will ligate to the adapters. Preferably the adapter has a variable label-tag sequence, and may also have a constant sequence for priming. Preferably, the constant sequence is 5' of the variable sequence. In some aspects, the adapter ligated mRNA may then be copied to form a first strand cDNA by, for example, random priming or priming using oligo dT. The cDNA may subsequently be amplified by, for example, PCR._T4 RNA ligase may also be used for ligation of a

DNA oligo to single stranded DNA. See, for example, Troutt *et al,* (1992) *Proc. Natl, Acad. Sci. USA,* 89, 9823-9825.

[0134] In other aspects, the ligated target-label-tag molecule may be enriched in the sample relative to other nucleic acids or other molecules. This enrichment may be, for example, by preferentially amplifying the target-label-tag methods, using for example, a DNA or RNA polymerase, or by degrading non target-label-tag molecules preferentially.

[0135] In one aspect, the target-label-tag molecule may be nuclease resistant while the unligated target and unligated label-tag molecules may be nuclease sensitive. A nuclease can be added to the sample after ligation so that ligated target-label-tag molecules are not digested but non-ligated molecules are digested. For example, the targets may be resistant to a 5' exonuclease (but not a 3' exonuclease) while the label-tags are resistant to a 3' exonuclease but not a 5' exonuclease. Ligating target to label-tag generates a molecule that is resistant to 5' and 3' exonuclease activity. After ligation the sample may be treated with a 5' exonuclease activity, a 3' exonuclease activity or both 5' and 3' exonuclease activities. For examples of nucleases see Rittie and Perbal, *J Cell Commun. Signal.* (2008) 2:25-45, which is incorporated by reference (in particular see Table 5). Exo VII, for example degrades single stranded DNA from both the 5' and 3' ends so the sample could be treated with Exo VII after ligation to degrade molecules that are not ligation products.

[0136] In another aspect amplification may include a rolling circle amplification (RCA) step. See for example, Baner et al. (1998) NAR 26:5073, Lizardi et al. (1998) *Nat. Genet.* 19:225, Fire and Xu, (1995) PNAS 92:4641-5, Zhao et al. *Angew Chem Int Ed Engl.* 2008; 47:6330-6337 and Nilsson et al. (2008), Trends in Biotechnology, 24:83-88. The targets may be ligated so that they have a label-tag and a universal priming (UP) sequence attached to the 5' end of the targets. The UP-label-target is then ligated to form a circle. A primer complementary to the UP is then hybridized to the circles and extended using a strand displacing polymerase. The resulting amplification product contains multiple copies of the complement of the circle, UP-target-L.

[0137] In another aspect, targets may be labeled in a copying step. For example, a primer having a 3' target specific region and a 5' variable label-tag region may be hybridized to the targets, either RNA or DNA, and extended to create a single complimentary copy of the target. Each extension product will have a different label-tag and the junction between the label-tag and the target specific region is known. The target specific region may be common to multiple targets, for example, if the targets are mRNA the target specific region may be oligo dT. Polyadenylated mRNA may

optionally be enriched by binding to oligo dT coated beads. The oligo dT region hybridizes to the polyA tail at the 3' end of the mRNA and serves as a primer. The label-tag is 5' of the oligo dT region and thus becomes part of the cDNA extension product. There is preferably an amplification primer sequence 5' of the label-tag region that also becomes part of the extension product and can be used in a subsequent amplification step. In preferred aspects the cDNA extension product is made double stranded in a subsequent step. A second strand cDNA can be synthesized, for example, in the presence of RNaseH and a DNA polymerase. The amplification primer may be, for example, a promoter sequence for an RNA polymerase such as T7 or SP6 or variants thereof. Multiple labeled aRNA copies can be generated by IVT. In other aspects the amplification primer may include a recognition site for a nicking enzyme, such as Nt.BspQI. The dsDNA can be nicked and the nick extended to create another copy and displacing the earlier copy in a strand displacing amplification step. The resulting amplified product in all cases can then be analyzed to detect the label-tag and enough of the target sequence to identify the target.

[0138] In another aspect the target specific region may be a short stretch of random sequence, e.g. N 6-label-tag. The extension may be performed in the presence of nuclease resistant nucleotides so that the extension product is resistant to nuclease but the unextended primers are not. After extension the reaction is treated with a 3'-5' exonuclease activity to digest unextended primer. Exonuclease I, for example, removes nucleotides from single stranded DNA in the 3' to 5' direction and Exo III removes nucleotides from the 3' termini of duplex DNA. Exonuclease T (or RNase T) is a single-stranded RNA or DNA specific nuclease that requires a free 3' terminus and removes nucleotides in the 3' to 5' direction. The extension products are then detected by hybridization to probes that are complementary to the primers and include the unique label-tag portion and the constant target specific portion. If the target is RNA it can be digested with RNase H after extension. The extension product may also be amplified before hybridization.

[0139] FIG. 4 shows a strategy for selecting probes for target fragments. Each of these junction regions as shown has a counter region 411 denoted by N's, a fixed sequence 413 that is defined by the restriction enzyme used for fragmentation and a target specific region 415. The region 415 is shown as N's but in preferred aspects it is a known or predictable sequence of the target that is adjacent to the selected restriction site and can be used to identify the target. In an array is used for detection the array probes may be complementary to at least a portion of 411, a portion of 415 and all of 413. For each target sequence-counter combination there are 4 different probes that could be

included on the array, according to the 4 possible junctions 401, 403, 405 and 407. For example, if the targets are 10 loci from each of 4 chromosomes and 4 probes per fragment are included for 1200 different label-tags (1000 specific plus 200 non-specific) the array could have 192,000 total probes (4 x 10 x 4 x 1200). If sequencing is used for detection it is preferable to obtain sequence information for the region 411 and enough of region 415 to determine the identity of the target being sequenced. The length of 415 that need be determined will depend upon the number of different targets being analyzed and the complexity of the sample being analyzed, e.g. the fewer possible targets the less sequence needed to make an identification.

[0140] In some aspects methods for selecting a collection of label-tags optimized for use in the disclosed methods is contemplated. For example, a list of all possible 14 mers may be used as a starting pool (4^{14} is ~268 million different sequences). Different label-tag lengths can be used resulting in different numbers of starting sequences. Once a list of starting sequences has been generated, that list may be processed using one or more of the following steps: eliminate all label-tags that are not at least 50% GC content; eliminate all label-tags that do not use each of the 4 possible nucleotides at least twice; eliminate all label-tags that have more than two Gs or Cs in tandem, e.g. a probe with GGG or CCC would be eliminated, or with more than three As or Ts in tandem, e.g. AAAA or TTTT would be removed; remove label-tags that contain a selected restriction site; remove label-tags having a Tm that is outside of a range (for example 38.5 to 39.5 °C, 38 to 40 °C, 38-39 °C, or 39-40 °C); and remove probes that have self complementarity beyond a selected threshold. Further processing steps may include: perform a hierarchical clustering to maximize sequence differences between label-tags to minimize cross hybridization, same label-tag to same probe and minimize self-complementarity within the collection to reduce tendency of two label-tags binding to each other.

[0141] FIG. 5 shows a counter adaptor 501 that includes a counter region 505, a constant region for priming 503 and a sticky end 507 for ligation to an overhang created, in the fragments, by restriction digestion of the sample, for example with BamHI. In step 5001 adaptors 501 are ligated to target fragments 509. After ligation of the adaptors 501 to the target fragment 509 there are typically two adaptors ligated to the target fragment, one at either end. It is probable that the counters on the two ends will be different, as shown, although there is a predictable probability of having the same counter ligated to both ends of the same fragment. After adaptor ligation the adaptor-ligated fragments 511 can be amplified in step 5002 by PCR using a common primer to the

503 region of the adaptor. The adaptor may first be filled in to make it double stranded using a DNA polymerase. The PCR amplification may be used to preferentially amplify fragments of a selected size range, for example, 300 to 2 kb. Smaller fragments are not amplified as efficiently because of self complementarity between the ends of the individual strands (capable of forming a panhandle structure that inhibits amplification) and longer fragments (longer than about 3 kb) also don't amplify well. For a discussion of one-primer multiplex PCR see, for example, Matsuzaki *et al. Genome Res.* 2004, 14(3):4 14-425, which is incorporated herein by reference in its entirety.

[0142] The PCR product 511 may be circularized by ligating the adaptor ends together at circularization point 515. After circularization, the uncircularized fragments can be digested using an exonuclease, for example. The circularized fragments can be amplified using target specific primers to generate amplification product 513. Step 5002 includes formation of the circularized product (not shown) followed by PCR amplification to generate product 513. In FIG. 5 the target specific primers are identified as "TS primer F" and "TS primer R". They are primers that are specific for the target sequences being counted. Whereas the primers used to amplify 511 are common to all adaptor ligated fragments and will amplify all fragments that are in the size range to be amplified using PCR, the TS primers are specific for selected targets to be analyzed and this amplification step will reduce the complexity of the sample by preferentially amplifying the targets to be counted. Non-targets are amplified in step 5001 but not efficiently in step 5002. The amplification product 513 has in the 5' to 3' direction, target specific sequence, overhang sequence, a first counter "Li", first adaptor sequence, circularization junction 515, second adaptor sequence, second counter "L_2", second overhang sequence and a second target specific sequence. The first and second counter are generally different (although they may be the same at a low probability depending on the number of species of counters) and the first and second target sequence are different. The product 513 or preferably fragments thereof can be detected by a variety of methods, for example, an array of probes as exemplified by probe 517 can be used or the product can be sequenced using any available method. As shown in FIG. 5 step 5003, the array probe 517 is complementary to a region of the target, the overhang region and the counter. When hybridized the target will have an overhanging single stranded region that corresponds to the adaptor sequence. A labeled probe 519 that is complementary to one strand of the adaptor can be hybridized and the ligated to the array probe as shown in step 8004, and as described below. The labeling of the array probe with biotin indicates the presence of that target-counter L1 combination and is indicative of

the presence of one occurrence of the target. The array has probes (probe features comprising multiple copies of the sample probe sequence) for each possible target-counter combination.

[0143] In some aspects the label-tags are generated within the target to be counted. For example, the label-tag may be a unique cleavage site in a target fragment as shown in FIG. 6. Each of the copies of the target to be counted 601 have a common sequence at one end identified in the figure as 603. This may be a common sequence that has been added to the targets through ligation or primer extension or it may be a naturally occurring sequence in the target. The targets are fragmented randomly, for example by shearing or sonication resulting in cleavage at the points indicated by the arrows to generate cleavage products 604. Cleavage is at a different and unique site in each of the fragments and results in a unique sequence in the target immediately to the left of the point of cleavage in the illustration (indicated by circles in fragments 607). This unique sequence can function as a label-tag for the disclosed methods. A second common sequence 605 may be attached to each of the targets immediately downstream of the cleavage point, through for example ligation of an adaptor sequence. The resulting targets 607 can be analyzed directly to determine how many unique sequences are present and using this number as an indication of the number of targets in the starting sample. This is illustrated for nucleic acids, but could similarly be applied to proteins or other contiguous strings of monomers or units that are assembled in a non repeating pattern.

[0144] An exemplary method for detection is diagramed in FIG. 7. The 3840 distinct label-tag oligos (counters) were single stranded oligos pooled from the DdeI TACL primer panel (40 primer plates by 96 wells per plate for 3840 different oligos). An example label-tag oligo 701 is shown:

(SEQ ID NO: 1964) 5'TCGATGGTTTGGCGCGCCGGTAGTTTGAACCATCCAT-3 '. The 48 different primers used as "targets" were synthesized using as target 48 different 21 nucleotide sequences from the Affymetrix TrueTag 5K_A array. An example target oligo 707 is shown (SEQ ID NO: 1965) 5'GCCATTTACAAACTAGGTATTAATCGATCCTGCATGCC-3 '.

The "label" or "counter" oligo has an 18 nt common sequence at the 5' end and a 15-28 nt "label" (or "counter") sequence at the 3' end. An example "label" 705 is shown. The universal primer 703 common to all or a group of the label-tag oligos has sequence 5' TCGATGGTTTGGCGCGCC-3 ' (SEQ ID NO: 1966) at the 5' end and each target oligonucleotide has common sequence 711 that is 5' AATCGATCCTGCATGCCA-3 ' (SEQ ID NO: 1967) at the 3' end as universal priming sequence. The target oligos vary in sequence at the 5' ends 709.

[0145] In another embodiment, illustrated schematically in FIG. 8, genomic DNA 301 is fragmented with a restriction enzyme, for example, BamHI, which generates a single stranded overhang for sticky ended ligation. The fragments 305 are ligated to adaptors 501 that include a label-tag 505 and a universal priming site 503. Different adaptors vary in the label-tag portion **505** but have a common priming site **503.** The label-tag is 3' of the universal priming site so that it is between the fragment and the universal priming site. The adaptor ligated fragments are amplified by PGR using primer **313.** The PCR product can be fragmented, labeled with a detectable label and hybridized to an array. The resulting strands are detected by hybridization to an array having target-label-tag probes to the junction between target and label-tag as illustrated in FIG. 7. The array may have a different probe feature for each target-label-tag combination. The figure shows a collection of features for each of Target 1 and Target 2 where each different probe feature is represented by a single vertical line. The PCR amplicons may be fragmented prior to array hybridization. Preferably the fragments are labeled, for example, by TdT incorporation of a nucleotide that can be detected, such as a biotin containing nucleotide.

[0146] The probes of the array are complementary to the junction between the label-tag and the restriction fragment. The sequences at the ends of the individual strands of the restriction fragments are predicted based on *in silico* digestion of the human genome. Also, fragments are targeted that are within the size range that is known to amplify efficiently by adaptor ligation PCR, for example, 200 bases to 2 kb. The adaptor **501** has two segments, a constant priming region **503** and a variable label-tag region **505.** Together **503** and **505** form the label-tag adaptor **501.** The primer **313** has the same sequence 5' to 3' as **503.** The schematic is drawn showing only one strand, but one of skill in the art would understand that in a preferred embodiment the genomic DNA is double stranded and the restriction fragments have two strands, which may be referred to as a top strand and a bottom strand. The convention is that the top strand is drawn 5' to 3' left to right and the bottom strand is the complement of the top strand and is drawn 3' to 5' left to right. Adaptors are preferably double stranded for at least a portion of the adaptor. They may have single stranded overhangs, for example to have "sticky ends" that facilitate hybridization and ligation to the overhang resulting from restriction digestion. In a preferred aspect, the same adaptor can be ligated to the two ends of a strand of a restriction fragment and may be ligated to one or both strands. The adaptor may be ligated to the ends of the top strand in opposite orientations as shown in FIG. 8, so that the label-tag **505** is internal to the priming site at both ends. The adaptor may have a top and a bottom strand and

the top strand may be ligated to the top strand of the fragment and the bottom strand ligated to the bottom strand of the fragment. The top and bottom strands of the adaptor may be complementary over the entire length, but often have single stranded regions at one end to facilitate sticky ended ligation to an overhang created by a restriction enzyme.

[0147] FIG. 9 shows a method for reading out the counter-labeled targets on arrays by ligation of biotin-labeled oligos 901 to the array probes if the cognate counter-labeled targets are present. The left panel shows the results when target region Gi ligated to label-tag L_1 to form G_1L_1 hybridizes to the complementary G_1Li probe on the array. The constant region (in white) can hybridize to its labeled complement so that the 3' end of the biotin-labeled oligo is juxtaposed with the 5' end of the Li region of the probe on the array and the ends can be ligated to label-tag the array probe. In the center panel the target hybridizing to the G_1L_1 probe is non-cognate; the label-tag region is L_2 and not L_1 so it does not hybridize to the Li region of the probe. The biotin-labeled oligo can hybridize to the partially hybridized target but it is not juxtaposed with the 5' end of the L_1 region of the probe so it should not ligate to the probe. In the right panel the target shown hybridized has the Li region and is complementary to the array probe at that region, but the array probe has a G region that is not G_1 so the G_1L_1 target does not hybridize. The labeled oligo can hybridize to the target but because the Li:L_1 region is short the duplex is not stable and the labeled oligo does not ligate to the end of the array probe efficiently. This allows for labeling of properly hybridized target-label-tag pairs with both hybridization and ligation discrimination.

[0148] In the collection of label-tag sequences, each label-tag species has a different sequence but multiple copies of each species are preferably present. All species may be present at the same amount or there may be variable amounts of some species, for example, some species may be present at 10 M and others at 1M. Other ratios are also possible, e.g. 1:100 or 1:1000 for species in the collection. It may be desirable to vary the relative ratios of species in the label-tag pool in accordance with the expected ratios of targets to be detected. In many aspects it is preferable that the number of different species of label-tags in the pool is at least 10 times the number of copies of the most abundant target to be counted. In a preferred aspect, the label-tags are a collection of known sequences such as a collection of all possible 6mers (N_6), (or 7-14mers) or a subset of all possible N-mers of a given length. Subsets may be selected to optimize or normalize hybridization conditions or to minimize cross hybridization. Sets of label-tags may be further subdivided into subsets of

[0149] In preferred aspects the label-tag sequences are covalently associated with the targets. Attachment, by for example, ligation, is random so that any given label-tag has about the same probability of ligating to any one target occurrence. So if there are 1000 different occurrences of a target each could be ligated to a different label-tag sequence and the probability that any two target occurrences will have the same label-tag ligated is preferably low and depends on the number of label-tags available. Because the attachment is a random stochastic process there is a known probability that if there are C copies of a given target and N different label-tags that any two copies of a target T will have the same label.

[0150] The relationship between the number of targets and the number of label-tags determines the probability that two C's will have the same L. In preferred aspects Y is greater than X for each target to be counted. This reduces the probability of undercounting due to double labeling. If C1 and C2 of T1 are both labeled with L3 both copies will be counted as a single occurrence, resulting in under counting. Undercounting can also be adjusted for by estimating the number of copies that are likely to be multiply labeled and adjusting the final count upwards to take those into account. For example, if there is a likelihood that 5 of 1000 copies will be labeled with the same label-tag then the final number should be adjusted up by 0.5%.

[0151] In preferred aspects, the detection is by hybridization to an array of probes. The array has a collection of features for each target that includes a different feature for each label-tag. For example, if there are X label-tags there are X features for each target, T1L1, T1L2,. ..T1LX and the same for target 2, T2L1, T2L2,.. .T2LX, out to TNL1, TNL2, ...TNLX. The number of features of the array is on the order of X times N. Each probe has a target complementary sequence and a label-tag complementary sequence. Within a set of probes for a given target the target segment of the probe would remain constant and the label-tag portion varies from feature to feature so that each label-tag sequence is represented by at least one feature for each target.

[0152] In one aspect, the methods may be used to count the number of copies of each of a plurality of targets in a sample. The amount of target containing sample mixed with the label-tags may be diluted so that the number of copies of each target to be counted is less than the number of label-tags. For example, if the targets to be counted are present at about 1,000 copies per cell and there are 10,000 label-tags you want to have the amount of sample in the mixture to be about the equivalent of one cell's worth of RNA. You can mix that with multiple copies of each label-tag, but you want to keep the absolute number of copies of target below the number of types of label-tag

sequences. Dilution of the sample and use of an appropriately small amount of starting material may be used. If a target sequence is present at low copy number per cell it is possible to use the nucleic acid from a larger number of cells. For example, to measure the DNA copy number of a chromosomal region relative to other chromosomal regions the expected copy number is low (e.g. 2 for normal) so if there are 10,000 different label-tags, the number of genomes that can be added to the sample for attachment of label-tags can be high, e.g. 500 to 1000.

[0153] In one aspect, the methods are used to identify regions of genomic amplification and chromosomal abnormalities. For example, the methods may be used to detect trisomy or other forms of aneuploidy. Trisomy is a type of aneuploidy where most of the chromosomal regions will be present in 2 copies per cell and the region of trisomy will be present in 3 copies per cell. Trisomy may also be partial, i.e. there is an extra copy of part of a chromosome. Trisomy 21 and 18 are the most common in humans. Trisomy may be indicated if 3:2 ratio is observed in a counting analysis performed using the disclosed methods. For example, if the sample contains about 500 genomes the observed count may be 1000 copies of most regions and 1500 copies of the trisomy regions. Small errors in the counting, resulting from undercounting, would have little or no effect on the conclusion.

[0154] In some aspects, controls of known copy number may be spiked in to a sample as an internal control or to determine accuracy.

[0155] Stochastic labeling of tl,N (collection of essential identical molecules of copy 1, 2....N of target 1) by $L_{1,m}$ (effectively an infinite reservoir of diversity m when m is much greater than N). This allows for complete or near complete resolution of members of $ti_{,N}$, by imparting separate identities to the members of the collection of $ti_{,N}$ (provided that M is sufficiently smaller than N in the labeling). This provides for a stochastic or random projection of $ti_{,N}$ onto $Li_{,m}$. In some aspects $Li_{,m}$ is a library and the members of the library that are associated with $ti_{,N}$ can be counted to determine the number of copies of the target. In some aspects the methods can be described as indexing the members of the target. This provides a method to follow individual molecules that are members of a type of molecule that would not otherwise be distinguishable one from another.

[0156] Because stochastic labeling can impart identi liability to otherwise non-identifiable molecules it can impart identifiability to any two targets that may be very similar, but different. Examples of targets that may be highly similar but could be separately counted using the disclosed methods, include, for example, alternative splice forms of a gene, and sequences that have one or

more variations, including a variation in a single base (e.g. SNP or indels (insertion or deletions of short regions, e.g. 1-5 bases). Counting alleles can be used to measure allelic ratios or mosaicism. For example, two populations of cells within the sample individual may have different genotypes resulting in different numbers of each allele of one or more SNPs. The methods allow for precise measurement of the numbers of copies of each allele of a given SNP in a sample. In some aspects the methods impart a clonal labeling, that allows a single copy to be separately detected and separately isolated from the solution. Variants that occur as the result of amplification after the label-tag has been attached, e.g. resulting from polymerase errors, can also be detected as they will have the same label-tag but different sequences.

[0157] Some nucleic acid sequencing reactions use methods that stochastically attach targets to a solid support followed by amplification of the attached target and analysis. The target attaches in an unknown location and the location can be determined by sequencing the amplified target at specific locations. In contrast, the disclosed methods provide for clonal amplification of known targets in a known location. The stochastic nature of the formation of the target-label-tag molecule provides a mechanism for isolating single occurrences of selected targets that can be subsequently amplified and analyzed. In some aspects the label-tag can be used as a handle for isolating clonal populations of targets. The labeling step generates an indexed library that has a variety of applications. For example, the indexed library could be used for sequencing applications. The method adds distinguishability to any set of molecules, even molecules that are not distinguishable by other mechanisms because they may share common regions or even been identical. The indexed library can be stored and used multiple times to generate samples for analysis. Some applications include, for example, genotyping polymorphisms, studying RNA processing, and selecting clonal representatives to do sequencing.

[0158] In some aspects the methods are used to stochastically label-tag a polyclonal antibody population. This may be used to identify different polyclonal populations.

[0159] The methods may be used to convert an analog readout of hybridization signal intensities on arrays into a measurable process that can be scored digitally on the arrays. The method leverages a random process where the tagging of assayed molecules is governed by stochastic behavior. In a random process, the more copies of a given target, the greater the probability of being tagged with multiple label-tags. A count of the number of incorporated label-tags for each target can

approximate the abundance level of a given target of interest. The ability to count label-tags on microarrays would be a clear cost-advantage over the other existing techniques.

[0160] A stochastic counting assay system as described herein can also be a sub-system within a much larger bio-analysis system. The bio-analysis system could include all the aspects of sample preparation prior to, for example, optical detection, the post processing of data collected in the optical detection phase and finally decision making based on these results. Sample preparation may include steps such as: extraction of the sample from the tested subject (human, animal, plant environment etc.); separation of different parts of the sample to achieve higher concentration and purity of the molecules under investigation; sample amplification (e.g. through PCR); attachment of detectable labels to different parts of the sample; and transfer of the sample or a portion of the sample into a reaction vessel or site on a substrate. The post processing of the collected data may include: normalization; background and noise reduction; and statistical analysis such as averaging over repeated tests or correlation between different tests. The decision making may include: testing against a predefined set of rules and comparison to information stored in external data-bases.

[0161] The applications and uses of the stochastic labeling and counting methods and systems described herein can produce one or more result useful to diagnose a disease state of an individual, for example, a patient. In one embodiment, a method of diagnosing a disease comprises reviewing or analyzing data relating to the presence and/or the concentration level of a target in a sample. A conclusion based review or analysis of the data can be provided to a patient, a health care provider or a health care manager. In one embodiment the conclusion is based on the review or analysis of data regarding a disease diagnosis. It is envisioned that in another embodiment that providing a conclusion to a patient, a health care provider or a health care manager includes transmission of the data over a network.

[0162] Accordingly, business methods relating to the stochastic labeling and counting methods and methods related to use thereof as described herein are provided. One aspect of the invention is a business method comprising screening patient test samples for the amount of a biologically active analyte present in the sample to produce data regarding the analyte, collecting the analyte data, providing the analyte data to a patient, a health care provider or a health care manager for making a conclusion based on review or analysis of the data regarding a disease diagnosis or prognosis or to determine a treatment regimen. In one embodiment the conclusion is provided to a patient, a health care provider or a health care manager includes transmission of the data over a network.

[0163] Applications for the disclosed methods include diagnosing a cancerous condition or diagnosing viral, bacterial, and other pathological or nonpathological infections, as described in U.S. Pat. No. 5,800,992. Additional applications of the disclosed methods and systems include, pathogens detection and classification; chemical/biological warfare real-time detection; chemical concentration control; dangerous substance (e.g., gas, liquid) detection and alarm; sugar and insulin levels detection in diabetic patients; pregnancy testing; detection of viral and bacterial infectious diseases (e.g. AIDS, Bird Flu, SARS, West Nile virus); environmental pollution monitoring (e.g., water, air); and quality control in food processing.

[0164] Any available mechanism for detection of the label-tags may be used. While many of the embodiments discussed above use an array readout form, it will be obvious to one of skill in the art that other methods for readout may be used. For example, sequencing may be preferred in some embodiments, but detection may be by hybridization, PCR, real time PCR or reverse transcription, optionally followed by PCR. Amplification may be by PCR but may also be by any method of amplification available, such as random priming by a strand displacing polymerase.

[0165] In some aspects the readout is on an array. The array may be a solid support having immobilized nucleic acid probes attached to the surface in an ordered arrangement. The probes may be, for example, synthesized in situ on the support in known locations using photolithography or the probes may be spotted onto the support in an array format. As discussed above, in some embodiments the array includes a probe feature for each possible label-target combination. A feature preferably includes many copies of a single probe sequence. The feature may also have some probes that are not full length, resulting from truncation of synthesis. The photo activation process may not be 100% efficient so some probes are terminated at each step without having subsequent bases added. These truncated probes have the sequence of a portion of the full length probe.

[0166] In many aspects the detection of the unique label-tag-target molecules can be done by using a sequencing readout. It is necessary only to determine the sequence of enough of the label-tag to unambiguously distinguish between other label-tags in the pool used and enough of the target to unambiguously distinguish between other targets being analyzed. After attachment of the label-tags to the targets in a stochastic manner, the targets may be amplified according to any of the methods disclosed herein prior to sequencing analysis of the product. Amplification is not required for some sequencing methods that are capable of sequencing single molecules, for example, methods that

sequence a molecule as it travels through a narrow opening or nanopore, the methods developed by Helicos and sequencing by synthesis in zero-mode wave-guides (Pacific Biosciences).

[0167] The methods of the invention can be used in conjunction with essentially any sequencing methodology. Suitable techniques include, for example, Pyrosequencing, FISSEQ (fluorescent in situ sequencing), MPSS (massively parallel signature sequencing) sequencing by ligation-based methods, sequencing by synthesis, sequencing by hybridization. Methods that uses arrays of chemical-sensitive field effect transistors, or FETs, to detect how nucleotides incorporate into growing strands of DNA by measuring changes in current or pH for sequencing may also be applied to the methods disclosed herein.

[0168] A number of alternative sequencing techniques have been developed and many are available commercially. For a review see, for example, Ansorge *New Biotechnology* 25(4): 195-203 (2009), which is incorporated herein by reference. These include the use of microarrays of genetic material that can be manipulated so as to permit parallel detection of the ordering of nucleotides in a multitude of fragments of genetic material. The arrays typically include many sites formed or disposed on a substrate. Additional materials, typically single nucleotides or strands of nucleotides (oligonucleotides) are introduced and permitted or encouraged to bind to the template of genetic material to be sequenced, thereby selectively marking the template in a sequence dependent manner. Sequence information may then be gathered by imaging the sites. In certain current techniques, for example, each nucleotide type is tagged with a fluorescent tag or dye that permits analysis of the nucleotide attached at a particular site to be determined by analysis of image data. As indicated above, any method of sequencing that is available could be applied to detection of the target-label-tags to count the number of unique target-label tag combinations to count the number of a given target in a sample.

[0169] In another aspect, mass spec analysis may be used to detect the label-tags and count the targets. The label-tags can be distinguishable based on size or other property that can be detected. Many of the examples provided herein identify the label-tag based on unique nucleic acid sequence but any distinguishable label-tag may be used, for example, the pool of label-tags may be label-tags that are differentially detectable based on fluorescence emission at a unique wavelength.

[0170] FIG. 9 shows a method for reading out the labeled targets on arrays. On the left, the target with G_1 ligated to Li, "GiL_1", is shown hybridizing to the complementary array probe over the entire length of the probe. On the right target Gi ligated to label-tag L_2 is shown partially

hybridized to the G1L1 probe on the array. On the left the biotin labeled constant segment can hybridize to the GiL_1 target and ligate to the 5' end of the GiL_1 array probe. The constant segment can hybridize to the L_2 segment but will not ligate to L_1. This allows for labeling of properly hybridized target-label-tag pairs with both hybridization and ligation discrimination. The lower panel shows an example where the target or G portion is not matching with the probe on the array. This will not ligate efficiently because it hybridizes less stably.

[0171] The left panel shows the results when target G_1 ligated to label-tag L_1 to form G_1Li hybridizes to the complementary G_1L_1 probe on the array. The constant region (in white) can hybridize to its labeled complement so that the 3' end of the labeled complement is juxtaposed with the 5' end of the L_1 region of the probe on the array and the ends can be ligated. In the center panel the target hybridizing to the G_1Li probe is non-cognate, the label-tag region is L_2 and not L_1 so it does not hybridize to the Li region of the probe. The labeled oligo can hybridize to the partially hybridized target but it is not juxtaposed with the 5' end of the Li region of the probe so it should not ligate to the probe. In the right panel the target shown hybridized has the L_1 region and is complementary to the array probe at that region, but the array probe has a G region that is not Gi so the G_1L_1 target does not hybridize. The labeled oligo can hybridize to the target but because the Ll :L1 region is short the duplex is not stable and the labeled oligo does not ligate to the end of the array probe.

[0172] The methods are broadly applicable to counting a population of molecules by performing a stochastic operation on the population to generate a stochastic population of identifiable molecules. The targets need not be identical. For example, the methods may be used to count the absolute number of members of a group. In one aspect, a sample having an unknown number of copies of a selected nucleic acid target is fragmented randomly so that on average each copy of the target has a different end resulting from a distinct fragmentation event. A common adaptor sequence can be ligated to the end of each fragment and used for amplification of the fragments. Each ligation event generates a new molecule having a junction formed by the end of the random fragment and the adaptor sequence. The new junction can be detected by, for example, sequencing using a primer complementary to the adaptor or a region of the adaptor. Because the fragmentation was a stochastic process the number of different ends detected is a count of the number of different starting target molecules, assuming one fragment per starting target molecule.

[0173] FIG. 26 shows another method for attaching label-tags. Polyadenylated RNA 4001 is reverse transcribed using a first primer [UR**1**_label] 4002. The primer may include several regions including an oligo dT region 4003 3' of a label-tag region 4005. A sequencing primer region 4007 may be 5' of the label-tag and another priming region **4008** may be 5' of that. The primer region **4007** may be used as a first round sequencing primer. The reaction time can be controlled to shorten transcript length in the first round synthesis reaction. The primer preferably terminates at the 3' end with at least one non-T base so that it can hybridize at the beginning of the polyA tail rather than at a random position within the tail. For example, if the primer has VN at the 3' end (as shown in the figure) where V is A, C or G but not T, and N is A, C, G or T, then the V should hybridize in the mRNA and not within the polyA tail. This serves to anchor the primer at the junction between the end of the mRNA sequence and the beginning of the polyA tail (as shown). The label-tag 4005 may be a series of N's 5' of the oligo dT region. The primer may optionally include a restriction site 5' of the label-tag and optionally one or more U bases (shown between the label-tag and the oligo(dT)) that can be subsequently made abasic by UDG. Abasic sites do not serve as efficient template for extension and can thus be used to block or mitigate amplification of the input primer.

[0174] The primer is extended to make a copy of the transcript. The extended region is shown by the dashed arrow. Exo I may be used to remove residual oligos that haven't been extended. The products are treated with RNase H and then subjected to poly dA tailing with TdT (step 3001). A second strand is synthesized using a second set of label-tag primers 4009. The second set of primers has from the 5' end a constant region **4010,** a 1st round sequencing primer 4011, a 5' label-tag **4013,** a poly(dT) **4003** stretch and a VN anchor. After the second set of label-tag primers is used to primer second strand synthesis, the sample is treated with Exo I and UDG to remove or disable the residual oligos and the reaction is amplified using PCR using the constant priming regions 4008 and 4009. The label-tags 4013 and 4005 are flanked by sequencing primers 4007 and 4011. The fragments may be separated by size, for example, by gel sizing, and subjected to sequencing using, for example, Illumina paired end sequencing. Efficiency of amplification may be affected by fragment size. Higher primer concentrations, e.g. greater than 3 uM, may be used. A first round sequencing primer (5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' SEQ ID NO. 1978) and a second round sequencing primer (5' CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3' SEQ ID NO. 1979) may be used

in subsequent steps to perform primer extension based sequencing using standard methods, for example, next generation sequencing methods commercialized by Illumina, Inc. , Life Technologies and Roche 454. The sample prep methods for these manufacturers use adaptor ligation and can be modified to include counters within the adaptors that are added during sample prep. Methods for sequencing have been reviewed, for example, in Metzker, *Nature Reviews Genetics* Vol. 11, pp31-46 (2010) which is incorporated herein by reference in its entirety. Methods include, for example, use of reversible terminator chemistry, virtual terminators and ligation based methods.

[0175] FIG. 27 shows another method for attaching label-tags. As in FIG. 26, polyadenyiated RNA 4001 is reverse transcribed using a first primer [URl_label] that has from the 5' end, a priming region 4008, a 2nd round sequencing primer 4007, a 3' label-tag 4005, a region of uracil bases, a poly(dT) region 4003 and a VN at the 3' end as described above where V is A, C or G but not T, and N is A, C, G or T. The [UR1 _label] primer is extended to make a copy of the transcript. Exo I may be used to remove residual oligos that haven't been extended. The products are treated with RNase H and then subjected to poly dG tailing with TdT. A second strand is synthesized using a second set of label-tag primers [UF Mabel]. The second set of primers has from the 5' end a constant region 4009, a 1st round sequencing primer 4011, a 5' label-tag 4013, a poly(dC) 4015 stretch and a DN anchor where D is any base but C. After the second set of label-tag primers is used to primer second strand synthesis, the sample is treated with Exo I and UDG to remove or disable the residual oligos and the reaction is amplified using PCR using the constant priming regions 4008 and 4009. The label-tags 4013 and 4005 are flanked by sequencing primers 4007 and 4011. The fragments may be separated by size, for example, by gel sizing, and subjected to sequencing using, for example, Illumina paired end sequencing. Unlike the method shown in FIG. 26 the use of polyG tailing after 1st strand synthesis reduces the self complementarity between the ends of the strands. Instead of poly A and polyT which are complementary the strands have polyG and polyT or polyC and poly A.

[0176] In another aspect shown in FIG. 28A the primer may have the following sequence in the following order (5' to 3'): oligo dT, a first sequence, a restriction site, a label-tag sequence, a second sequence that is complementary to the first sequence, another dT region, 1-4 U's, V and N. Such a sequence is shown in FIG. 28A (SEQ ID No. 1977). The first and second complementary sequences for the step of a hair pin and the label-tag and the restriction site (Not I in the example

shown), form the loop. The formation of the stem-loop structure allows for a shorter length of oligo dT between the label-tag and the target mRNA.

[0177] The primer is hybridized to the mRNA and extended using the hybridized mRNA as template. The mRNA can then be digested and the cDNA resulting from the extension of the primer is tailed at the 3' end, for example, by the addition of a run of poly (dA) using polyA polymerase (PAP) or tailing using TdT. Primers that have not been extended can be digested with a 3' to 5' single stranded exonuclease like Exo I. A second strand cDNA can be synthesized by extending a primer complementary to the added 3' tail, for example polyT. The now double stranded products can be randomly fragmented with DNase, and digested with the restriction enzyme (e.g. Not I). This is shown schematically in FIG. 28B. Adaptors can be ligated to the fragments and the fragments can be amplified. The adaptors may contain priming sites for sequencing. The ligation of an adaptor at the Not I cleavage site positions a sequencing primer near the label-tag and facilitates sequencing of the label. Prior to the PGR amplification step the sample can be treated with UDG to remove excess unincorporated primer with label-tags. The UDG treatment removes the uracils from the label-tag primer extension product so that the strand is not copied 5' of that region. The oligo dT regions 3203 flank the stem regions 3205 which flank the label-tag 3007 and the restriction site 3009. The target region 3211 is between the region added by tailing 3213 and the second oligo dT region 3203b. The full length product is amplified by PCR, fragmented with DNAse to generate fragments with blunt ends. Digested with the restriction enzyme to generate a site for adaptor ligation adjacent to the label. Adaptors 3215 are ligated, resulting in a sequencing primer being positioned near the label. The sequencing is performed through the label-tag, the second step region 3205b, the second oligo dT region 3203b and into the target 3211.

[0178] The oligon dT region, a region containing 1 or more uracil bases, a 3' label-tag region and a constant region for subsequent analysis. An exemplary primer may be, (SEQ ID NO. 1976) NVTTTTTTTTTTTTTTUUUCANNNNNNNNNNNNgatctagccttctcgccaagtcgtccttacggctctggctaga gcatacggcagaag

[0179] Exo I is used to remove the residual oligos and RNase H plus poly dA tailing with TdT is used to add a 3' poly A tail to the first strand cDNA. A second strand cDNA is synthesized using a primer having a second label-tag, an oligo dT region and a second constant region. Exo 1 and UdG are used to remove residual oligos. In the oligos N is A, C, G or T and V is A, C or G but no T.

This facilitates the primer docking at the start of the A homopolymer rather than further down the run of A's. A 72 base single read or paired end read sequencing run can be performed to provide 39 bases of the 3' mRNA end ahead of the polyA site.

[0180] In some aspects the probability that any two targets are labeled with the same label-tag may be decreased by using two or more labeling steps. For example, a first labeling step where each target has a label-tag selected from a set of label-tags followed by a second labeling set using the same set of label-tags. The first labeling event will be independent of the second so the probability that the first and second labeling events will both be the same in two independent targets is the product of the probability of two targets having the same label-tag in either step. If there are N possible label-tags, and the first target is labeled first with label-tag N1 and then with label-tag N4, the probability that a second target will be labeled also with N1 and then N4 is $1/N^2$. So if there are 100 different label-tags, the probability that two targets will be labeled with the same label-tag in the first round and the same label-tag in the second round is 1/10,000.

[0181] In another aspect a first round of labeling may be done with 16 probes (for example, all possible 2 base combinations) and then a second round of labeling is done using the same 16 probes. The chance of any one probe attaching to a given target occurrence in the first round is 1 out of 16, the chance that the same probe will attach to the second target is 1/16 and the chance that the same two probes will attach is 1/16 X 1/16 or 1/256.

[0182] In another aspect reversible terminators are used to add a sequence to the end of each target being counted. For example, a 6 base sequence may be added and the chance of two being the same is 1 in 4^6 or 1 in 4096. See, for example, WO 93/06121 and US 6,140,493 which disclose stochastic methods for synthesizing random oligomers.

[0183] There is a finite set of label-tags, Li_{-X} and each target to be detected is present in the sample at a certain integer occurrence (Tl_{1-t}^{1}, $T2i._t^2$, ...$TN\,i_{-t}^{n}$). In a preferred aspect, the method is used to count the number of each of the different targets, (e.g. how many occurrences of T1, how many of T2,. ..how many of TN) in the sample. The targets are independently labeled with the label-tag molecules. Labeling is stochastic, so that any given target occurrence can be labeled with any one of the label-tags. For example, T1-1/L689, T1-2/L3, T1-3/1.4,567 and so on. For Target 2, any given occurrence can also be labeled with any of the label-tag molecules. This might generate, for example, (T2-1, L5), (T2-2, LI98), (T2-3, L34) and so on. There are multiple copies of each label-tag so T2-1 might be labeled with L5 and Tl-500 may also be labeled with L5.

[0184] The methods disclosed herein may be used to measure random cell-to-cell variations in gene expression within an isogenic population of cells. Such variation can lead to transitions between alternative states for individual cells. For example, cell-to-cell variation in the expression of comK in B. subtilis has been shown to select cells for transition to the competent state in which genes encoding for DNA uptake proteins are expressed. See, Maamar *et al. Science* 317:526-529 (2007) which is incorporated herein by reference.

[0185] The methods disclosed herein may be used to count the number of transcripts or DNA targets of a single type in a single cell (or small group of cells) and to separately count many different transcript types from a single cell (or small group of cells). The transcriptome or genome from a single cell or a few cells can thus be compared to the transcriptome or genome from another cell or group of cells. This comparison may be between different cell types, the same cell type at different developmental stages or under different environmental circumstances or between different portions of a tissue that is a mosaic.

[0186] Stochastic fluctuations in the levels of cellular components may play an essential role in cellular activities and may facilitate probabilistic differentiation strategies. For a review see Eldar and Elowitz, *Nature* 467, 167-173 (2010), which is incorporated herein by reference. Pooling of the nucleic acids from a larger number of cells may mask differences between individual cells or clusters of cells. Single cell analysis methods have been reported previously. See, for example, Aumann et al., (2008) Exp. Neurol. 213:419-430, Cauli et al., (1997) J. Neurosci. 17:3894-3906, Neuhoff et al. (2002) J. Neurosci. 22:1290-1302, Stahlberg and Bengtsson (2010) PGR Methods 50:282-288 and Esumi et al. Neurosci Res. 2008, 6(4):439-51, Other methods for analysis or the transcriptome of single cells that do not provide unique label-tags are impacted by amplification bias, for example, Tang et al. (Nature Protocols 5, 516-535 (2010) uses deep sequencing after 29 cycles of PCR to analyze the transcriptome of single mouse oocytes. Targets that did not amplify efficiently in the initial rounds of amplification may go undetected by sequencing.

[0187] Methods for analyzing gene expression at the single cell level is difficult because of the relatively small amount of mRNA available, sometimes being less than about 1 pg. Standard methods of mixing microliter volumes such as shaking, triturating or vortexing can fail to product sufficient turbulence at small scales so other methods of mixing may be desirable. Acoustic microstreaming uses sound waves in the audible spectrum to rapidly and effectively mix solutions in volumes of about 10 to about 100 ul (see Petkovic-Duran et al. (2009) Biofechniques 47:827-

834 and US Patent 20090034360). Methods for improving the yield of cDNA from reverse transcription of small amounts of RNA, such as those amounts that might be obtained from a single cell have been developed and may be used in conjunction with the reverse transcription methods for associating unique identifiers with individual molecules that are disclosed herein. In one aspect methods for improved mixing of the reverse transcription reaction may be used. One such method, acoustic microstreaming, is described in Boon et al., BioTechniques 50(2), 116-1 19. Acoustic microstreaming is a phenomenon where sound waves propagating around a small obstacle create a mean flow near the obstacle. Boon et al. describe using acoustic microstreaming at audio frequencies by ensuring the system has a liquid-air interface with a small radius of curvature that causes the entire drop to oscillate. This method was most effective at RNA concentrations of 0.1 to 1 pg/ul).

[0188] In one aspect, polyadenylated RNA from a single cell is analyzed by the methods disclosed herein. After cell lysis the poly A RNA may be enriched by capture on a solid support, such as a bead, having oligo dT attached or the amplification can be performed on the lysate. A labeled-cDNA copy of the RNA is made by hybridizing a primer that has an oligo dT region and a label-tag region. The label-tag region being 5' of the oligo dT region. Preferably there is an amplification sequence that is 5' of the label-tag region so that the label-tag region, which is variable between primers, is between a 5' common amplification primer sequence and a 3' oligo dT region. Second strand cDNA is then synthesized using standard methods, for example use of RNaseH and DNA polymerase. The resulting dsDNA can then be linearly amplified depending on the amplification primer sequence. For example, if the amplification primer sequence is a T7 RNA polymerase promoter sequence, antisense RNA can be generated by IVT using T7 RNA pol. If the amplification prime sequence includes a site for s nicking enzyme (e.g. Nt. BspQl), nicking enzyme strand displacement can be used to generate DNA copies of the RNA targets. The copies can then be modified to include sequencing primers at one or both ends and the products can be sequenced. Sequence information is collected for the tag and enough of the adjacent sequence to provide an identification of the target.

[0189] The examples provided herein demonstrate the concept of using a stochastic labeling strategy in the high sensitivity detection and counting of individual DNA molecules. The difficult task of quantifying single nucleic acid molecules is converted into a simple qualitative assay that leverages the statistics of random probability; and at the same time, the requirement of single

molecule detection sensitivity is achieved with PCR for the robust amplification of single DNA molecules. In some aspects improved methods for amplification will be used. For example, linear amplification methods may be used to mitigate the representation distortions created by exponential cycling in PCR. Given the lack of available techniques for single molecule counting, and the increasing need for its use, the new concept of stochastic labeling is likely to find numerous applications in the near future.

[0190] The examples of the present invention presented below are provided only for illustrative purposes and not to limit the scope of the invention.

EXAMPLES

[0191] To demonstrate stochastic labeling, an experiment was performed to count small numbers of nucleic acid molecules in solution. Genomic DNA from a male individual with Trisomy 21 was used to determine the absolute and relative number of DNA copies of chromosomes X, 4 and 21, representing 1, 2 and 3 target copies of each chromosome, respectively. Genomic DNA isolated from cultured B-Lymphocytes of a male Caucasian with Trisomy 21 was purchased from The Coriell Institute for Medical Research (Catalog # GM01921). The DNA quantity was determined by PICOGREEN dye (Invitrogen) measurements using the lambda phage DNA provided in the kit as reference standard. DNA quality was assessed by agarose gel electrophoresis.

[0192] The DNA concentration in the stock solution was measured by quantitative staining with picogreen fluorescent dye, and dilutions containing 3.62 ng, 1.45 ng, 0.36 ng and 0.036 ng were prepared. In each dilution, the number of copies of target molecules in the sample was calculated from a total DNA mass of 3.5 pg per haploid nucleus (see, T. R. Gregory *et al, Nucleic Acids Res* 35, D332 (2007) , and represent approximately 500, 200, 50 and 5 haploid genomes. The absolute quantity of DNA in the sample was determined by optical density measurements and quantitative staining with PICOGREEN fluorescent dye (Invitrogen) prior to making dilutions.

[0193] As outlined in FIG. 3, the genomic DNA sample 301 was first digested to completion with the *BamHI* restriction endonuclease to produce 360,679 DNA fragments 305. A diverse set of label-tags consisting of 960 14-nucleotide sequences was synthesized as adaptors harboring *BamHI* overhangs (SEQ ID Nos. 44-1963). Genomic DNA was digested to completion with *BamHI* (New England BioLabs, NEB) and ligated to a pool of adaptors consisting of an equal concentration of 960 distinct label-tags (as shown in FIG. 3). Each adaptor consists of a universal PCR priming site,

a 14 nucleotide long counter sequence and a *BamHI* overhang (similar to the form of the adaptor shown in FIG. 4. The sequence of the label-tag adaptors SEQ ID Nos. 44-1963 were selected from an all-possible 4^{14} nucleotide combination to be of similar melting temperature, minimal self-complementation, and maximal differences between one-another. Homopolymer runs and the sequence of the *BamHI* restriction site were avoided. Each pair, for example, SEQ ID Nos. 44 and 45, form an adaptor pair that has a region of complementarity starting at base 12 in SEQ ID No. 44 and base 5 in SEQ ID No. 45:

SEQ ID 44 5' CGACAGACGCCTGATCTTTTGTTAGCCGGAGT 3'

3' ACTAGAAAACAATCGGCCTCACTAG5 SEQ ID 45

[0194] The adaptors have a 5' overhang of 11 bases in the even numbered SEQ IDs and 4 bases (GATC) in the odd numbered SEQ IDs. Oligonucleotides were synthesized (Integrated DNA Technologies) and annealed to form double-stranded adaptors prior to pooling. For ligation, the digested DNA was diluted to the desired quantity and added to lOOpmoles (equivalent to 6×10^{13} molecules) of pooled label-adaptors, and 2×10^{6} units (equivalent to 1×10^{13} molecules) of T4 DNA ligase (NEB) in a 30µl reaction. The reaction was incubated at 20°C for 3 hours until inactivation at 65°C for 20 minutes.

[0195] In some aspects of the stochastic labeling reaction, each DNA fragment-end randomly attaches to a single label-tag by means of enzymatic ligation of compatible cohesive DNA ends to generate labeled fragments. High coupling efficiency is achieved through incubation with a large molar excess of label-tags and DNA ligase enzyme (~10^{13} molecules each). At this stage, the labeling process is complete, and the samples can be amplified as desired for detection. A universal primer may be added either by including as part of the adaptor or by extension of a primer containing the universal priming region in a subsequent step, and the entire population of labeled DNA fragments may be PCR amplified. The PGR reaction preferentially amplifies approximately fragments in the 150 bp-2 kb size range. Larger fragments, greater than about 9000 bases, for example, do not amplify efficiently under the selected PCR conditions. There are approximately 80,000 such fragments when the human genome is fragmented with *BamHI.* (FIG. 19). Adaptor-ligated fragments were amplified in a 50µl reaction containing IX TITANIUM Taq PCR buffer (Clontech), 1M betaine (Sigma-Aldrich), 0.3mM dNTPs, 4µM PCR004StuA primer (SEQ ID No. 1974), 2.5U Taq DNA Polymerase (USB), and IX TITANIUM Taq DNA polymerase (Ciontech).

An initial PCR extension was performed with 5 minutes at 72°C; 3 minutes at 94°C; followed by 5 cycles of 94°C for 30 seconds, 45°C for 45 seconds and 68°C for 15 seconds. This was followed by 25 cycles of 94°C for 30 seconds, 60°C for 45 seconds and 68°C for 15 seconds and a final extension step of 7 minutes at 68°C. PCR products were assessed with agarose gel electrophoresis and purified using the Q1AQUICK PCR purification kit (Qiagen).

[0196] The purified PCR product was denatured at 95°C for 3 minutes prior to phosphorylation with T4 polynucleotide kinase (NEB). The phosphorylated DNA was ethanol precipitated and circularized using the CIRCLIGASE II ssDNA Ligase Kit (Epicentre). Circularization was performed at 60°C for 2 hours followed by 80°C inactivation for 10 minutes in a 40µ1reaction consisting of IX CIRCLIGASE II reaction buffer, 2.5 mM $MnCl_2$, 1M betaine, and 200U CIRCLIGASE II ssDNA ligase. Uncirculated DNAs were removed by treatment with 20U Exonuclease I (Epicentre) at 37°C for 30 minutes. Remaining DNA was purified with ethanol precipitation and quantified with OD_{260} measurement.

[0197] Amplification of gene targets. Three assay regions were tested: One on each of chromosomes 4, 21 and X. The genomic location (fragment starting and ending positions are provided), of the selected fragments are as follows: Chr4 106415806_106416680 (SEQ ID No. 1), Chr21 38298439J8299372 (SEQ ID No. 2), and ChrX 133694723_1 33695365 (SEQ ID No. 3). The lengths are 875, 934 and 643 bases respectively. The circularized DNA was amplified with gene specific primers (SEQ ID Nos. 4-9) in a multiplex inverse PCR reaction. PCR primers were picked using Primer3 (available from the FRODO web site hosted by MIT) to yield amplicons ranging between 121 and 168 bp. PCR was carried out with 1X TITANIUM Taq PCR buffer (Clontech), 0.3mM dNTPs, 0.4µM each primer, IX TITANIUM Taq DNA Polymerase (Clontech), and ~200ng of the circularized DNA. After denaturation at 94°C for 2 minutes, reactions were cycled 30 times as follows: 94°C for 20 seconds, 60°C for 20 seconds, and 68°C for 20 seconds, with a 68°C final hold for 4 minutes. PCR products were assessed on a 4-20% gradient polyacrylamide gel (Invitrogen) and precipitated with ethanol.

[0198] The amplified DNA was fragmented with DNase I, end-labeled with Biotin, and hybridized to a whole-genome tiling array which spans the entire non-repetitive portion of the genome with uniform coverage at an average probe spacing of -200 nt (see Matsuzaki *et al., Genome Biol* 10, R125 (2009) and Wagner *et at Systematic Biology* 43, 250(1994)). In FIG. 19, probe intensity ("Array Intensity") from a whole-genome tiling array (y-axis) is grouped into 200 nt bins by the

length of the *BamHI* fragment on which it resides. The sizes and sequences of restriction fragments from a selected genome can be predicted and binned according to size. High probe intensity demonstrates the preferential amplification of fragments in the 600bp ~ 1.2kb size range (x-axis, log-scale). The computed size distribution of *BamHI* restricted fragments in the reference genome sequence (NCB1 Build 36) is shown by the curve labeled "Number of Fragments". After circularization of the amplified products to obtain circles as shown in FIG. 3, three test target fragments were isolated using gene-specific PCR; one on each of chromosomes X, 4, and 21, and prepared for detection.

[0199] The three labeled targets were counted using two sampling techniques: DNA microarrays and next-generation sequencing. For the array counting, a custom DNA array detector capable of distinguishing the set of label-tags bound to the targets was constructed by dedicating one array element for each of the 960 target-label-tag combinations. Each array element consists of a complementary target sequence adjacent to one of the complements of the 960 label-tag sequences (as shown in FIG. 2C).

[0200] Array Design: For each gene target assayed, the array probes tiled consist of all possible combinations of the 960 counter sequences connected to the two *BamHI* genomic fragment ends (FIG. 5). An additional 192 counter sequences that were not included in the adaptor pool were also tiled to serve as non-specific controls. This tiling strategy enables counter detection separately at each paired end, since each target fragment is ligated to two independent counters (one on either end).

[0201] Arrays were synthesized following standard Affymetrix GENECHIP manufacturing methods utilizing lithography and phosphoramidite nucleoside monomers bearing photolabile 5'-protecting groups. Array probes were synthesized with 5' phosphate ends to allow for ligation. Fused-silica wafer substrates were prepared by standard methods with trialkoxy aminosilane as previously described in Fodor *et al,* Science 25 1:767 (1991). After the final lithographic exposure step, the wafer was de-protected in an ethanolic amine solution for a total of 8 hrs prior to dicing and packaging.

[0202] Hybridization to Arrays: PCR products were digested with Stu I (NEB), and treated with Lambda exonuclease (USB). 5μg of the digested DNA was hybridized to a GeneChip array in 112.5μ1of hybridization solution containing 80μg denatured Herring sperm DNA (Promega), 25% formamide, 2.5pM biotin-labeled gridding oligo, and 70μ1hybridization buffer (4.8M TMAC1,

15mM Tris pH 8, and 0.015% Triton X-100). Hybridizations were carried out in ovens for 16 hours at 50°C with rotation at 30 rpm. Following hybridization, arrays were washed in 0.2X SSPE containing 0.005% Trition X-100 for 30 minutes at 37°C, and with TE (10 mM Tris, 1 mM EDTA, pH 8) for 15 minutes at 37°C. A short biotin-labeled oligonucleotide (see 519 in FIG. 5) was annealed to the hybridized DNAs, and ligated to the array probes with *E.coli* DNA ligase (USB). Excess unligated oligonucleotides were removed with TE wash for 10 minutes at 50°C. The arrays were stained with Streptavidin, R-phycoerythrin conjugate (Invitrogen) and scanned on the GCS3000 instrument (Affymetrix).

[0203] In order to maximize the specificity of target-label-tag hybridization and scoring, a ligation labeling procedure was employed on the captured sequences (FIG. 5). Thresholds were set to best separate the intensity data from the array into two clusters, one of low intensity and one of high intensity to classify label-tags as either being used or not (FIGS. 20, 21 and 25). A label-tag was scored as "present" and counted if its signal intensity exceeded the threshold. To count label-tags thresholds for the array intensity, or the number of sequencing reads were set. Appropriate thresholds were straightforward to determine when used and un-used label-tags fall into two distinct clusters separated by a significant gap. In situations where a gap was not obvious, the function normalmixEM in the R package mixtools was used to classify label-tags. This function uses the Expectation Maximization (EM) algorithm to fit the data by mixtures of two normal distributions iteratively. The two normal distributions correspond to the two clusters to be identified. The cluster of label-tags with a high value is counted as "used", and the other as "not used". The average of the minimum and maximum of the two clusters, $(I_{\text{rain}} + I_{\text{max}})/2$, was applied as the threshold for separating the two clusters.

[0204] Sampling error calculation. A sampling error can be introduced when preparing dilutions of the stock DNA solution. This error is a direct consequence of random fluctuations in the number of molecules in the volume of solution sampled. For example, when exactly 10µl of a 100µl solution containing 100 molecules is measured, the actual number of molecules in the sampled aliquot may not be exactly 10. The lower the concentration of the molecules in the entire solution, the higher the sampling error, and the more likely the actual abundance in the sampled aliquot will deviate from the expected abundance (n = 10). To calculate sampling errors, the number of molecules for each chromosome target in the stock DNA solution was determined and numerical simulations were performed to follow the dilution steps in preparing the test samples (3.62ng, 1.45ng, 0.36ng and

0.036ng). To illustrate, if the dilution step is sampling 1μl of a 25μl solution containing 250 molecules, 25 bins are created and each of the 250 molecules is randomly assigned into one of the bins. A bin was chosen at random and the number of molecules assigned to that bin was counted to simulate the process of sampling 1/25th of the entire solution. If a serial dilution was performed, the simulation process could be repeated accordingly. For each dilution, the observed copy number ratios of Chr 4:X or 21:X for 10,000 independent trials are summarized as observed medians, along with the 10th and 90th percentiles and shown in FIGS. 12 and 13.

[0205] As an alternate form of detection, the labeled samples can be sequenced using second-generation sequencing methods. This was demonstrated by submitting samples to two independent DNA sequencing runs using SOLID sequencing (ABI). The arrangement and position of the adaptors and PCR primers used to convert the DNA sample hybridized to microarrays into sequencing templates are shown schematically in FIG. 10. For additional detail and exemplary sequences see also, Fig. S5 of Fu et al. PNAS, vol. 108(22):9026-9031 (2011), which is incorporated herein in its entirety by reference. The circularized junction 515 is located between the two counter label-tags. PCR primers (3116-3118) that have restriction sites are used to amplify two fragments. Exemplary PCR primers are as follows "Fsac" 3116, "PCR004StuA_Bgl, 3117 and "Rsac" 3118. The PCR amplification introduces restriction sites at the ends of the fragments. The fragments are digested with the restriction enzymes to generate ends compatible with ligation to sequencing adaptors "P2 Sac I adaptor" and "P1 Bgl II adaptor" which also may include an encoder sequence to identify different samples. In the example the digestion is with Bgl II and Sac I because those are the sites that were included in the primers 3116-3118, but a variety of restriction enzymes could be used. The sequencing adaptors, including the priming sites for the sequencing reaction, are ligated to the ends to generate fragments that have a label-tag sequence and a portion of the target that is about 48 to 94 base pairs in length flanked by sequences for use with SOLID sequencing. In one embodiment the sequence of the entire label-tag is determined, for example 14 bases, and enough of the target to determine identity of the target. Read lengths may be, for example, 35 to 50 bases. The number of bases of sequence needed to unambiguously assign an identity to each read of a fragment will vary depending on the number of possible targets and the difference between targets. Reads of 6 to 21 bases of the target were used in the present examples. If fragments are very similar, for example, different alleles of a variant, such as a SNP or indel, it may be necessary to sequence the variable region or a portion of the variable region.

[0206] Validation by DNA sequencing (First SOLID run). DNA targets that were used for hybridization to arrays were converted to libraries for sequencing on the SOLID instrument (ABI). P1 and P2 SOLID amplification primers were added to the DNA ends through adaptor ligation and strand extension from gene-specific primers flanked by P1 or P2 sequences (FIG. 10). Each sample received a unique ligation adaptor harboring a 4-base encoder (SEQ ID Nos. 34-43) that unambiguously identifies the originating sample of any resulting read. Each adaptor includes two strands, SEQ ID Nos. 34 and 35, 36 and 37, 38 and 39, 40 and 4 1 or 42 and 43, that hybridize to form a double stranded region of 29 base pairs and a single stranded 4 base overhang (GATC). Individual libraries were prepared for each sample, and quantified with picogreen before equal amounts of each sample was combined into a single pooled library. DNA sequencing was performed on SOLID v3 by Cofactor Genomics. A total of ~46 million 50 base reads were generated. Each read is composed of three segments, corresponding to the sample encoder, label-tag sequence and gene fragment (FIG. 10). Reads were removed if: uncalled color bases were present, the average Quality Value (aQV) of the whole read < 10, the aQV of the sample encoder < 20, or the aQV of the label-tag sequence < 18. 40% of the raw reads were removed. Filtered reads were mapped to reference sequences using the program Short Oligonucleotide Color Space (SOCS), available from ABI with a maximum tolerance of 4 color mismatches between the first 45 color bases in each read and reference sequences (the last 5 color bases on 3'end of each read were trimmed in alignment). About 64.3% reads were uniquely mapped to reference sequences, of which 89.5% (16 million) have high mapping quality, i.e., with no mismatch in the sample encoder and at most 1 mismatch in the label-tag sequence. These high-quality reads, accounting for ~35% of the total reads generated, were used in subsequent counting analysis.

[0207] Sequencing replication (Second SOLID run). An aliquot of the exact same DNA library originally sequenced by Cofactor Genomics was subsequently re-sequenced by Beekman Coulter Genomics. Approximately 50 million 35 base reads were generated, and processed following the same rules. Approximately 61% of the raw reads passed quality filters, of which 81% uniquely mapped to a reference sequence with a maximum tolerance of 3 color mismatches (An adjusted mismatch tolerance was applied in the alignment step to account for the shorter length of these reads). Of the mapped reads, 91% (22.5 million) are of high mapping quality, i.e., with perfect match in the sample encoder and at most 1 mismatch in the label-tag sequence. These high-quality reads (45% of the total raw reads generated) were used for counting analysis.

[0208] Between several hundred thousand to several million reads were used to score the captured label-tags. Table 1 shows the number of mapped reads from SOLID DNA sequencing.

Table 1

DNA sample			5ng	2ng	0.5ng	0.05ng	0ng
Chr4	Left side	1st SOLiD run	709,076	252,211	237,380	316,629	1,204
		2nd SOLiD run	621,372	73,962	189,937	237,520	411
	Right side	1st SOLiD run	1,724,955	1,662,958	1,114,246	2,201,078	3,353
		2nd SOLiD run	1,422,673	1,359,512	839,775	980,616	2,386
Chr21	Left side	1st SOLiD run	1,615,416	1,474,208	832,234	1,428,540	1,851
		2nd SOLiD run	1,296,685	1,038,456	622,429	930,461	840
	Right side	1st SOLiD run	1,124,772	886,421	551,192	849,204	821
		2nd SOLiD run	910,298	522,358	367,207	479,621	224
ChrX	Left side	1st SOLiD run	444,960	316,975	254,386	515,213	744
		2nd SOLiD run	266,606	157,860	137,706	220,121	5
	Right side	1st SOLiD run	1,227,047	921,214	777,033	1,064,531	64
		2nd SOLiD run	1,043,475	768,296	559,038	695,873	43

[0209] Thresholds were set for the number of sequencing reads observed for each label-tag. and score a label-tag as "present" and counted if the number of sequencing reads exceeded the threshold. Label-tag usage summaries from experimental observations or from the stochastic modeling are shown in Table 2. The number of attached label-tags, *k*, detected for each target in each dilution either by microarray counting or sequence counting is presented in Table 2, and plotted in FIG. 12.

[0210] Several dilutions (3.62 ng, 1.45 ng, 0.36 ng and 0.036 ng) of DNA isolated from cultured lymphoblasts of a Trisomy 21 male individual were processed for microarray hybridization (FIG. 12 left) and DNA sequencing (FIG. 12 right). Three gene targets were tested from chromosome X, 4 and 21, and observed numbers of detected label-tags are shown (lines 1201). The number of target molecules for each sample was determined from the amount of DNA used, assuming a single haploid nucleus corresponds to 3.5 pg. For comparison, the calculated number of label-tags expected to appear using a stochastic model are also plotted, lines 1202. Numerical values are provided in Table 4. Relative copy ratios of the three gene targets (FIG. 13): ChrX (right bar), Chr4 (left bar) and Chr21 (center bar) representing one, two and three copies per cell, respectively. The calculated number of target molecules was determined from the number of label-tags detected on

microarrays (Table 4, column 9) or from the SOLiD sequencing data. For each sample dilution, the copy number ratio of each gene target relative to ChrX is shown for the microarray (FIG. 13 left) and for SOLiD sequencing (FIG. 13 right). For comparison, FIG. 13 also shows relative copy ratios obtained from *in silico* sampling simulations, where circles indicate the median values from 10,000 independent trials and error bars indicate the 10th and 90th percentiles. The 90th percentile values of the relative copy ratios at the lowest concentration (0.036 ng) are provided (4.5 and 6.67).

Table 2

DNA sample			3.62ng	1.45ng	0.36ng	0.036ng	0ng
Chr4	*Stochastic Model*		*633*	*336*	*98*	*10*	*0*
	Left side	Microarray	501	260	102	14	0
		1st SOLiD run	513	251	101	14	0
		2nd SOLiD run	516	273	102	14	0
	Right side	Array	525	256	107	14	0
		1st SOLiD run	544	291	103	13	1
		2nd SOLiD run	557	307	103	13	1
Chr21	*Stochastic Model*		*769*	*457*	*143*	*15*	*0*
	Left side	Microarray	651	335	160	20	0
		1st SOLiD run	678	381	152	20	0
		2nd SOLiD run	665	358	161	18	0
	Right side	Microarray	627	341	157	20	0
		1st SOLiD run	650	381	146	19	0
		2nd SOLiD run	653	379	146	19	0
ChrX	*Stochastic Model*		*400*	*186*	*50*	*5*	*0*
	Left side	Microarray	281	148	50	11	0
		1st SOLiD run	290	149	43	11	0
		2nd SOLiD run	300	150	45	11	0
	Right side	Microarray	306	133	48	10	1
		1st SOLiD run	336	153	50	12	0
		2nd SOLiD run	344	167	43	11	0

[0211] The counting results span a range of approximately 1,500 to 5 molecules, and it is useful to consider the results in two counting regimes, below and above 200 molecules. There is a striking agreement between the experimentally observed number of molecules and that expected from

dilution in the first regime where the ratio of molecules to label-tags *(n/m)* < 0.2 (Table 2). Below 200 molecules the data are in tight agreement, including the data from the lowest number of molecules, 5, 10 and 15 where the counting results are all within the expected sampling error for the experiment (The sampling error for 10 molecules is estimated to be 10 ± 6.4, where 10 and 6.4 are the mean and two standard deviations from 10,000 independent simulation trials).

[0212] In the second regime above 200 molecules, there is an approximate 10-25% undercounting of molecules, increasing as the number of molecules increases. This deviation may be due to a distortion in the amplification reaction. PCR-introduced distortion occurs from small amounts of any complex template due to the differences in amplification efficiency between individual templates *(2, 3).* In the present case, stochastic labeling will produce only one (at low *n/m* ratios), and increasingly several copies (at higher *n/m* ratios) of each template. Modeling suggests that simple random dropout of sequences (PCR efficiencies under 100%) generate significant distortion in the final numbers of each molecule after amplification. At any labeling ratio, random dropout of sequences due to PCR efficiency will result in an undercount of the original number of molecules. At high *n/m* ratios, the number of label-tags residing on multiple targets will increase and have a statistical survival advantage through the PCR reaction causing greater distortion. In support of this argument, a wide range of intensities on the microarray and a wide range in the number of occurrences of specific sequences in the sequencing experiments were observed (FIGS. 20 and 25). For additional plots of label-tags observed in the microarray experiments and by sequencing see Figures S4A and S4B of Fu et al. PNAS, vol. 108(22):9026-9031 (201 1). This effect can be reduced by carrying out the reaction at *n/m* ratios near or less than 0.2, increasing the number of label-tags *m*, further optimization of the amplification reaction, or by employing a linear amplification method. Other factors, such as errors from inaccurate pipetting, could also contribute.

[0213] The lymphoblast cell line used in this study provides an internal control for the relative measurement of copy number for genes residing on chromosomes X, 4 and 21. FIG. 13 presents the relative number of molecules from all three chromosomes normalized to copy number 1 for the X chromosome. As shown, the measurements above 50 molecules all yield highly precise relative copy number values. At low numbers of molecules (0.036 ng), uncertainty results because the stochastic variation of molecules captured by sampling an aliquot for dilution are significant. Numerical simulations were performed to estimate the sampling variation, and summarized medians, along with the 10th and 90th percentiles of the copy number ratios and are shown in FIG.

13 as circles and range bars, respectively. At the most extreme dilutions, where ~5, 10, and 15 molecules are expected for the chromosome X, 4 and 2 1 genes, the copy number ratios fall within the expected sampling error.

[0214] Overall, the identity of label-tags detected on the microarrays and in sequencing are in good agreement, with only a small subset of label-tags unique to each process (Table 7). Despite a high sequencing sampling depth (Table 1), a small number of label-tags with high microarray intensity appear to be missing or under-represented in the sequencing results. In contrast, label-tags that appear in high numbers in the sequencing reaction always correlate with high microarray intensities. No trivial explanation could be found for the label-tags that are missing from any given sequencing experiment. While under-represented in some experiments, the same label-tags appear as present with high sequence counts in other experiments, suggesting that the sequences are compatible with the sequencing reactions.

[0215] PGR was used as an independent method to investigate isolated cases of disagreement, and demonstrated that the label-tags were present in the samples used for the sequencing runs. PCR was used to detect the presence of 16 label-tag sequences (Table 3) which were either observed as high or low hybridization intensity on microarrays, and observed with either high or low numbers of mapped reads in SOLID sequencing. The Chr4 gene target was PCR amplified with 3 dilutions (0. lpg, lpg, and lOpg) of the 3.62ng NA01921 sample, using the DNA target that was hybridized to microarrays, or the prepared SOLID library template. PCR products were resolved on 4% agarose gels and fluorescent DNA bands were detected after ethidium bromide staining.

Table 3

Label-tag ID	Label-tag Sequence	1st SOLiD reads	2nd SOLiD reads	Microarray intensity	Microarray target template	SOLiD library template
112	AGATCTTGTGTCCG	0	2	15,907	1	2
182	ATCTTCGACACTGG	0	1	10,304	3	4
779	TCGAGATGGTGTTC	0	4	9,411	5	6
782	TCGGATAGAGAGCA	0	0	6,716	7	8
783	TCGGTACCAACAAC	1	4	13,132	9	10
290	CCAAGGTTTGGTGA	1	17	10,777	11	12
780	TCGCAAGAGGTAAG	1	1	8,915	13	14
570	GGAGTTACGGCTTT	1	2	8,252	15	16
741	TCAACCAGTAAGCC	794	400	466	**17***	**18***
424	CTGTAAACAACGCC	1,191	1,292	527	**19***	**20***
242	CACGATAGTTTGCC	905	781	1,103	**21***	22
859	TGTACTAACACGCC	920	892	1,107	**23***	**24***
83	ACGCTAACTCCTTG	8,629	7,704	19,500	25	26
383	CGTTTACGATGTGG	7,278	6,402	19,022	27	28
804	TCTTAGGAAACGCC	0	0	70	**29***	**30***
834	TGCAATAGACGACC	0	1	72	**31***	**32***

[0216] Table 3. PCR detection for the presence of label-tag sequences in the processed DNA sample that was hybridized to microarray, or in the DNA sequencing library. Each PCR contained O.lpg of template, which represents approximately 1×10^6 DNA molecules. The number of mapped sequencing reads and the microarray intensity of each of the 16 label-tags for this selected gene target (Chr4, 3.62ng) are listed. The last 2 columns show the gel lane number containing the indicated sample. Those numbers indicated by an * correspond to reactions where PCR failed to detect the label-tag sequence in the sample.

[0217] Although their presence in the sequencing libraries is confirmed, it is unclear why these label-tags are missing or under-represented in the final sequencing data.

[0218] To test the stochastic behavior of label-tag selection, the results of multiple reactions at low target concentrations (0.36 and 0.036 ng), where the probability that a label-tag will be chosen more than once is small, were pooled. FIG. 14 shows that the number of times each label-tag is used closely follows modeling for 1,064 data points obtained from microarray counting. The graph is a

comparison between experimentally observed label-tag usage rates (microarray results) with those predicted from stochastic model (stochastic model). At low target molecule numbers, the chance of multiple target ligations to the same label-tag sequence is low. It is therefore reasonable to consider data from experiments with low target numbers (0.036ng and 0.36ng of DNA), from those experiments, a total of 1,064 label-tags were observed, with the total frequency of label-tag usage ranging from 0 to 6. The theoretically expected label-tag usage frequency for 1,064 target molecules was obtained by performing 5000 simulation runs, with multiple independent reactions simulated in each run. The error bars indicate one standard deviation from the corresponding means.

[0219] Furthermore, since each end of a target sequence chooses a label-tag independently, the likely hood of the same label-tag occurring on both ends of a target at high copy numbers can be compared. Table 4 columns 10-1 1 present the experimentally observed frequency of label-tags occurring in common across both ends of a target and their expected frequency from numerical simulations. No evidence of non-stochastic behavior was observed in this data.

Table 4

Gene target	Genomic DNA amount (ng)	Estimated # of molecules at either end	Expected # of label-tags at either end	Expected # of label-tags in common across paired-ends	Microarray observed # of label-tags			# of molecules inferred from microarray observed # of label-tags	Expected # of label-tags in common across paired-ends	Microarray observed # of label-tags in common across paired-ends
					L	R	Avg			
Chr4	3.62	1034	633	417.68 ± 11.35	501	525	513	733	273.96 ± 10.24	303
	1.45	414	336	117.92 ± 7.83	260	256	258	300	69.22 ± 6.43	63
	0.36	103	98	9.93 ± 2.81	102	107	104	110	11.26 ± 2.99	20
	0.036	10	10	0.11 ± 0.32	14	14	14	14	0.20 ± 0.44	0
	0	0	0	0	0	0	0	0	0	0
Chr21	3.62	1551	769	616.74 ± 11.94	651	627	639	1051	425.28 ± 11.37	453
	1.45	620	457	217.36 ± 9.51	335	341	338	416	118.79 ± 7.83	130
	0.36	155	143	21.37 ± 3.98	160	157	158	172	25.86 ± 4.38	32
	0.036	15	15	0.24 ± 0.48	20	20	20	20	0.40 ± 0.62	0
	0	0	0	0	0	0	0	0	0	0
ChrX	3.62	517	400	166.63 ± 8.81	281	306	294	351	90.14 ± 7.08	103
	1.45	207	186	36.26 ± 4.98	148	133	140	151	20.34 ± 3.94	23
	0.36	51	50	2.58 ± 1.52	50	48	49	50	2.45 ± 1.51	4
	0.036	5	5	0.03 ± 0.16	11	10	10	10	0.10 ± 0.31	2
	0	0	0	0	0	1	0	0	0	0
1	2	3	4	5	6	7	8	9	10	11

Label-tags detected on microarray experiments are quantified in Table 4. Indicated quantities (col. 2) of genomic DNA derived from a Trisomy 21 male sample were tested on 3 chromosome targets (col. 1). The estimated number of copies of target molecules (or haploid genome equivalents, col. 3), the number of label-tags expected by the stochastic model (col. 4), and the actual number of label-tags detected on microarrays (col. 6-8) are summarized. Because each gene target fragment paired-end consists of random, independent label-tag ligation events at the left (L) and the right (R)

termini, the number of identical label-tags expected (col. 5) can be predicted from computer simulations, and compared to the number actually detected (col. 11). Given the number of label-tags detected (col. 8), the corresponding number of copies of target molecules (col. 9) in the stochastic model, and the predicted occurrences of identical label-tags across paired-ends (col. 10) were obtained. The numbers in col. 5 and 10 are the means from 5,000 independent simulation runs along with one standard deviation of the corresponding means, given the number of label-tags at either end (col. 4 and col. 9).

[0220] The detailed column information for Table 4 is as follows: column 1: name of tested gene targets; column 2: estimated number of target molecules at either left or right end, this number is determined by PICOGREEN dye measurement (Molecular Probes, Inc.), the DNA concentration is also listed in this column; column 3: number of label-tags expected to be observed/used at either end (predicted by theoretical models), given the estimated number of target molecules in 2nd column; column 4: number of label-tags expected to be observed in common across the paired-ends (predicted by theoretical models), given the estimated number of target molecules in 2nd column; column 5: empirically observed number of label-tags used at the left end of gene target; column 6: empirically observed number of label-tags used at the right end of gene target; column 7: empirically observed number of label-tags used in common across the paired-ends; column 8: number of target molecules predicted by theoretical models, based on the empirically observed number of label-tags used (i.e., number in 7th column); column 9: number of label-tags expected to be observed in common across the paired-ends, given the number of target molecules in 8th column; column 10: empirically observed number of label-tags that were used in common across the paired-ends of the gene target.

[0221] An array was designed having 48 target sequences. Each target was paired with one of 3840 label-tags or "counters" for a total of 48 x 3840 or 184,320 probes. The probes were 30 mers (30 nucleotides in length) and the assay was designed to test whether or not the 30 mer imparts sufficient discrimination. Of the 30 bases, 15 bases are from the label-tags and the other 15 bases are derived from the targets. The probes were assayed to determine if each label-target combination hybridizes specifically. A phage RNA ligase was used to join label-tags with targets. Universal priming sites of 18 bases were included on the 5' end of the label-tags and the 3' end of the targets, facilitating PCR amplification of the joined label-targets.

[0222] To test the method illustrated in FIG. 7, a 1:1 dilution of each of the 3840 counters was mixed with various dilutions of each of the 48 target oligos to simulate different expression levels under ligation conditions so that the 5' end of the target oligos can be ligated to the 3' end of the label-tag oligos. In preferred aspects T4 RNA ligase may be used to join the ends of the single stranded oligos. The 5' and 3' ends of the target oligos are phosphorylated and the 5' and 3' ends of the label-tag oligos are hydroxylated. After the ligation the products are amplified by PCR using primers to the universal priming sequences. Only those fragments that have both universal priming sequences 703 and 711 will amplify efficiently.

[0223] Each of the 48 target sequences may be tiled with each of the 3,840 counters, resulting in a total number of features on array = 48 x 3,840 = 184,320. This is the number of different possible combinations of target with label. The product of the ligation and amplification reactions is hybridized to the array. For each target, the number of features that light up is determined and can be compared to the known copy number of each target in the input sample.

[0224] To test the digital counting methods, also referred to as stochastic labeling a collection of label-tag sequences was provided. Each has a common 5' universal priming sequence, preferably 15-20 bases in length to facilitate amplification, and a 3' label-tag sequence, preferably 17-21 bases in length. Each type of primer in the collection has the same universal priming sequence but each type has a label-tag sequence that is different from all of the other types in the collection. In one aspect there are about 4,000 to 5,000 different types of label-tag sequences in the collection to be used. For testing the method, a set of 50 target sequences was synthesized. The target sequences each have a universal priming sequence at the 3' end (5'GCTAGGGCTAATATC-3 'SEQ ID NO: 1968, was used in this experiment). Each of the 50 oligo target sequences that were generated has a different 21 base sequence from the GENFLEX array collection of sequences, for example, 5' GCCATTTACAAACTAGGTATT'3' SEQ ID NO: 1970. The collection of label-tag oligos and the collection of target oligos was mixed. Various dilutions of the different targets were used in the mixture of targets to simulate a mixed population present at different levels, for example, different expression or copy number levels. T4 RNA ligase was added to ligate the label-tag oligos to the target oligos. There are 5000 different types of label-tag oligos and 50 different types of target oligos so the majority of the target oligos of the same type will be labeled with a type of label-tag oligo that is different from all of the other target oligos of that type. So target oligo type 1, occurrence 1 will be labeled with a label-tag oligo type A (11A) and target oligo type 1, occurrence

2, will be labeled with a different label-tag oligo, label-tag oligo type B (12B). There is a finite and calculable probability that two or more occurrences of the same target type will be labeled with the same label-tag oligo (11A and 12A), but that probability decreases as the number of different types of label-tag oligos increases relative to the number of occurrences of any given type of target.

[0225] The ligated target/label-tag oligos are then amplified using primers to the universal priming sites. Label-tags can be incorporated during amplification. The labeled amplification product is then hybridized to an array. For each different possible combination of target (50) and label-tag (5000) there is a different probe on the array that targets that junction of the target ligated to the label. There will therefore be 50 X 5000 different probes on the array or 250,000 different probes.

[0226] Scanned images of the 48x3840 array were analyzed and compared to expected results. A total of 8 of the 48 targets were ligated to a pool of 3840 label-tags (counters). The assay was as shown in FIG. 3. The conditions were single strand deoxyoligonucleotide ligation using a phage RNA ligase to join the label-tags with targets. Universal priming sites on the targets and label-tags were included to enable PCR amplification of the joined label-targets. The ligation conditions were essentially as described in (Tessier, D.C. *et al.* (1986) *Anal Biochem. 158, 171-178,* 50mM Tris-HC1, pH 8, lOmM MgC12; lOug/mL BSA, 25% PEG, ImM HCC, 20uM ATP; 5:1 acceptor (label-tags) to donor (the 8 targets) ratio at 25C overnight. The products were amplified using PCR, purified, biotin labeled with TdT, hybridized to the array, washed, stained, and scanned. The expected 8 blocks show hybridization to the array in the expected patterns.

[0227] Different ligation conditions were also tested by ligating either a single target or a pool of 48 targets to the 3,840 counters. The concentrations of the targets used in the experiment were high as in the previous experiment so most counters will be ligated to targets. In ligation 1 a single target was ligated to 3,840 label-tags. In ligation 2, 48 targets at 1:1 copy number were ligated to 3,840 label-tags. Ligation 3 is a negative control for PCR so no DNA was added. PCR with the pair of universal primers was performed using the ligation products as template and the products separated on a gel. As expected a band was observed from ligations 1 and 2, but not 3. The PCR products were labeled and hybridized to the array and the scan images after array hybridization were analyzed. As expected no signal was observed for ligation 3, all of the targets were observed for ligation 2 and the single expected target was observed for ligation 1.The single target lights up in the correct region of the chip, but background signal was also observed in unexpected locations.

Increased stringency of hybridization conditions can be used to minimize hybridization to unexpected probes of the array.

[0228] In another example, conditions for optimization of hybridization to decrease cross hybridization were tested. The products used were as described above and hybridization was performed with formamide and with or without non-specific competitor (herring sperm DNA). The non-specific signal is significantly decreased in the presence of formamide, with and without non specific competitor. This demonstrates that even though the targets and counters alone have 15 bases of complementarity to probes on the array, the combination of target plus counter and the resulting increase to 30 bases of complementarity to the probes, results in specific hybridization. Within the block of 3,480 probes, there is heterogeneity in the hybridization intensity. Preliminary sequence analysis shows a strong correlation of GC content with high signals. To minimize this array probes may be selected to have similar melting temps for the counters or the target-counter combination may be optimized to obtain similar hybridization stabilities. For example, if two targets are to be analyzed the portions of each target that are to be part of the probe may be selected to have similar TMs.

[0229] To test the efficiency of T4 RNA ligase in the ligation of label-tags to targets, DNA ligase from *E. coli* was tested. This required a slight modification of the sample prep (as depicted in FIG. 17A) by creating an overhang site for duplex ligation. The target in this example has a double stranded target specific portion and a single stranded overhang. The overhang may be, for example, a run of inosine bases, for example 6 to 9, or a string of random bases, for example, N6-16. DNA ligase is used to close the gap and generate a ligated product that includes the target strand and a label/counter from the pool of 3,840 counters. The PCR is carried out in the same manner as above using common primers.

[0230] The expected targets were observed, but some non-specific bands were also detected in the amplified DNA, even in the absence of the target. This suggests that the some of the 3,840 label-tags are combining with each other when this method is used. Selection of an optimized pool of label-tags may be used to mitigate such interference.

[0231] In another example random primed PCR was tested. Instead of a ligation step, the targets have a 3' random region, that can be, for example, a degenerate region or an inosine region. The label-tags hybridize to the random region and the random region is used as a primer for extension through the label-tag during the PCR step to append a copy of the label-tag and the universal

priming site at the 5' end of the label-tag oligo to the 3' end of the target. The extended target has a copy of the label-tag sequence and the universal priming sequence and can be amplified by PCR.

[0232] In another example, a purification method for removing excess un-ligated counters was tested. The method is shown schematically in FIG. 18. The counters **101** and the targets **2103** are ligated to form counter-target molecules as shown previously. A support bound probe that is complementary to the universal primer at the end of the target oligonucleotides **2105,** is used to separate counter-targets and targets from un-ligated counters. The support **2109** may be, for example, a magnetic bead. A second separation can be used to separate counter-targets from un-ligated targets. The second separation uses a support bound probe complementary to the universal priming sequence at the end of the counters 2107. The single capture reduces background amplification. A double round of capture may also be used.

[0233] To test the method illustrated in FIG. 8 several adaptors were synthesized. The test adaptor has PCR002 (SEQ ID No. 1969) as top or sense strand and BamAdaAS (SEQ ID No. 1970) as bottom or antisense strand.

PCR002 5' ATTATGAGCACGACAGACGCCTGATCT (1969)

BamAdaAS 3' AATACTCGTGCTGTCTGCGGACTAGACTAG 5' P (1970)

[0234] The single stranded region on the right is the BamHI single stranded overhang. The adaptor also has a half Bgl II site. The "full length-label" adaptor has SEQ ID No. 1972 as top or sense strand and SEQ ID No. 1973 as bottom or antisense strand.

Sense 5' ATTATGAGCACGACAGACGCCTGATCTNNNNNNNNNNNNNNT

AntiSense 3' AATACTCGTGCTGTCTGCGGACTAGANNNNNNNNNNNNNNACTAG

[0235] A 5' phosphate may be added to one or both strands of the adaptor using, for example, T4 polynucleotide kinase. In some aspects a truncated adaptor, such as the one shown in FIG. 4, may be used. The top or sense strand is SEQ ID No. 1974 and the bottom or antisense strand is SEQ ID No. 1975. SEQ ID NOs. 1974 and 1975 are subsequences of SEQ ID NOs. 1972 and 1973 respectively. The N's in SEQ ID Nos. 1972-1975 indicate a variable sequence in the adaptor that is different for each different label. For example, if there are 1,920 different label-tags to be used then the Ni4 represents the 1,920 different label-tags. In the examples provided, the full length adaptor is 87 bases while the truncated is 57 bases. It is more economical to use shorter sequences particularly when considering that for each different label-tag 2 oligos must be synthesized (e.g. 1,920 label-tags

requires 3,840 different oligos). The separate oligos are preferably annealed together prior to being combined into a pool for ligation to fragments. The primer used for amplification may be, for example, SEQ ID NO. 1969 or the 5' 17 bases of SEQ ID No. 1974.

[0236] In another example, a truncated label-tag adaptor was used (SEQ ID Nos. 1974 and 1975). The adaptor ligated fragments were extended to fill in the ends with polymerase prior to PCR. Hybridization was done in duplicate to either the CNV-type array or HG49 design C. Fragmented DNA and non-fragmented DNA were plotted. The intensity of the DNA that was not fragmented prior to hybridization is less than the intensity of the fragmented DNA. The peak of the intensity for both plots is at a fragment size of about 900 base pairs.

[0237] FIG. 12 shows counting results for DNA copy number titrations using microarray hybridization on the left or DNA sequencing on the right. Dilutions (3.62ng, 1.45ng, 0.36ng and 0.036ng) of a DNA sample isolated from cultured lymphoblasts of a Trisomy 21 male individual were processed for microarray hybridization (left) and DNA sequencing (right). Three chromosome targets were tested and observed numbers of counters (Y-axis) are shown (curve **1201).** The number of target molecules for each sample (X-axis) was determined from the amount of DNA used, assuming a single cell corresponds to lOpg. For comparison, the theoretical counter usage rates from the stochastic model equation are also plotted **1202.** Numerical values are provided in Table 4.

[0238] BamHI cuts the human genome into an estimated 360,679 fragments with a size distribution of 6 bp to 30,020,000 bp. The median size is 5142 bp and the mean is 8320 bp. There are 79,517 fragments in the size range of 150 bp to 2 kb. For testing it may be desirable to choose fragments that meet selected criteria, for example, within a selected size range, select fragments that have more than 1 probe on the HG49m array, exclude fragments that are in known CNV regions, or exclude fragments having a SNP in the first or last 20-30 bases.

[0239] The upper panel of FIG. 23 shows the intensities of 1,152 array probes associated with one gene target on chromosome 4, chr4_01s. The data are from the array with 5ng DNA, i.e., 1000 copies of the tested gene target. The 1,152 probes shown share the same genomic sequence portion, but have different label-tag sequences. Each black dot represents one label-tag sequence. The left 960 dots (on the left side of the dashed vertical line) correspond to specific label-tags (i.e., label-tags used in ligation reaction), and the 192 dots to the ri8ght of the dashed vertical line correspond to non-specific label-tags (i.e., label-tags not used in ligation reaction). The probe intensities were

plotted in natural log scale on the y-axis. The horizontal dashed line is the threshold determined by analysis algorithm, which has a value of 3,800.

[0240] The array design for the experiment represented in FIG. 23 is as follows. For each gene target assayed, the array probe consists of all possible combinations of the 960 label-tag sequence and either of the two BamHI genomic fragment ends. An additional 192 label-tag sequences that were not included in the adaptor pool were also tiled to serve as non-specific controls. This tiling strategy enables consistency check on the number of label-tags used at the paired ends, since each target fragment is ligated to two independent label-tags (one on either end), and for the same target fragment, the counts on the left and right side should be very similar.

[0241] The lower panel of FIG. 23 shows the histogram of the intensity data corresponding to 960 specific label-tags. Also shown in the figure are the 2 fitted normal distributions. The fitted distributions have the mean and standard deviation of 1447 ± 680 and 12186 ± 3580, respectively. The vertical dashed line in the lower panel is the threshold, which has the same value as the horizontal dashed line shown in the upper panel. Based on such threshold, 501 probes (i.e., label-tags) were counted as "used".

[0242] FIG. 24 shows the number of times observed for each of the 960 specific label-tags. Empirically, 349 label-tags were not observed in any of the 20 cases. By model, we would expect to observe 643.05 ± 9.96 label-tags at least once, which means we expect not to observe 307-327 label-tags. This result shown was obtained by grouping label-tags used in independent ligation reactions together. To more accurately estimate the frequency of usage of label-tags, only data from experiments with low concentrations (0.05ng and 0.5ng of DNA ligation amount) were considered. Under each concentration, 5 different gene targets independently ligated to label-tags at both ends. Therefore, a total of 20 independent reactions (2 concentrations x 5 gene targets x 2 ends) were grouped together. Of these reactions, 1,064 label-tags were observed; some were observed more often than the others, the frequency of usage of label-tags ranges from 0 to 6.

[0243] This suggests that the differential usage of label-tags may be observed before PCR is started. Such difference in the amount of molecules associated with different label-tags will carry on as PCR process goes on.

[0244] For the PCR simulations, we defined n copies of a gene fragment T, each ligated to a single counter randomly selected from an infinite pool of m unique counters to generate a collection of k resulting counter-ligated gene target molecules $T^* \leftarrow \{tl_i, i \leftarrow 1,2,\cdots,£\}$. We assumed that each

counter-ligated gene target molecule tl_i replicates randomly and independently of other target molecules; and that the replication probability p (i.e., amplification efficiency) of different molecules, tl_i, remains constant throughout the PCR process. For each tl_i, we denote the number of molecules at PCR cycle c as N_i^c. When $c = 0$, N_i^0 is the initial number of tl_i. The PCR process at cycle $c+1$ can be modeled as a series of N_i^c independent trials that determine the replicability of each of the N_i' molecules with replication probability p. Let AN_i^c represent the number of molecules replicated at cycle $c+1$, then the number of molecule tl_i after cycle $c+1$ is $N_i^{c+1} = N_i^c + AN_i^c$, where the probability of AN_i^c is $p(AN_i^c \mid N_i^c) = \binom{N_i^c}{\Delta N_i^c} p^{\Delta N_i^c} (1-p)^{N_i^c - \Delta N_i^c}$. We determined the relative abundance of different counter-ligated gene target molecules tl_i upon completion of the simulated PCR run for $n = 500$, 50, or 5, and $p = 0.8$, 0.7 or 0.6 (Table 5). In each case, we performed 1,000 independent runs to simulate 30 cycles of adaptor PCR, followed by 30 cycles of gene-specific PCR. Table 5 shows summary statistics drawn from 100 independent simulation runs modeling PCR, ligation at each end of targets is considered.

Table 5.

Initial copy number	N = 5		N = 50		N = 500	
Side	Left	Right	Left	Right	Left	Right
# of labels observed	**5**	**5**	**48.61 0.99**	**48.64 1.09**	**388.91 6.85**	**389.27 7.18**
Max	(1.43 0.19) * 10^11	(1.43 0.19) * 10^11	(2.18 0.49) * 10^10	(2.13 0.47) * 10^10	(4.52 0.59) * 10^9	(4.44 0.55) * 10^9
Min	(5.73 2.27) * 10^10	(5.73 2.27) * 10^10	(2.35 1.06) * 10^9	(2.35 1.06) * 10^9	(1.15 0.49) * 10^8	(1.23 0.49) * 10^8
Ratio btw max & min	**3.13 2.44**	**3.13 2.44**	**14.10 11.41**	**13.87 13.42**	**43.42 25.66**	**44.92 30.04**
Mean	(1.00 0.16) * 10^11	(1.00 0.16) * 10^11	(1.03 0.06) * 10^10	(1.03 0.06) * 10^10	(1.27 0.03) * 10^9	(1.27 0.03) * 10^9
Standard deviation	(3.44 1.02) * 10^10	(3.44 1.02) * 10^10	(3.98 0.52) * 10^9	(3.94 0.54) * 10^9	(6.75 0.41) * 10^8	(6.69 0.40) * 10^8
Coef. of variation	**0.36 0.13**	**0.36 0.13**	**0.39 0.05**	**0.38 0.05**	**0.53 0.03**	**0.53 0.03**

[0245] Focusing on the experiments with concentrations of 0.5 and 0,05ng, (3rd and 4th in each group of 5), which provide the most accurate count of label-tags, there are 20 different opportunities

for a given label-tag to be observed (2 concentrations x 5 amplicons x 2 sides (left or right)). We observed 1,064 label-tags over the 20 opportunities.

[0246] To observe the distortion of the relative abundance of DNA molecules in the reaction resulting from the PCR process, dispersion in the quantitative distribution of PCR amplified DNA molecules was analyzed. A model of the PCR process was generated to understand the dispersion in the distribution of amplified molecules (FIG. 22, Table 6). A series of 1,000 independent simulation runs were performed to simulate the replication of 500 uniquely labeled target molecules through PCR processes. For each run, the distribution of the final amount of PCR products was determined and the dispersion of distribution was quantified using two measures: ratio of the maximal to the minimal amount, and coefficient of variation of final PCR products. For a library of 960 counters and 500 target molecules the expectation is that about 390 counters will ligate at various redundancies (1, 2, 3 and 4 are shown). For each of the 390 counters the relative ratio of its abundance to that of the counter with the lowest abundance is plotted (y-axis) for the onset of PCR (panel A), or after 5, 10 or 15 cycles of amplification (panels B-D respectively). The x-axis represents the different labeled molecules. The ratio of the most abundant to the least abundant is 30. This demonstrates that the degree of dispersion increases with the incidence of replicate use of identical counters, which may be in-part responsible for the deviation observed when assaying high target copy numbers. Table 6 lists the ratio and CV for distributions corresponding to different concentrations and replication probabilities (one sided ligation considered).

Table 6

	replication probability	$n = 5$	$n = 50$	$n = 500$
Ratio of max to min amount of PCR amplified product	p = 0.6	5.69 ± 4.95	26.16 ± 23.78	124.18 ± 88.04
	p = 0.7	4.59 ± 8.03	16.22 ± 15.53	71.55 ± 55.13
	p = 0.8	2.82 ± 1.51	11.54 ± 9.53	42.24 ± 27.49
Coefficient of Variation (CV)	p = 0.6	0.48 ± 0.16	0.51 ± 0.06	0.62 ± 0.03
	p = 0.7	0.41 ± 0.14	0.44 ± 0.05	0.57 ± 0.02
	p = 0.8	0.34 ± 0.12	0.36 ± 0.05	0.52 ± 0.02

[0247] Example 1 of a method for selecting a collection of label-tags starting with all possible 14 mers (4^{14} or ~268 million possible label-tags). Step 1: clustering based on the last 7 bases: all sequences with the same last 7 bases are grouped together; within each cluster, randomly pick one sequence, this gives us 11,025 sequences, denoted by set A. Step 2: clustering based on the first 7 bases: all sequences with the same first 7 bases are grouped together; within each cluster, randomly pick one sequence, this gives us 13,377 sequences, denoted by set B. Step 3: get the union set of set A and B, the combined set has 24,073 sequences. Then do clustering based on the middle 6 bases, randomly pick one sequence out of every cluster, this gives us 3,084 sequences, denoted by set C. Step 4: calculate the all-against-all alignment score of set C, which gives us a 3,084 X 3,084 self-similarity score matrix, denoted by S. Step 5: filter based on the score matrix. If an element of the score matrix S(i,j) has a high value, that means, the corresponding sequences i and j are very similar to each other. Starting from the elements with top self-similarity score, randomly pick one and discard the other; repeat this process until the number of retained sequences < 2000. Until this step, 1,927 sequences were retained.

[0248] For the retained 1,927 sequences, an all-against-all complement score was calculated for each. This gave a 1,927 x 1,927 cross complement score matrix. A step similar to step 5 was performed, to avoid label-tags with maximal cross-complement with other label-tags. Starting from the pairs with top cross-complement score, one was randomly pick and the other discarded. This process was repeated until the number of retained sequences was 1920. Next the 1920 label-tags were split into 2 sets, with one set (denoted by set A) consisting of sequences that are maximum different from one-another; and the other set (denoted by set B) consisting of the remaining sequences. The procedure used to split sequences was as follows. Starting from the original 1920 by 1920 similarity score matrix, for each sequence, first, sum up all its similarity scores with the rest of the sequences in the pool, that is, for each sequence, calculate a total similarity score. Second, sort the total similarity scores of all sequences and select the sequence with the lowest total score, and move it to set A. Third, remove the row and column corresponding to the selected sequence, i.e., both the number of rows and columns in the similarity score matrix are reduced by 1. Repeat steps 1-3, until the number of rows and columns in the similarity score matrix reaches 960 or half of the original. The selected sequences belong to set A and the remaining sequences belong to set B.

[0249] In another embodiment a collection of label-tags is selected using the following steps. Starting with all possible 14 mers (4^{14} or ~268 million possible label-tags) eliminate all that do not have 50% GC content. Eliminate those were each nucleotide does not occur at least twice. Eliminate those that have more than two G/C in tandem or more than three A/T in tandem. Eliminate those that contain a selected restriction site. That reduces the original set to ~33 million or 12.43% of the original set. From that set select those that have a Tm within the range of 38.5°C to 39.5°C. This step results in a set of ~ 7 million or 2.73% of the original set. Remove those that have regions of self-complementarity. The resulting set in this example was now 521,291. A hierarchical clustering was performed to identify a set that has maximum sequence difference between one-another. The resulting set contained 1,927 label-tags. Label-tags were removed if the sequence had a tendency to bind to other label-tags in the set. This reduced the set to 1,920 label-tags. A final set of 960 label-tags was selected from the 1,920 as being maximally different for the "specific" label-tags and 192 additional counters to tile on the array as "non-specific" controls.

[0250] Selection of Targets and design of test array. Regions selected to assay as targets included Chr X, Chr Y, Chr 4 as a reference and Chr 21 for Trisomy. Locations on the chromosomes for assaying were selected to avoid centromeres and telomeres. Fragments were selected based on Bam HI fragments of between about 400 and 600 base pairs. Fragment intensity was checked using HG49 array hybridization. The first and the last 26 nucleotides of the fragments (from and including the Bam HI site) were tiled. Repeats were avoided and GC% was optimized.

[0251] The array format was 100/25. Feature size is Sum. There are 436 x 436 features or 190,096 features. Synthesis was NNPOC, 43 mer probes, 5' up, no phosphate. The chip name is STCL-test2. The gridding probe used was the same as the HG49. No quality control probes were included.

[0252] Aside from reducing whole chromosomes into 360,679 smaller molecular weight DNA pieces more suitable for ligation reactions, restriction digestion also serves to reduce the overall sequence complexity of the sample, as only an estimated 79,517 fragments reside in the 150bp-2kb size range that is effectively amplified by PCR. To detect and quantify counters that have been selected by the target molecules, the labeled genomic target fragments may be circularized and PCR amplified to prepare for analysis, for example, using microarray hybridization or DNA sequencing. A representative *BamHI* target fragment was sampled for each of the three test chromosomes. Simultaneous measurements of all three chromosomes serve as an internal control independent of

dilution or other systematic errors. A suitable DNA array detector capable of distinguishing the set of counters bound to copies of the target molecules was constructed using photolithography (S. P. Fodor *et ah, Science* 251, 767 (Feb 15, 1991). Each array element for a target to be evaluated consists of a target complementary sequence adjacent to one of the complements to the 960 counter sequences. For increased specificity, a ligation-readout was performed on the microarray after hybridization to avoid false positive detection of the cross-hybridization of identical targets with different counters. As a means of validation through a secondary measure, samples hybridized to microarrays were subsequently sampled by DNA sequencing (FIGS. 10 and 25).

[0253] An equation to model the stochastic labeling of target molecules with a small library of 960 counters, and validate our equation model with numerical simulations is disclosed. In the model, the diversity of counters selected by, and ligated to target molecules in the reaction solution (simplified as 'used') is dictated by the number of copies of molecules present for each target fragment in the reaction (FIG. 12). Under conditions where the size of the counter library is much greater than the number of copies of a given target molecule, the counting efficiency is high, and counting the number of counters used is equivalent to counting the number of copies of the original target molecules (FIG. 16). When the number of target copies approaches and exceeds the number of different counters in the reaction, any counter in the library is more likely to be used multiple times (FIG. 15). The number of counters used at least once is an important measure because it serves as the basis for drawing yes/no conclusions in our digital readout on microarrays. Under stochastic labeling conditions, we expect that the absolute quantity of single DNA molecules can be accurately determined by proxy counts of labeling events. Indeed, microarray experiments demonstrate a high degree of correlation between the number of copies of target molecules added to the reaction and the number of counters used, as detected on microarrays (FIG. 12). In particular, counter usage precisely profiles the number of target molecules under conditions of high counting efficiency. Subtle deviations from the model may represent minor dilution errors in the preparation of the sample. However, within that sample dilution, the relative counter ratios of the three internally built-in controls are highly accurate (FIG. 13). FIG. 13 shows comparison of relative copy ratios of the three gene targets tested: ChrX, Chr4 and Chr21 representing genetic material of one, two and three copies per cell. Different dilutions (5ng, 2ng, 0.5ng and 0.05ng) of a DNA sample isolated from cultured lymphoblasts of a Trisomy 2 1 male individual were processed for microarray hybridization and DNA sequencing. The calculated number of target molecules (see, Table 4,

column 9) was inferred from the number of counters detected on microarrays (A), and was also calculated for the SOLID sequencing data (B). For each sample dilution, the target copy number ratio of each gene target relative to ChrX is shown.

[0254] On the other hand, when target copies exceed -100, detected labeling events appear to indicate fewer than actual molecules in solution (FIG. 12 inset in graph on left). This deviation was reproducible and consistently observed across multiple microarray experiments, and was also observed in the DNA sequencing experiments (FIG. 12 inset in graph on right). Under-counts of expected labeling events may originate from inadequate detection sensitivity of the microarray platform or from other systematic or indeterminate deficiencies in the sample preparation procedure. PGR, for example, is prone to amplification bias (T. Kanagawa. *J Biosci Bioeng* 96, 317 (2003) *and* M. F. Polz, C. M. Cavanaugh, *Appl Environ Microbiol* 64, 3724 (1998)), which could hinder the comprehensive detection of labeling events that may be genuinely stochastic.

[0255] To confirm the microarray results, a digital sequence counting of individual molecules in the DNA samples hybridized to microarrays was used as a means of validation, and to detect the presence of any false negatives that may have escaped microarray detection. Analysis of mapped sequence reads resulted in counts in agreement to the microarray observations. Furthermore, a second, independent sequencing run was repeated with similar findings (Table 3).

[0256] An additional feature of digital sequence counting is that unlike the microarray intensity data (FIGS. 20 and 21), which may provide lower resolution into the measurement of the concentration dispersion of PGR amplified molecules, sequence counting clearly demonstrates significant variation in the representation of amplified targets (FIG. 25), This is consistent with the computed PCR model. Overall, detected counters on the microarray and sequencing experiments correlate well, but a small subset of counters appear to be unique to each process (Table 7). The observed number of label-tags in common between the microarray and the two sets of sequencing experiments are summarized in the table. The number of label-tags in each category is included. The categories are as follows: A+1+2 for label-tags detected in each of the 3 experiments, 1+2 for label-tags detected only in sequencing runs 1 and 2, 1+A for label-tags detected in sequencing run 1 and by array, and so on for the amounts of DNA shown in column 3.

Table 7.

DNA sample			A+1+2	1 + 2	1+A	2+A	1	2	A
Chr4	Left side	0.036ng	13	0	0	0	0	0	1
		0.36ng	96	3	0	1	2	2	5
		1.45ng	228	13	4	22	6	10	6
		3.62ng	484	23	2	3	4	6	12
	Right side	0.036ng	14	0	0	0	0	0	0
		0.36ng	100	1	0	0	2	2	7
		1.45ng	249	25	2	0	15	33	5
		3.62ng	511	22	2	1	9	23	11
Chr21	Left side	0.036ng	18	0	2	0	0	0	0
		0.36ng	150	0	2	4	0	7	4
		1.45ng	324	17	8	1	32	16	2
		3.62ng	637	14	10	0	17	14	4
	Right side	0.05ng	18	0	1	1	0	0	0
		0.36ng	144	0	2	2	0	0	9
		1.45ng	330	34	2	3	15	12	6
		3.62ng	615	29	1	7	5	2	4
ChrX	Left side	0.036ng	11	0	0	0	0	0	0
		0.36ng	42	0	0	0	1	3	8
		1.45ng	137	3	1	5	8	5	5
		3.62ng	274	12	0	2	4	12	5
	Right side	0.036ng	10	1	0	0	1	0	0
		0.36ng	43	0	3	0	4	0	2
		1.45ng	127	15	0	0	11	25	6
		3.62ng	298	12	3	3	24	31	2

[0257] For the reverse scenario, high numbers of mapped sequence reads were always observed to correlate with high microarray intensities in these examples. No systematic or sequence correlations, or explanations were identified for the counters that are absent from any given sequencing experiment for which the microarray readout demonstrates a strong signal. While obviously underrepresented in some experiments, the same counters are sometimes present in high sequence counts in other experiments, suggesting that they are available for sequencing. PGR was used to resolve these isolated cases of disagreement and demonstrate these were false negatives in

the sequencing experiments (Table 3). Despite their presence in the sequencing library, it is unclear why the counters were not observed or were underrepresented in the original sequencing run, and also in the subsequent replicate sequencing run.

[0258] Aside from the comparative analysis of absolute and relative counts of the numbers of target molecules and counter label-tags, additional ways to assess the stochasticity of the labeling process were evaluated. First, if the labeling process is random, the frequency of incorporation of identical counters in independent events across the paired left and right termini of target fragments should closely resemble outcomes from numerical simulation. Observed counts on microarrays do in fact match closely with numbers obtained from computer simulations (Table 4, columns 10-1 1). Second, if the target molecules are labeled randomly with an equal likelihood of incorporation for any member of the 960 counters in the library, we would expect the number of repeated observations of counters to follow a stochastic nature. For this analysis, we accumulated a total of 1,064 counter observations over several microarray experiments restricted to low target copy numbers. Exclusion of data from high copy targets was necessary to avoid undercounting labeling events from multiple incidences of identical counters attaching individually to numerous target copies. As a further and final demonstration of stochastic labeling, results show that the frequency of label-tag usage follows a pattern consistent with outcomes from numerical simulation.

[0259] Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

[0260] All publications, patents and patent applications mentioned in this specification arc indicative of the level of skill of those skilled in the art to which this invention pertains, and 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.

We claim:

1. A method for counting individual occurrences of at least one species of target molecule in a mixture of nucleic acids having an unknown number of target molecule occurrences, the method comprising:

(a) combining the mixture with a pool of label-tags, wherein the pool of label-tags comprises multiple copies of each of a plurality of different label-tag sequences;

(b) attaching label-tags from the pool of label-tags to individual occurrences of the at least one species of target molecule in a random manner to obtain label-tag-target molecules, wherein different occurrences of a species of target molecule attach to different label-tag sequences from the plurality of different label-tag sequences, thereby obtaining a plurality of unique label-tag-target molecules;

(c) optionally amplifying the unique label-tag-target molecules;

(d) detecting each type of unique label-tag-target molecule; and

(e) counting the number of unique label-tag-target molecules that are detected to obtain a count of the target molecule occurrences of the at least one species of target molecule in the mixture.

2. The method of claim 1 wherein the collection of label-tags comprises a number of different label-tag sequences that is at least twice the number of each target molecule to be estimated.

3. The method of claim 1 wherein the collection of label-tags comprises between 900 and 10,000 different label-tag sequences

4. The method of claim 1 wherein the unique label-tag-target molecules are amplified by PCR in a multiplex amplification reaction so that there are a plurality of copies of each unique label-tag-target molecule that are detected in step (d).

5. The method of claim 1 wherein between 2 and 100 different species of target molecules are counted.

6. The method of claim 1 wherein between 100 and 10,000 different species of target molecules are counted.

7. The method of claim 1 wherein between 10,000 and 50,000 different species of target molecules are counted.

8. The method of claim 1 wherein the mixture of nucleic acids is a genomic DNA sample, the method further comprising:

fragmenting the genomic DNA sample to obtain fragments comprising target molecules prior to step (a),

ligating a first adaptor comprising a common priming sequence to a first end of the fragments;

attaching the label-tags by ligating a second adaptor comprising a label-tag and a second common priming sequence to the second end of the fragments, wherein the second adaptor is randomly selected from a pool of second adaptors that comprises the pool of label-tags, wherein the label-tag is between the target fragment and the second common priming sequence;

thereby obtaining the plurality of unique label-tag-target molecules; and

amplifying the unique label-tag-target molecules by PCR using a primer or a pair of primers to the first and second common priming sequences prior to detecting step (d).

9. The method of claim 1 wherein the number of different label-tags is at least an order of magnitude larger than the expected number of target molecules.

10. The method of claim 1 wherein label-tags are attached to targets by ligation.

11. The method of claim 1 wherein the pool of label-tags comprises a pool of label-tag primers that each consist of at least one label-tag and a priming sequence that is complementary to a species of target molecule and wherein the attaching step comprises: (i) hybridizing individual label-tag primers to individual occurrences of the target and (ii) extending the hybridized primers using the target as template to obtain unique primer extension products each comprising a different label-tag and each corresponding to a single occurrence of the target molecule.

12. The method of claim wherein 11 wherein the target is RNA and the extending step is by reverse transcriptase.

13. The method of claim wherein 11 wherein the target is DNA and the extending step is by a DNA dependent polymerase.

14. The method of claim 1 wherein the ratio of target molecule occurrences to number of different label-tags is less than 1:10.

15. The method of claim 1 wherein the ratio of target molecule occurrences to number of different label-tags is less than 1:100.

16. The method of claim 1 wherein the ratio of target molecule occurrences to number of different label-tags is less than 1:1000.

17. The method of claim 1 wherein the ratio of target molecule occurrences to number of different label-tags is less than 1:10,000.

18. The method of claim 1 wherein the step of detecting each type of unique label-tag-target molecule is by hybridizing at least a portion of the label-tag-target molecules to an array of probes comprising a plurality of features wherein each feature contains a different probe sequence and the probe sequence in each feature is specific for a different label-target combination, wherein the array comprises a plurality of features that are complementary to a predicted junction between the label-tag and a region of the target and wherein features that are hybridized to label-tag-target molecules are detectably labeled.

19. The method of claim 18 wherein each feature is a different bead.

20. The method of claim 19 wherein the step of counting comprises counting the number of beads that are detectably labeled.

21. The method of claim 19 wherein the beads are present in the array at random locations and the sequence of the probes on each bead is unknown.

22. The method of claim 1 wherein the step of detecting each type of unique label-tag-target molecule is by sequencing at least a portion of the label-tag-target molecules, wherein enough of the label-tag is sequences to unambiguously identify the label-tag and enough of the target is sequenced to unambiguously identify the target species.

23. The method of claim 22 wherein sequencing is by a method selected from sequencing by synthesis, sequencing by ligation, pyrosequencing, and sequencing by hybridization.

24. The method of claim 22 wherein sequencing is by a method that sequences single molecules as they pass through a nanopore.

25. The method of claim 1 wherein the mixture of nucleic acids is the total mRNA from a single cell.

26. The method of claim 1 wherein the mixture of nucleic acids is the nucleic acid from 10 to 1,000 cells, the nucleic acid from 10 to 1,000 cells or the RNA from a 10 to 1,000 cells.

27. The method of claim 1 wherein the step of amplifying comprises a method selected from PCR, reverse transcription, in vitro transcription, rolling circle amplification, and isothermal strand displacing amplification.

28. An array comprising a plurality of feature sets, including a feature set for each of a plurality of different target species, wherein each feature set comprises a plurality of different features each feature comprising a plurality of oligonucleotide probes of the same sequence, and wherein the features of a feature set have a common target specific sequence and a variable label-tag sequence, wherein each different feature in the set has the same target specific sequence and a different label-tag specific sequence and wherein each feature set has a feature for each different label-tag in a set of label-tags.

29. The array of claim 28 wherein the array has a first feature set for a first target species, a second feature set for a second target species and a third feature set for a third target species and wherein each feature set comprises at least one feature for each label-tag in a pool of label-tags.

30. The array of claim 28 wherein the array has between 3 and 100 different feature sets, each feature set being specific for a single different target species and each feature set comprising at least one feature for each label-tag in a pool of label-tags.

31. The array of claim 28 wherein the array has between 100 and 10,000 different feature sets, each feature set being specific for a single different target species and each feature set comprising at least one feature for each label-tag in a pool of label-tags.

32. The array of claim 30 wherein the label-tag set has a diversity of label-tags comprising 1,000 to 5,000 different label-tags.

33. The array of claim 30 wherein the label-tag set has a diversity of label-tags comprising 5,000 to 100,000 different label-tags.

34. The array of claim 30 wherein the label-tag set has a diversity of label-tags comprising 100,000 to 1,000,000 different label-tags.

35. A method of determining a number of target polynucleotides, the method comprising the steps of:

providing for each target polynucleotide a plurality of nucleic acid probes specific for the target polynucleotide, each nucleic acid probe having a different oligonucleotide tag;

combining in a reaction mixture the plurality of nucleic acid probes with the target polynucleotides so that substantially every target polynucleotide associates with a nucleic acid probe to form a probe-target complex, the plurality of nucleic acid probes having a number sufficiently greater than the number of target polynucleotides so that substantially every probe-target complex has a unique oligonucleotide tag;

treating the sample to enrich the probe-target complexes; and

determining the number of different oligonucleotide tags in a sample of enriched probe-target complexes by hybridization thereby determining the number of target polynucleotide in the mixture.

36. The method of claim 35 wherein the target polynucleotide is a restriction fragment having a first single strand sequence overhang and a second, different single strand overhang, and wherein a first adaptor has a single stranded overhang that is perfectly complementary to the first overhang and a second different adaptor has a single stranded overhang that is perfectly complementary to the second overhang.

37. The method of claim 36 wherein the restriction fragment is formed by digesting genomic DNA with at least one restriction endonuclease.

38. A method for determining a value for n for a plurality of different target species, wherein n is the number of occurrences of a first species of target molecule that are present in a sample, the method comprising:

attaching to each occurrence of the first species of target molecule, a label-tag from a set of diverse label-tags, wherein the set of diverse label-tags comprises m different label-tags and label-tags are chosen at random, thereby generating for each occurrence of the first species of target molecule, a labeled target molecule that comprises a single occurrence of the first species of target molecule and a label-tag from the set of diverse label-tags, wherein at least 90% of the labeled target molecules have a label-tag that is different from the label-tags on all of the other labeled target molecules of the first species of target molecule;

detecting each labeled target molecule by determining at least a partial sequence of the labeled target molecule, wherein at least X bases of the target molecule and at least Y bases of the label-tag are determined; and

counting the number of new molecules, thereby counting the number of molecules of the first target molecule in the sample.

39. The method of claim 38 wherein the ratio of m to n is greater than 50.

40. The method of claim 38 wherein the ratio of m to n is greater than 100.

41. The method of claim 38 wherein the ratio of m to n is greater than 500.

42. The method of claim 38 wherein the ratio of m to n is greater than 1000.

43. The method of claim 38 wherein the target molecules are proteins or peptides.

44. The method of claim 38 wherein target molecules are nucleic acids and the detecting step comprises sequencing.

45. The method of claim 44 wherein sequencing comprises sequencing by extension or sequencing by ligation.

46. The method of claim 44 wherein sequencing comprises sequencing using reversible terminator chemistry.

47. The method of claim 38 wherein the sample consists of a single cell, the nucleic acid from a single cell, the RNA from a single cell or a crude cell extract from a single cell.

48. The method of claim 38 wherein the sample consists of 10 to 1,000 cells, the nucleic acid from 10 to 1,000 cells or the RNA from a 10 to 1,000 cells.

49. The method of claim 38 wherein individual diverse label-tags from the set of diverse label-tags are attached to an occurrence of the first species of target molecules by extension of a primer that comprises the diverse label-tag using template dependent primer extension by an enzyme selected from: DNA dependent RNA polymerase, a DNA dependent DNA polymerase and an RNA dependent RNA polymerase.

50. The method of claim 49 wherein the DNA dependent RNA polymerase is selected from T7 RNA polymerase and SP6 RNA polymerase.

51. A method for counting the number of occurrences of each of a plurality of different target molecules in a sample, the method comprising the steps of:

(a) providing a plurality of conjugates each comprising a label-tag selected from a pool of label-tags, and a target molecule, wherein the label-tag is adjacent to a known target sequence in the conjugate so that a label-tag-target junction is formed and wherein more than 90% of the target molecule are attached to a label-tag, the pool of oligonucleotide label-tags comprising a plurality of different label-tag sequences and wherein the probability that any two target molecules is labeled with the same label-tag is less than a selected threshold value;

(b) amplifying the conjugates in a multiplex amplification reaction to obtain amplified conjugates comprising label-tags;

(c) detecting each different amplified conjugate by detecting different label-tags;

(d) counting the number of different lable-tags and conjugates detected in step (c); and,

(e) determining the number of target molecules in the sample from the number of different label-tags and conjugates counted in step (d).

52. The method of claim 51 wherein:

(i) the pool of label-tags has m different label-tags;

(ii) the first target species has n occurrences; and

(iii) following the covalent attaching step there are k first targets labeled with a label-tag that is unique in the set of labeled first targets; and

wherein the step of counting comprises empirically determining k and calculating n using $k = m(1- e^{-(n/m)})$.

53. The method of claim 52 wherein the number of different label-tags in the pool of label-tags is n and the number of occurrences of the first target is m and wherein n/m is less than 0.10.

54. The method of claim 53 wherein n/m is less than 0.01 .

55. The method of claim 53 wherein n/m is less than 0.005.

56. The method of claim 53 wherein n/m is less than 0.001.

57. The method of claim 51wherein label-tags are selected at random in the step of ligating a label-tag to individual occurrences of first and second target nucleic acid species.

58. The method of claim 51 wherein the label-tags in the pool of label-tags are DNA, the first and second target species are DNA and the ligation comprises ligation with a ligase selected from T4 DNA ligase and E. coli DNA ligase.

59. The method of claim 51 wherein detecting step is by hybridization to an array of probes that are complementary to the label-tag-target junctions.

60. The method of claim 51 wherein the step of detecting and counting comprises:

(a) hybridizing the plurality of amplified labeled first target species and the plurality of amplified labeled second target species to an array of probes comprising a first plurality of probe features each feature complementary to a different first target-label-tag junction and a second plurality of probe features each feature complementary to different second target-label-tag junctions;

(b) detecting hybridized features; and

(c) counting the hybridized features.

61. The method of claim 60 wherein the array of probes comprises a glass support having probes complementary to the junctions.
62. The method of claim 60 wherein the array of probes comprises a plurality of beads having probes complementary to the junctions.
63. The method of claim 60 wherein the array of probes comprises a plurality of microspheres.
64. The method of claim 60 wherein the array of probes comprises a plurality of encoded microparticles where the microparticles for the first target are encoded with a first code and the microparticles for the second target are encoded with a second code.
65. The method of claim 51 wherein the step of detecting and counting comprises:

sequencing amplified labeled first target species and amplified labeled second target species, wherein at least a portion of the label-tag and at least a portion of the target specific region is sequenced, wherein the number of different sequences obtained for each target is used to determine the count of each target.

66. The method of claim 65 wherein the targets to be counted are the transcriptome of a single cell or a collection of cells of a single type.
67. The method of claim 65 wherein the targets to be counted are the number of chromosomes of a single type in a genome or the number of copies of an allele of a gene in a genome.
68. A kit comprising a pool of label-tags wherein the diversity of the pool of label-tags is selected from between 100 and 1,000, between 1,000 and 10,000, between 10,000 and 100,000, between 100,000 and 1,000,000 and greater than 1,000,000 different types of label-tags.
69. The kit of claim 66 wherein the label-tags in the pool of label-tags comprise a variable region that is the same length in all label-tags in the pool and the length is between 6 and 14 bases.
70. The kit of claim 69 wherein the label-tags in the pool of label-tags further comprise a recognition site for a restriction enzyme, a common priming site or an RNA polymerase promoter sequence.
71. The kit of claim 69 wherein the label-tags in the pool of label-tags are double stranded adaptors comprising a common priming sequence that is present in all label-tags in a pool of lable-tags.
72. The kit of claim 66 further comprising an oligo dT primer, one or more sequencing primers, and a reverse transcriptase.

FIG. 1

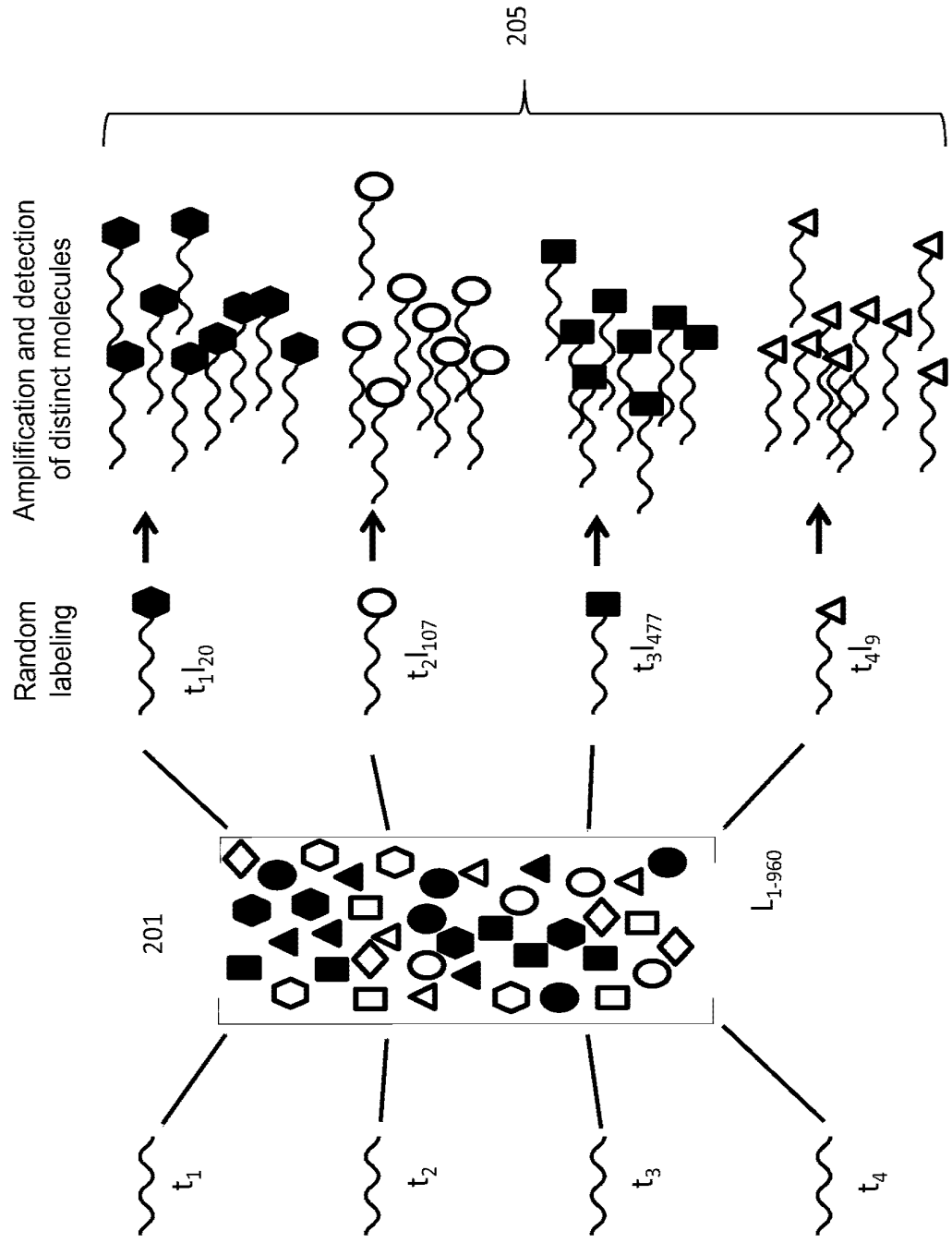

205
Amplification and detection of distinct molecules
Random labeling
t_1l_{20}
t_2l_{107}
t_3l_{477}
t_4l_9
201
$L_{1\text{-}960}$
t_1
t_2
t_3
t_4

FIG. 2A

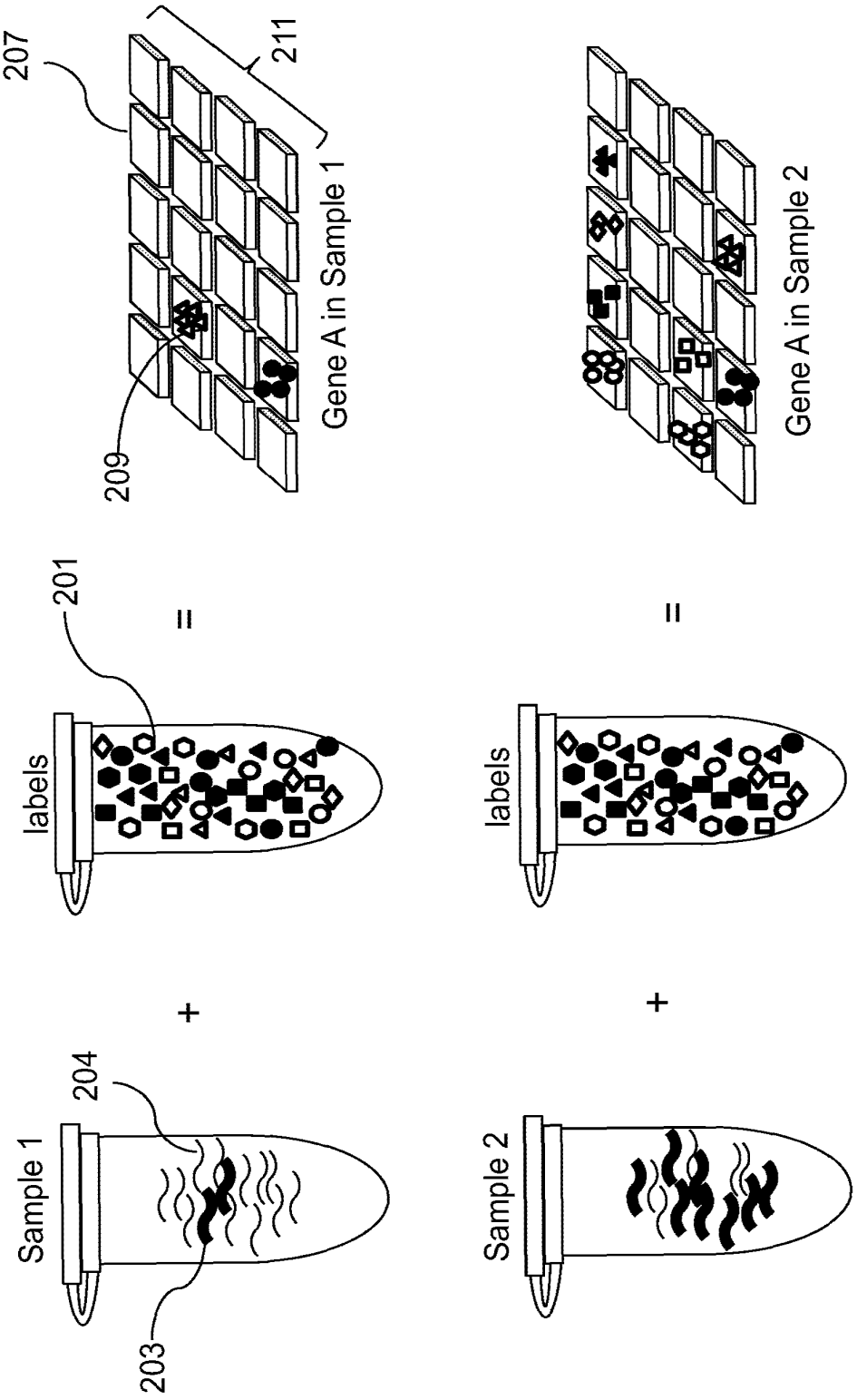

Sample 1
204
203
+
labels
201
=
207
209
211
Gene A in Sample 1
Sample 2
+
labels
=
Gene A in Sample 2

FIG. 2B

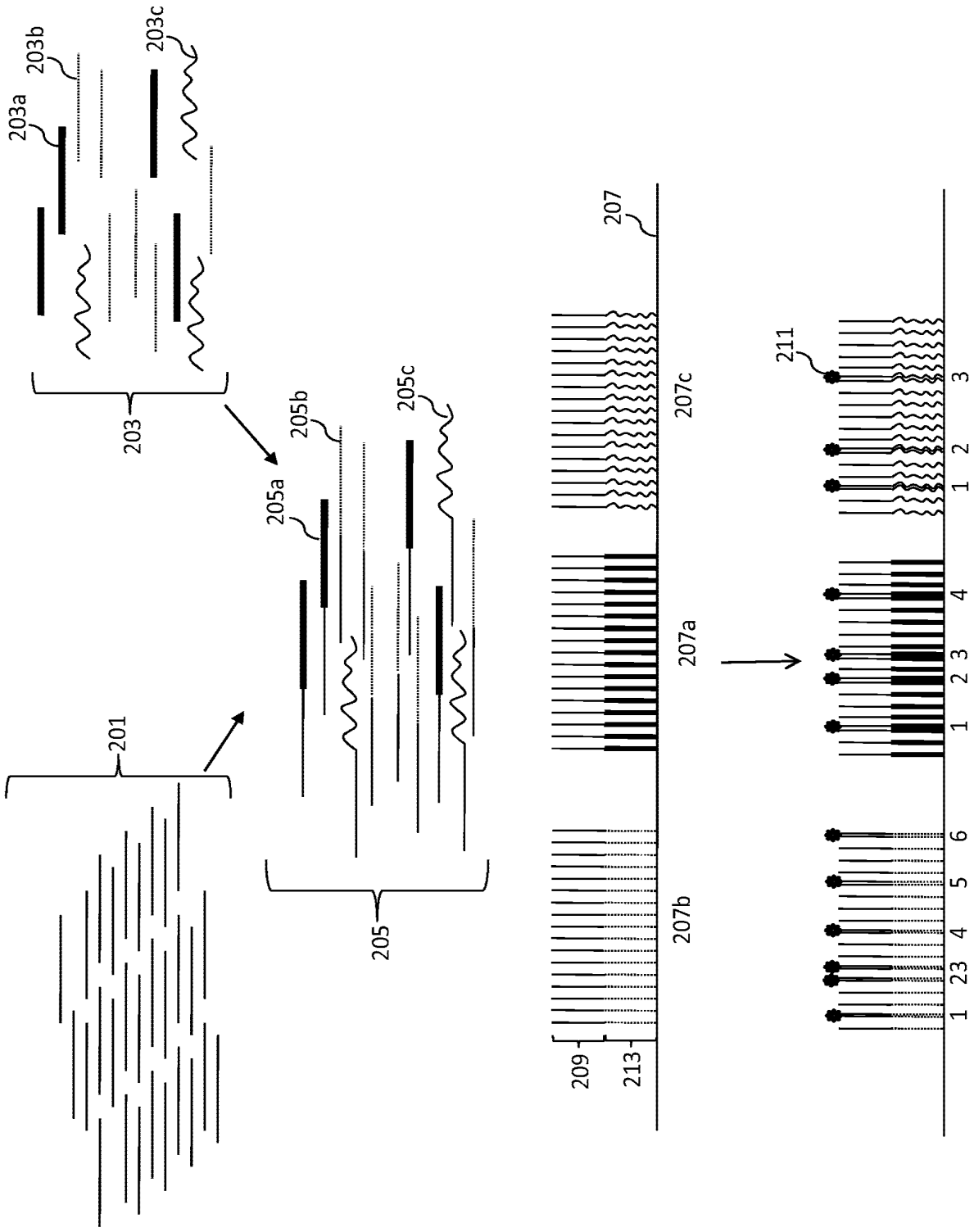

201
203
203a
203b
203c
205
205a
205b
205c
207
207a
207b
207c
209
211
213
1 23 4 5 6
1 2 3 4
1 2 3

FIG. 2C

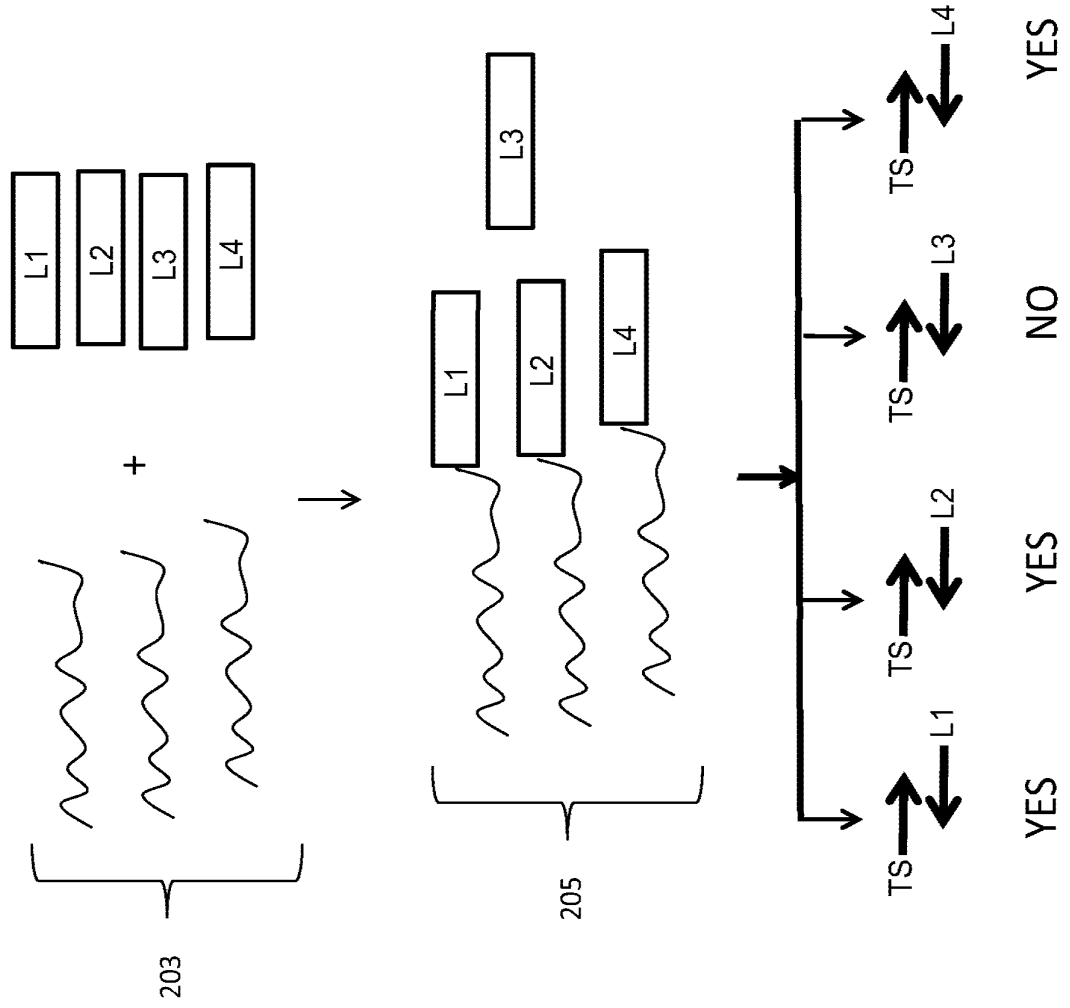


FIG.3

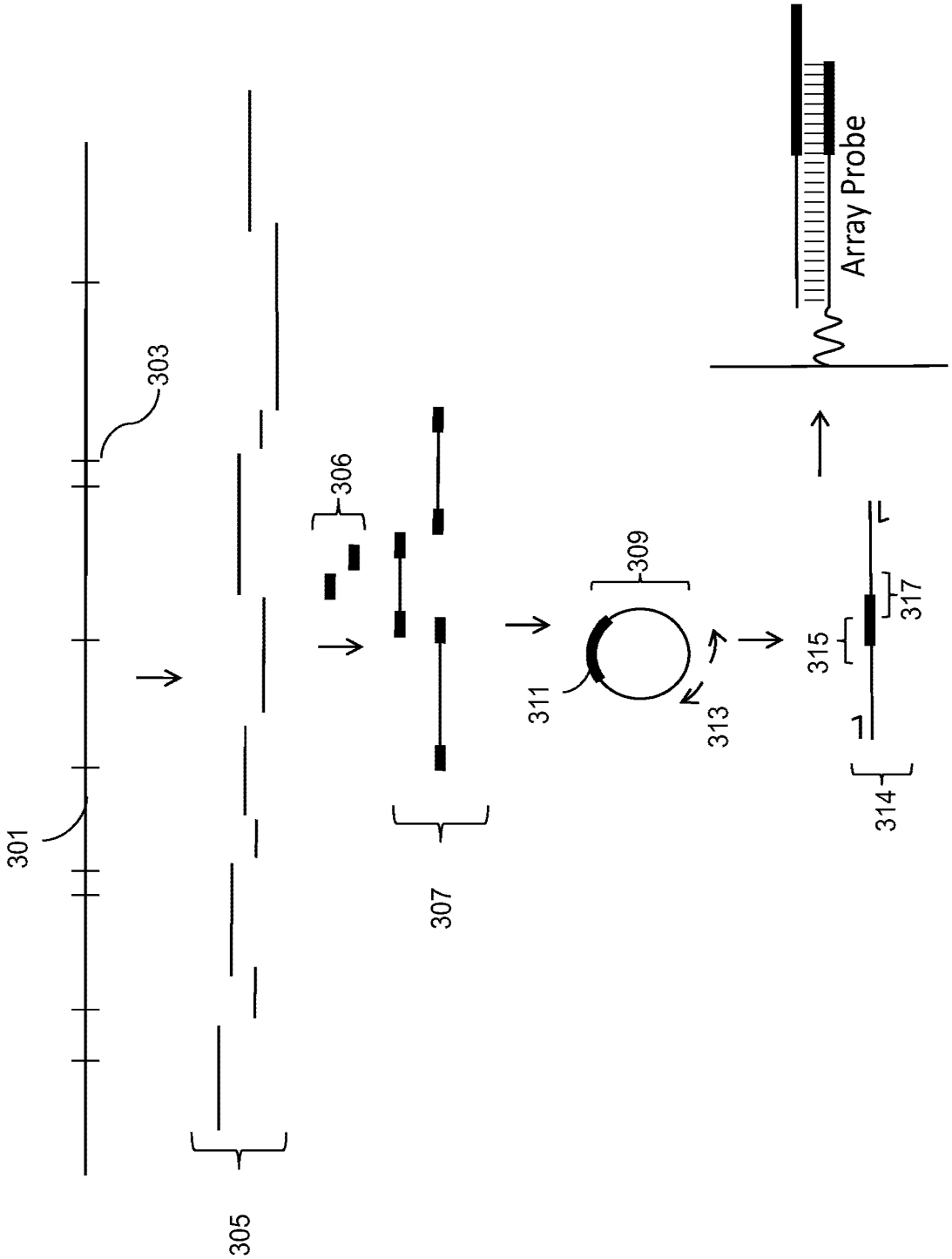


FIG. 4

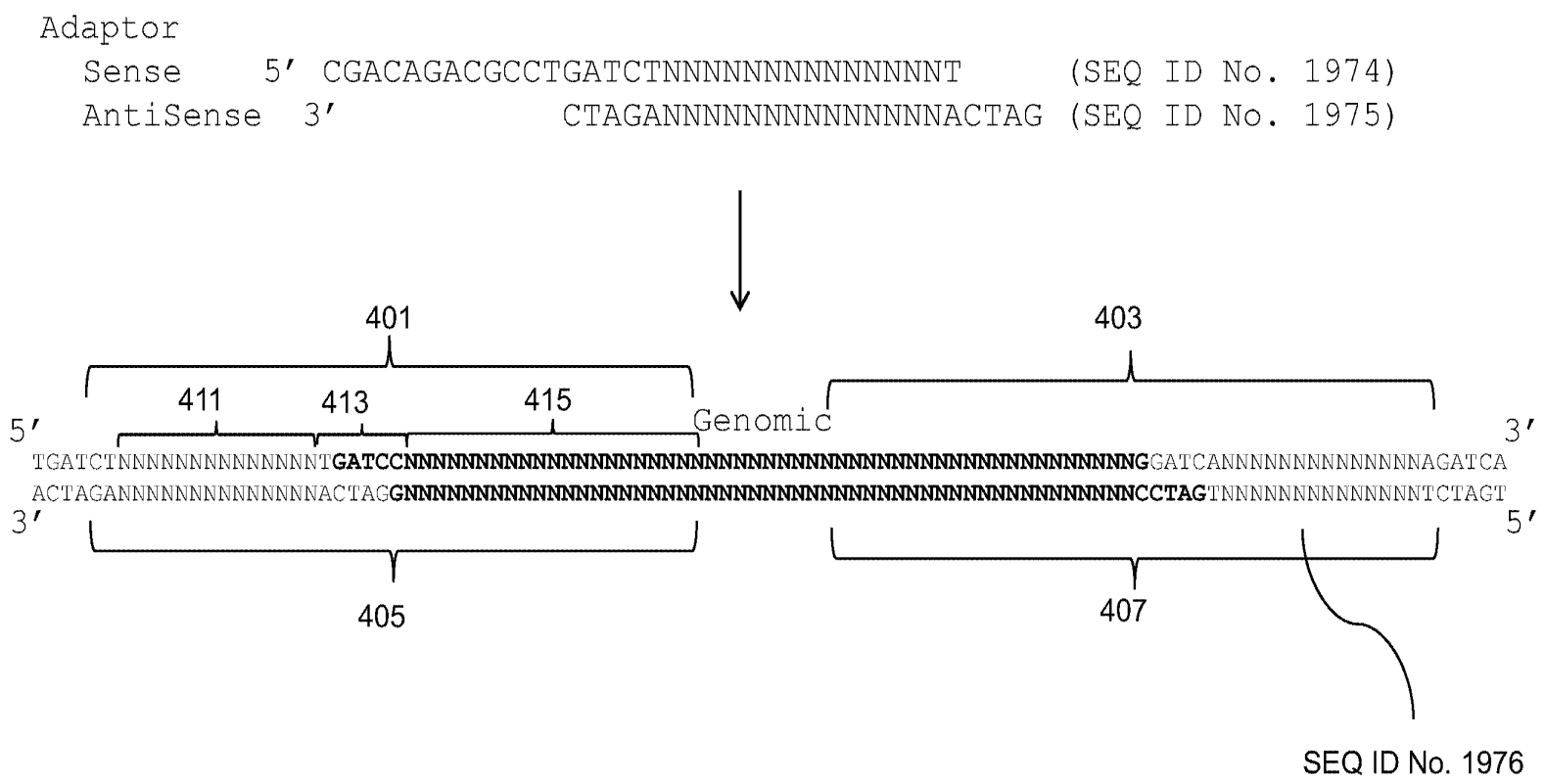

Adaptor
Sense 5′ CGACAGACGCCTGATCTNNNNNNNNNNNNNNT (SEQ ID No. 1974)
AntiSense 3′ CTAGANNNNNNNNNNNNNNACTAG (SEQ ID No. 1975)
401
403
411
413
415
5′
Genomic
3′
TGATCTNNNNNNNNNNNNNNTGATCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGATCANNNNNNNNNNNNNNAGATCA
ACTAGANNNNNNNNNNNNNNACTAGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCTAGTNNNNNNNNNNNNNNTCTAGT
3′
5′
405
407
SEQ ID No. 1976

FIG. 5

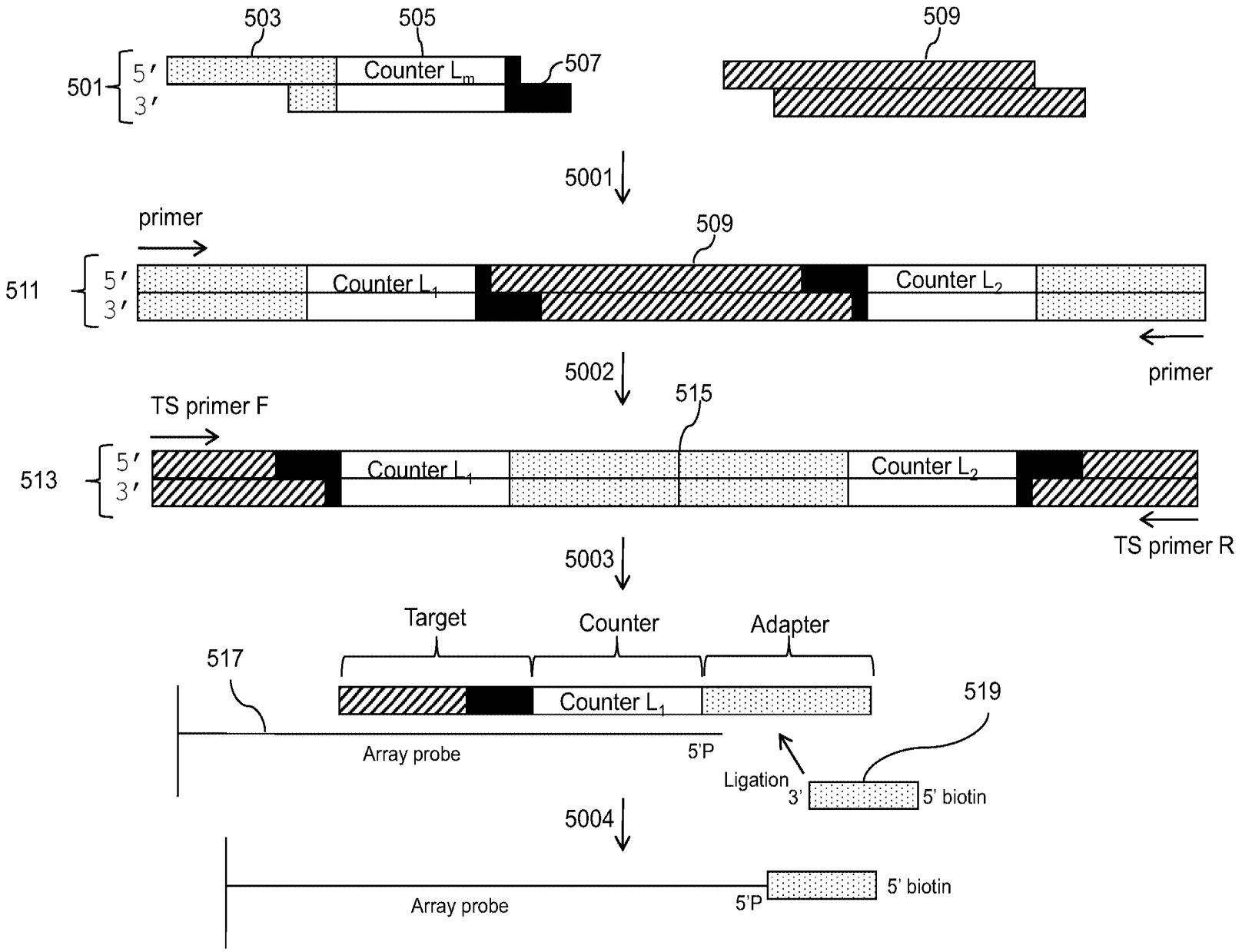

503
505
509
501
5′
3′
Counter L_m
507
5001
primer
509
511
5′
3′
Counter L_1
Counter L_2
primer
5002
515
TS primer F
513
5′
3′
Counter L_1
Counter L_2
TS primer R
5003
Target
Counter
Adapter
517
Counter L_1
519
Array probe
5'P
Ligation
3'
5' biotin
5004
Array probe
5'P
5' biotin

FIG. 6

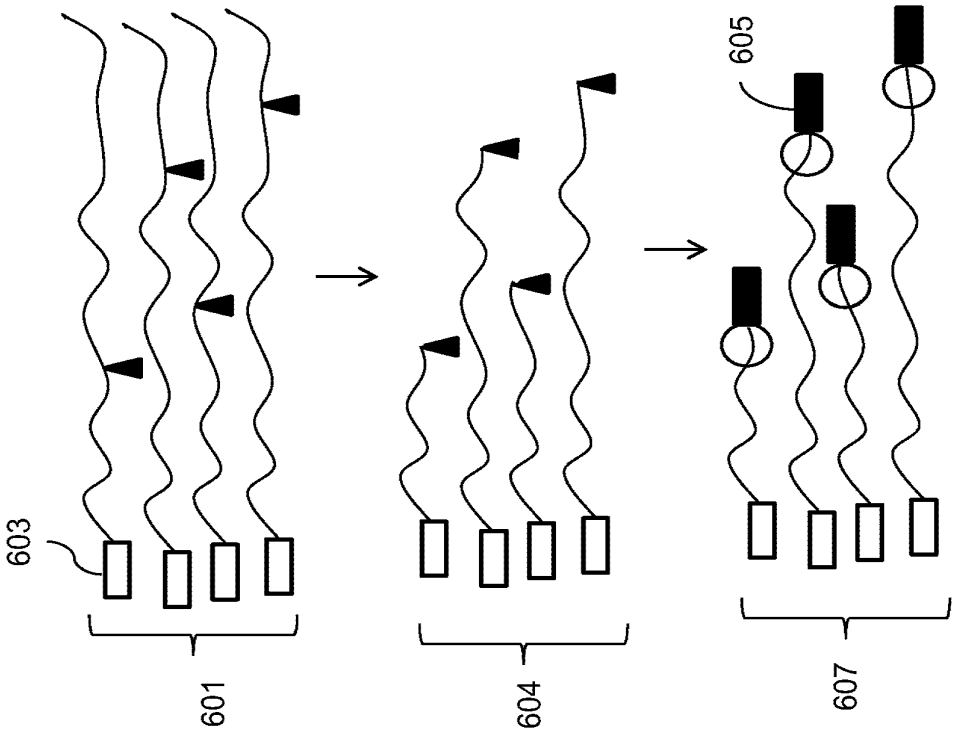


FIG. 7

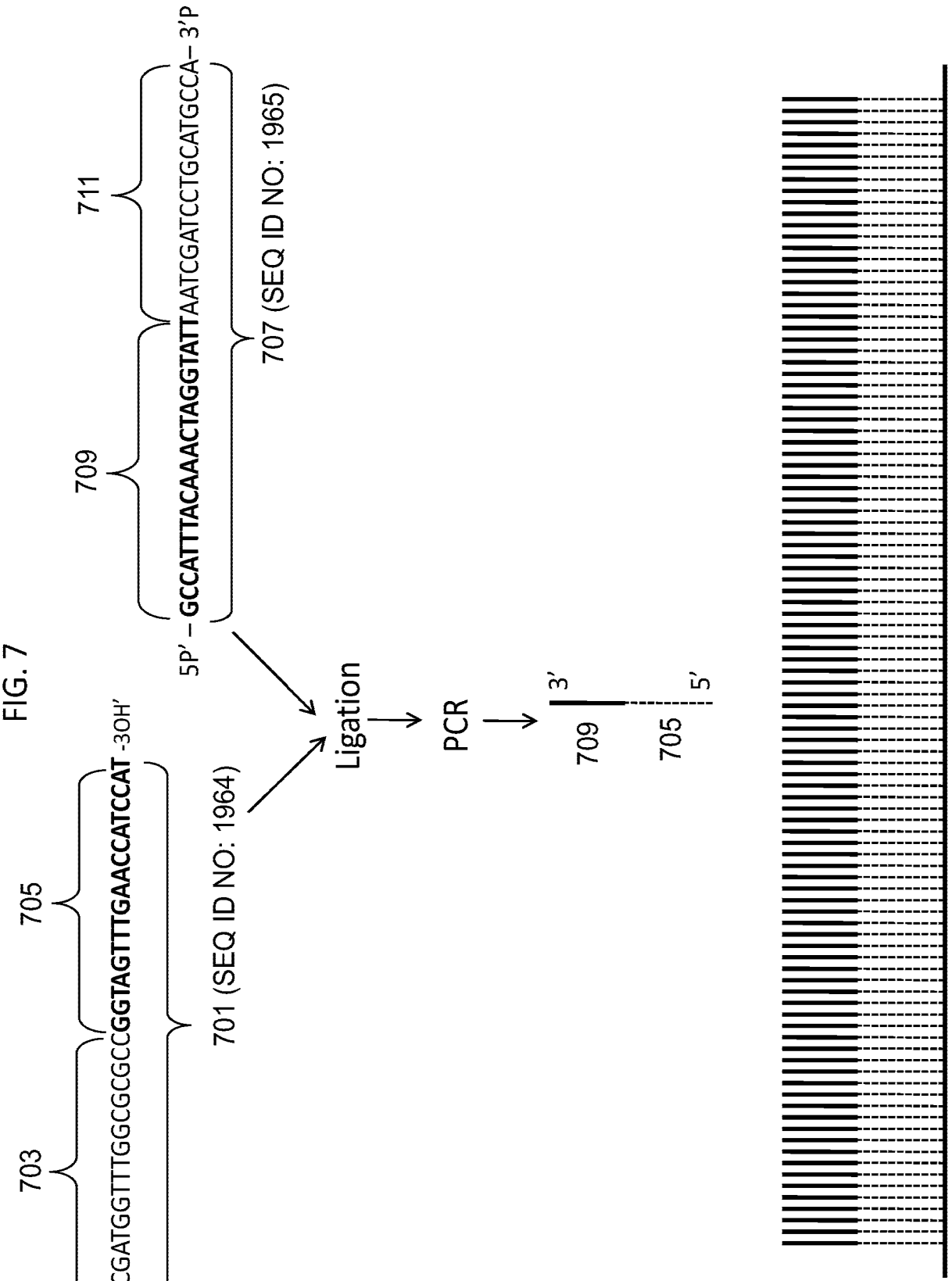

703
705
5'OH- TCGATGGTTTGGCGCGCCGGTAGTTTGAACCATCCAT -3OH'
701 (SEQ ID NO: 1964)
709
711
5P' – GCCATTTACAAACTAGGTATTAATCGATCCTGCATGCCA– 3'P
707 (SEQ ID NO: 1965)
Ligation
PCR
3'
5'
709
705

FIG. 8

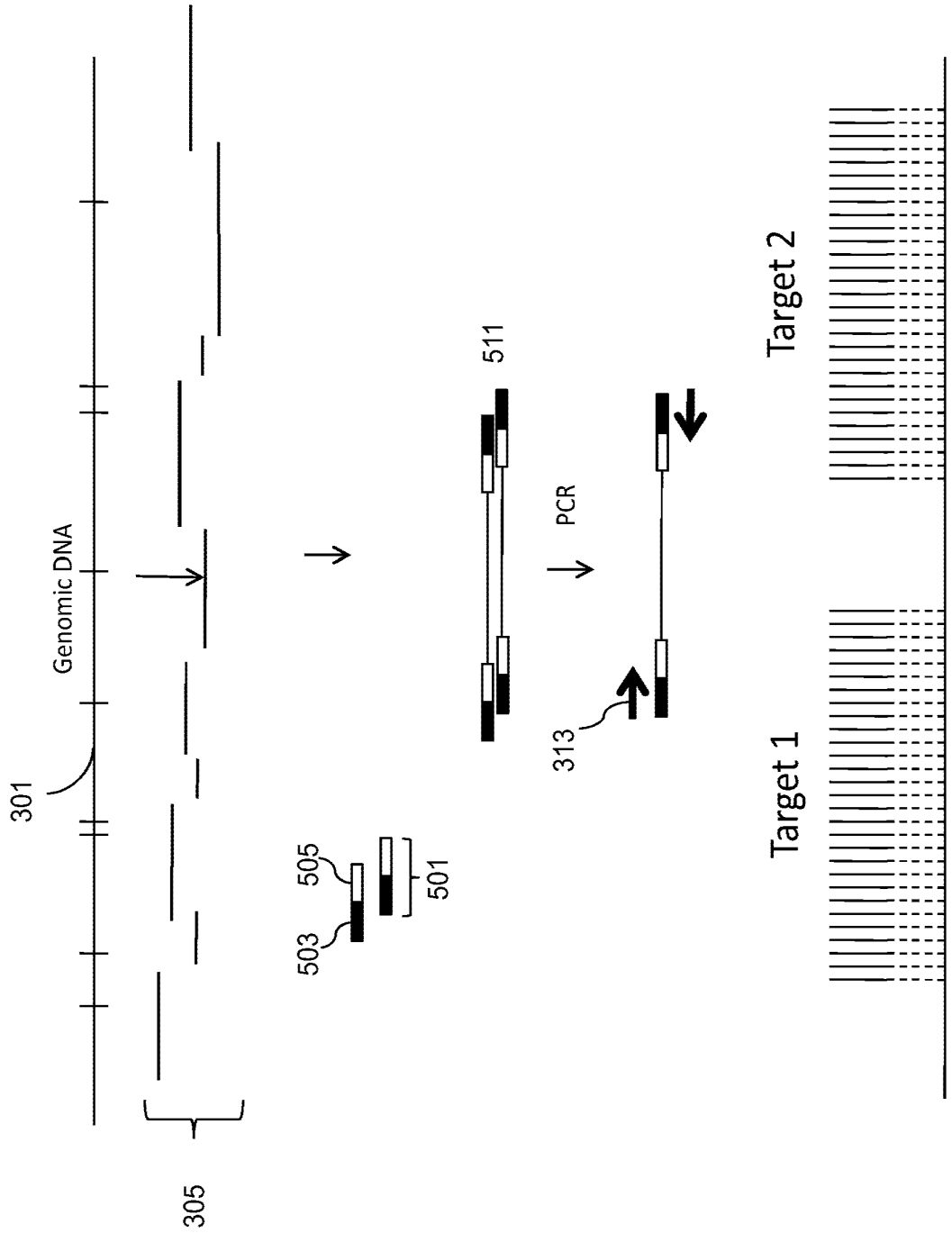


FIG. 9

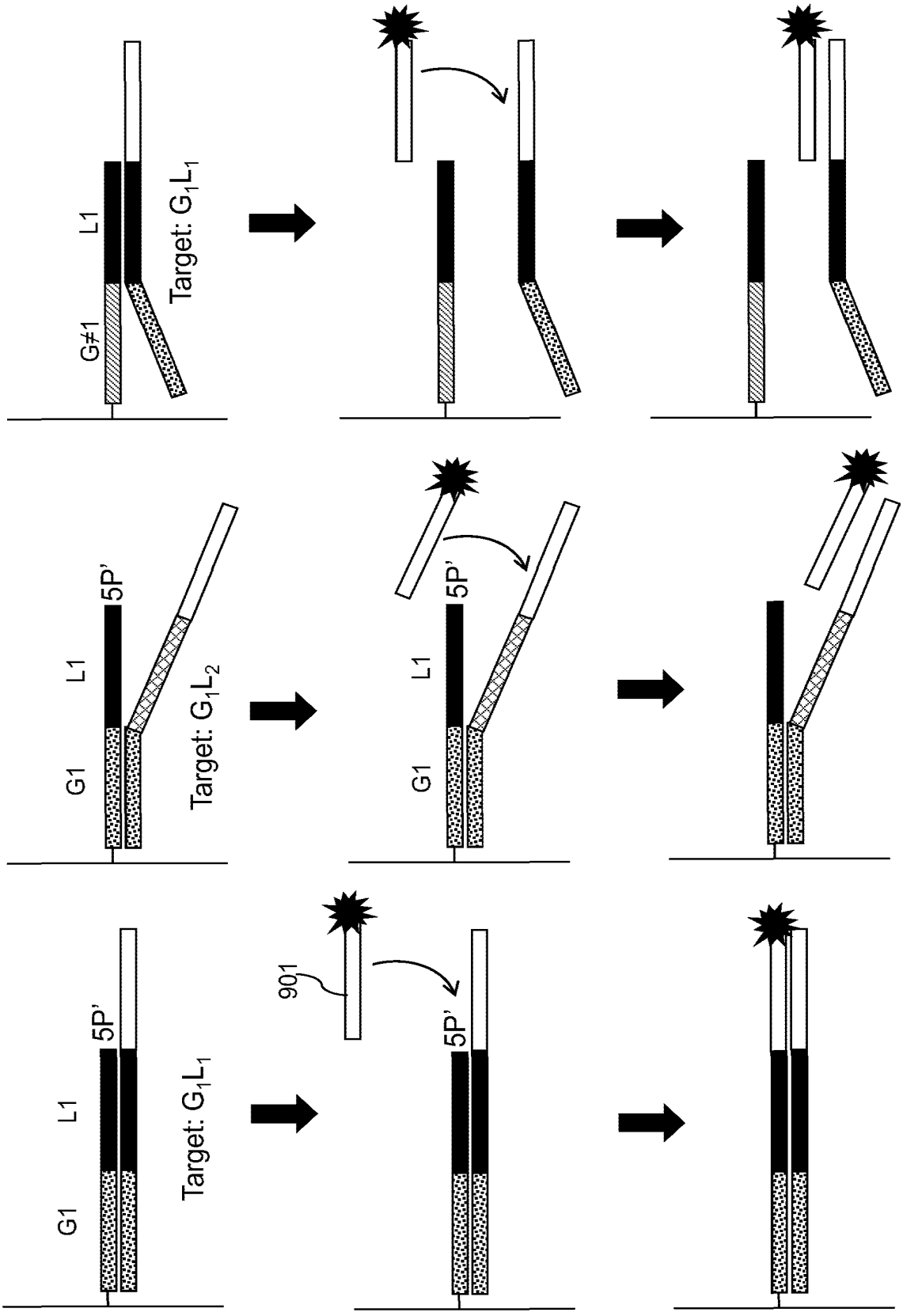

L1
G≠1
Target: G_1L_1
5P'
L1
G1
Target: G_1L_2
5P'
L1
G1
5P'
L1
G1
Target: G_1L_1
901
5P'

FIG. 10

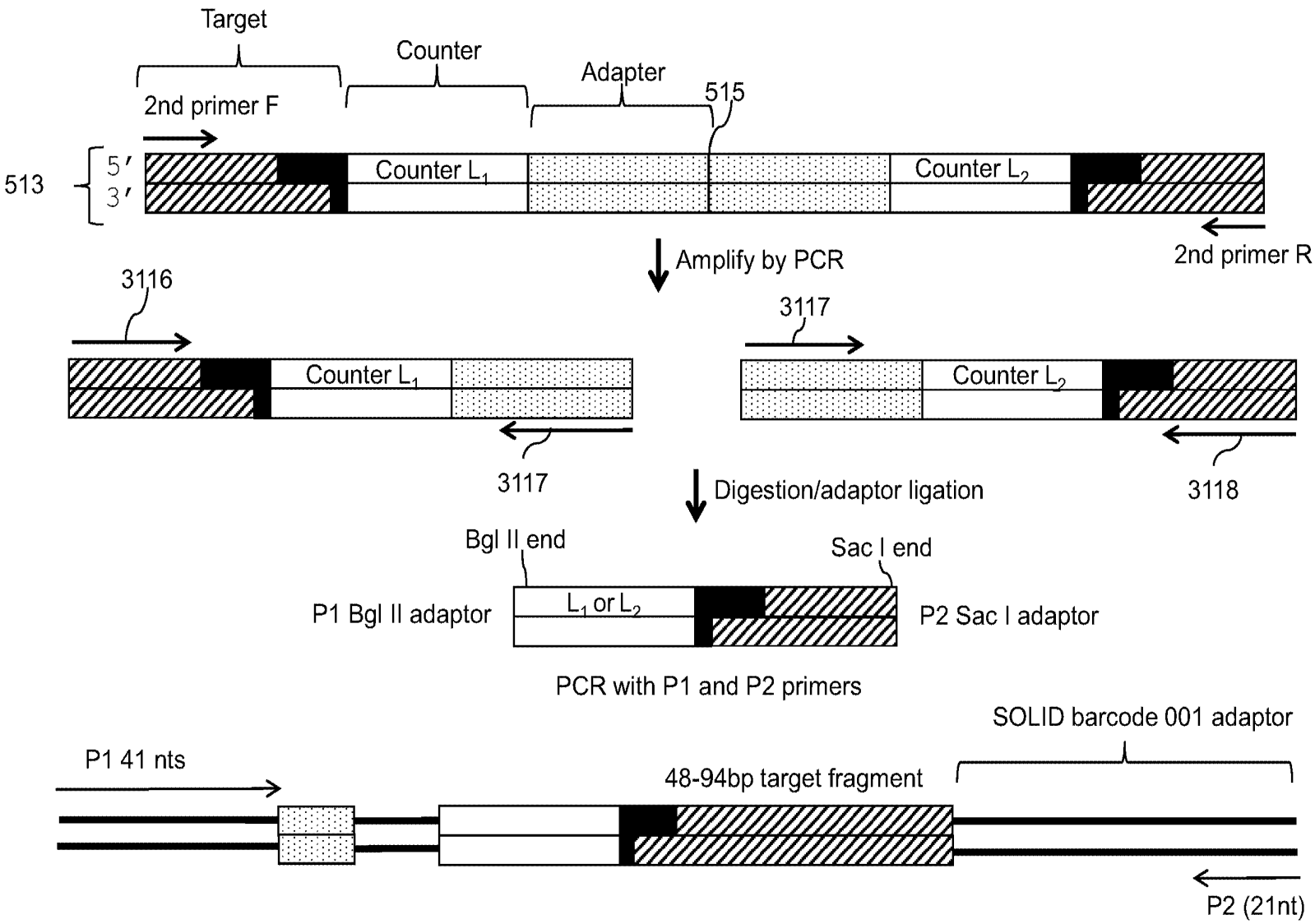

Target
Counter
Adapter
515
2nd primer F
513
5′
3′
Counter L1
Counter L2
2nd primer R
Amplify by PCR
3116
3117
Counter L1
Counter L2
3117
3118
Digestion/adaptor ligation
Bgl II end
Sac I end
P1 Bgl II adaptor
L1 or L2
P2 Sac I adaptor
PCR with P1 and P2 primers
SOLID barcode 001 adaptor
P1 41 nts
48-94bp target fragment
P2 (21nt)

FIG. 11

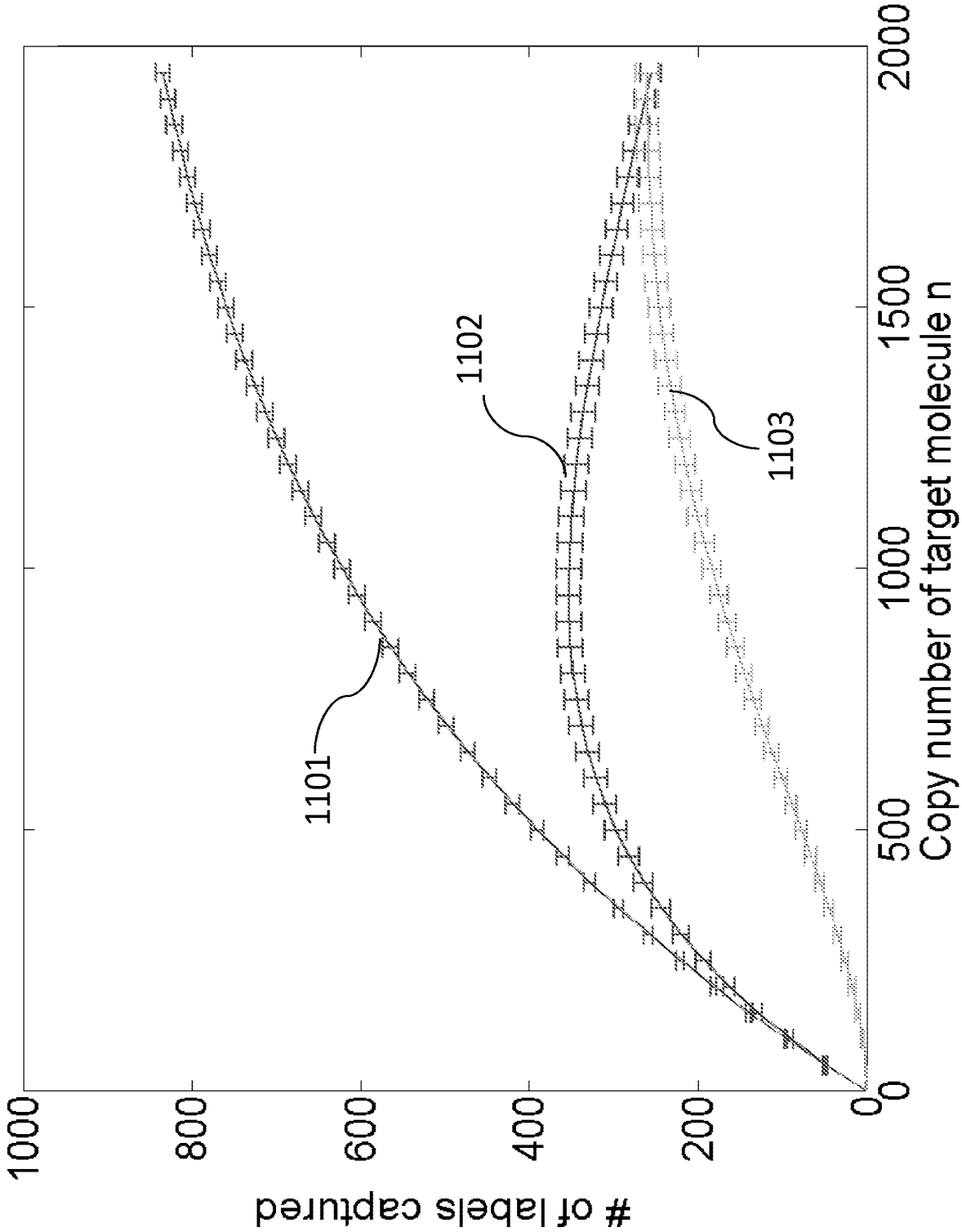

1000
800
600
400
200
0
# of labels captured
0
500
1000
1500
2000
Copy number of target molecule n
1101
1102
1103

FIG. 12

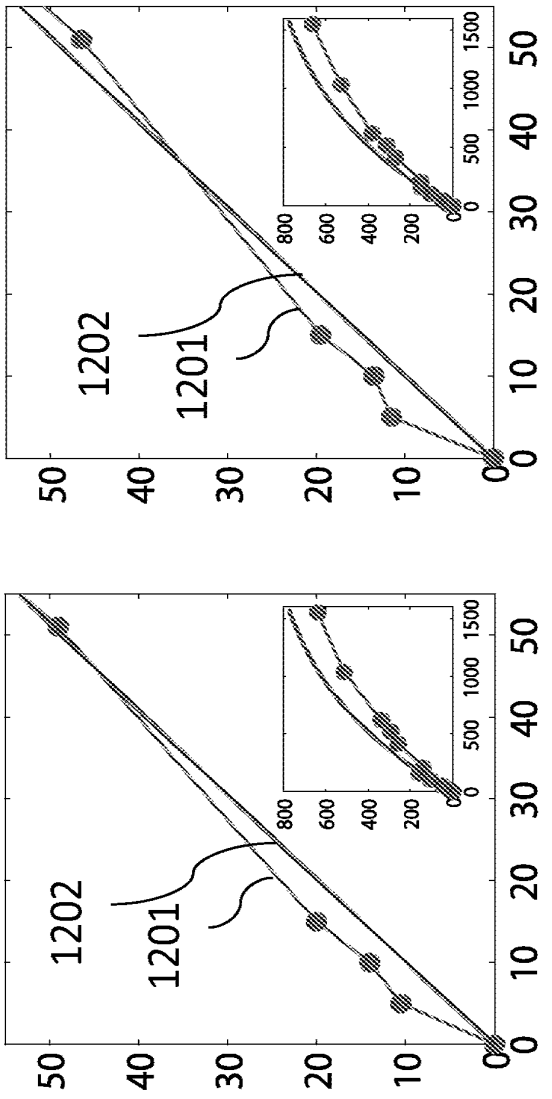


FIG. 13

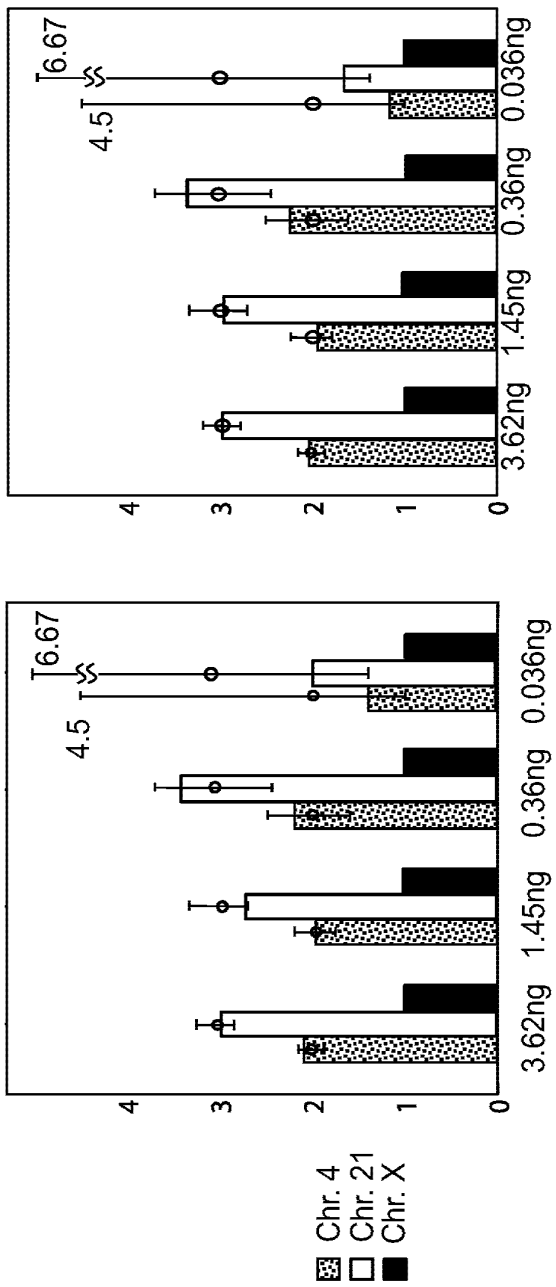


FIG. 14

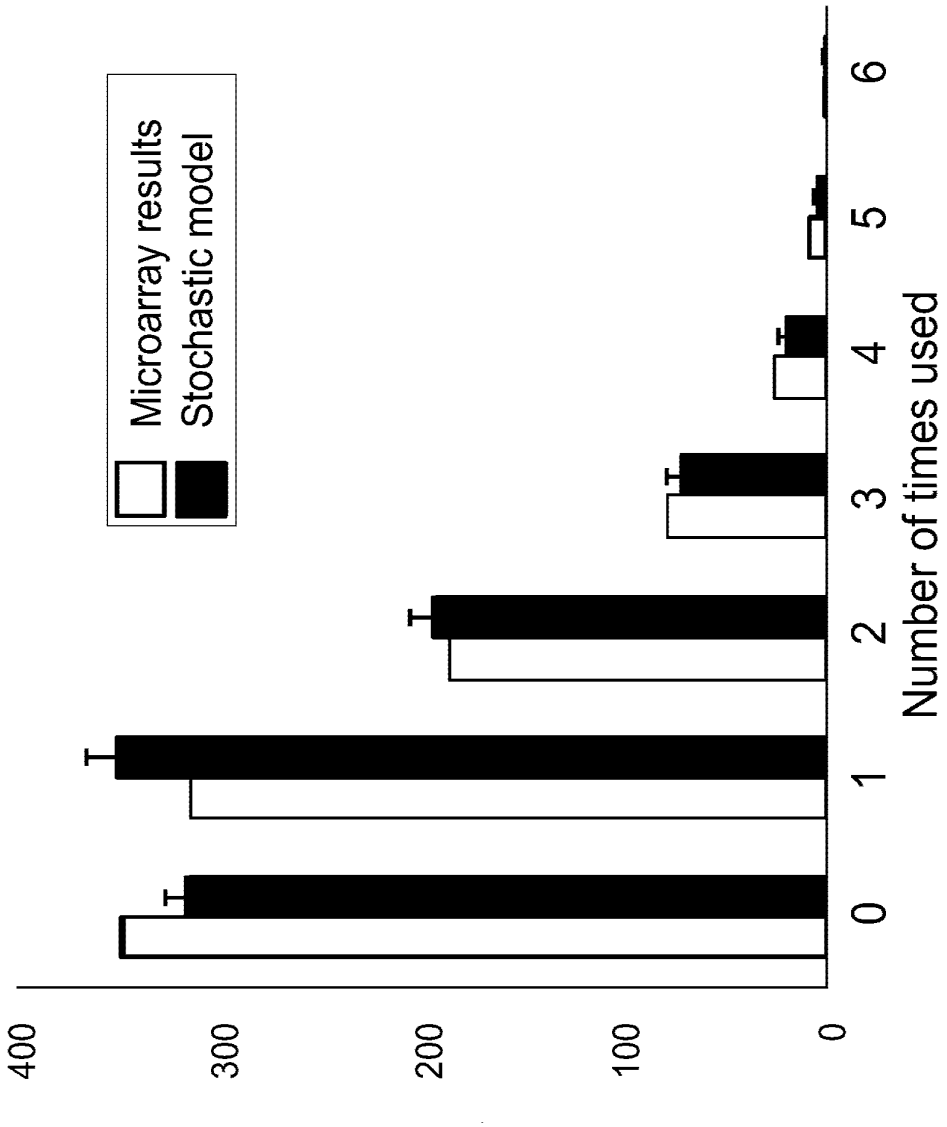

Microarray results
Stochastic model
400
300
200
100
0
0
1
2
3
4
5
6
Number of times used

FIG. 15

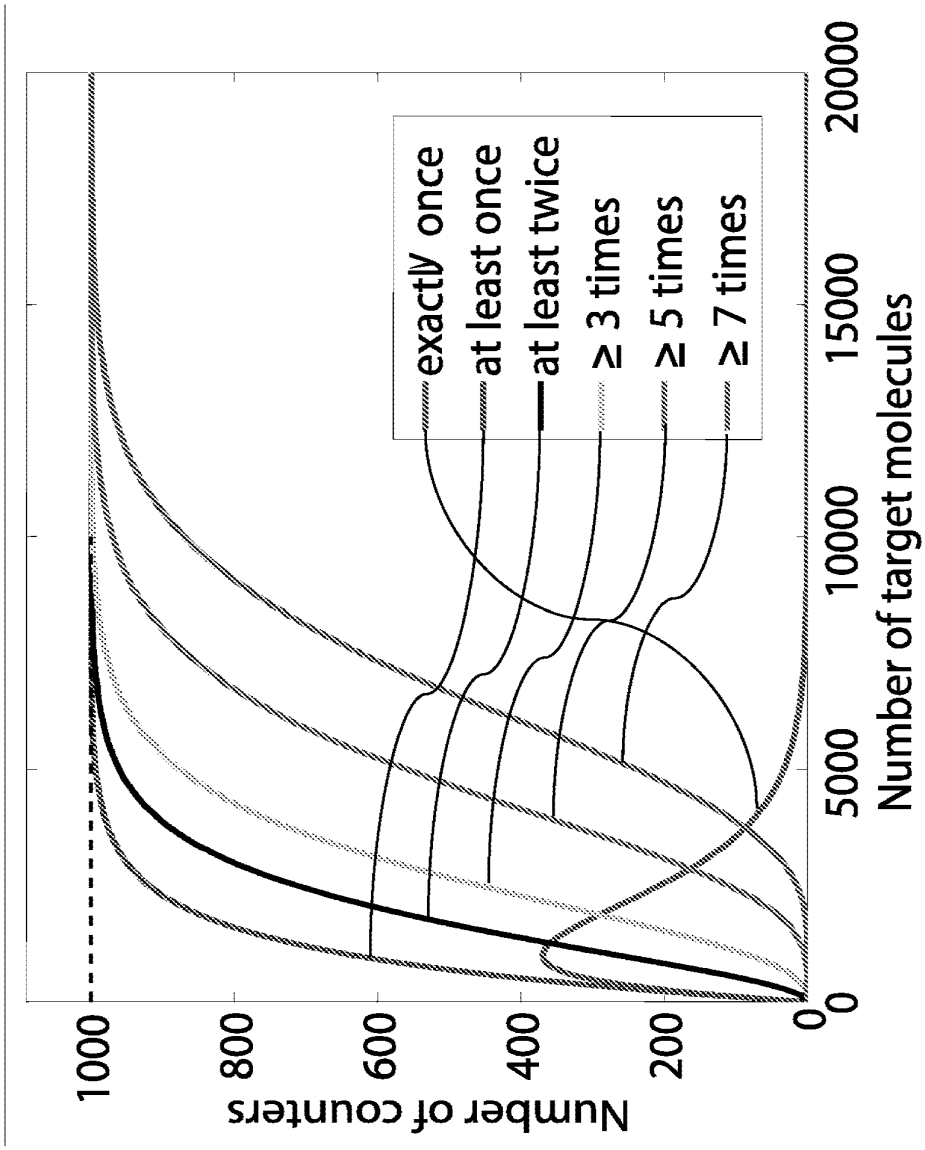

Number of counters
1000
800
600
400
200
0
0
5000
10000
15000
20000
Number of target molecules
exactly once
at least once
at least twice
≥ 3 times
≥ 5 times
≥ 7 times

FIG. 16

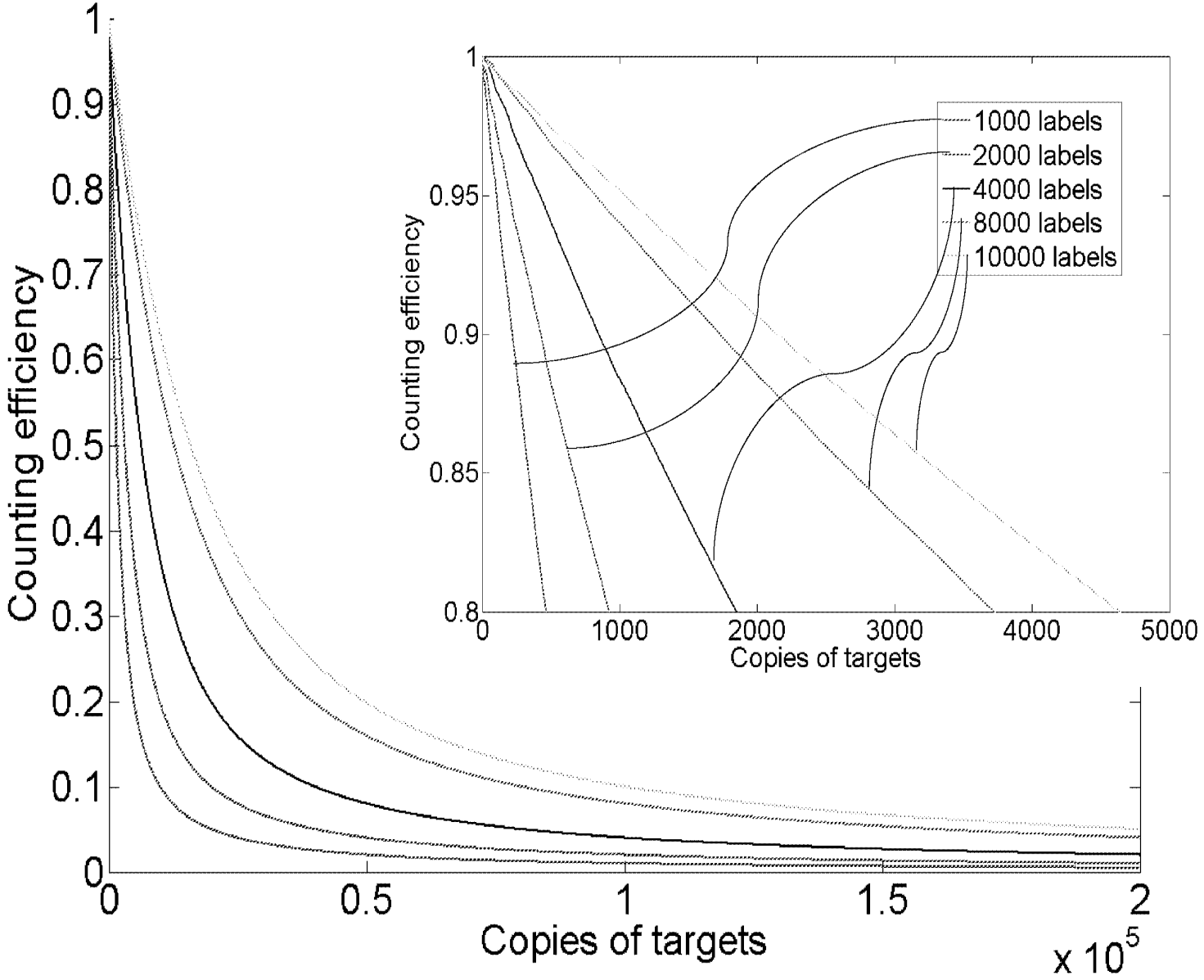

Counting efficiency
Copies of targets
x 10^5
0
0.5
1
1.5
2
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Counting efficiency
Copies of targets
0
1000
2000
3000
4000
5000
0.8
0.85
0.9
0.95
1
1000 labels
2000 labels
4000 labels
8000 labels
10000 labels

FIG. 17

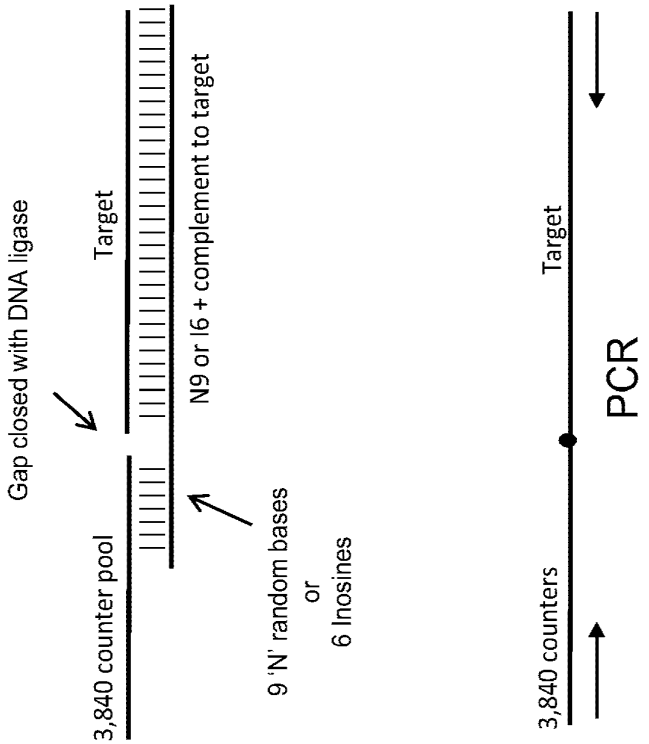

Gap closed with DNA ligase
Target
N9 or I6 + complement to target
3,840 counter pool
9 'N' random bases
or
6 Inosines
Target
PCR
3,840 counters

FIG. 18

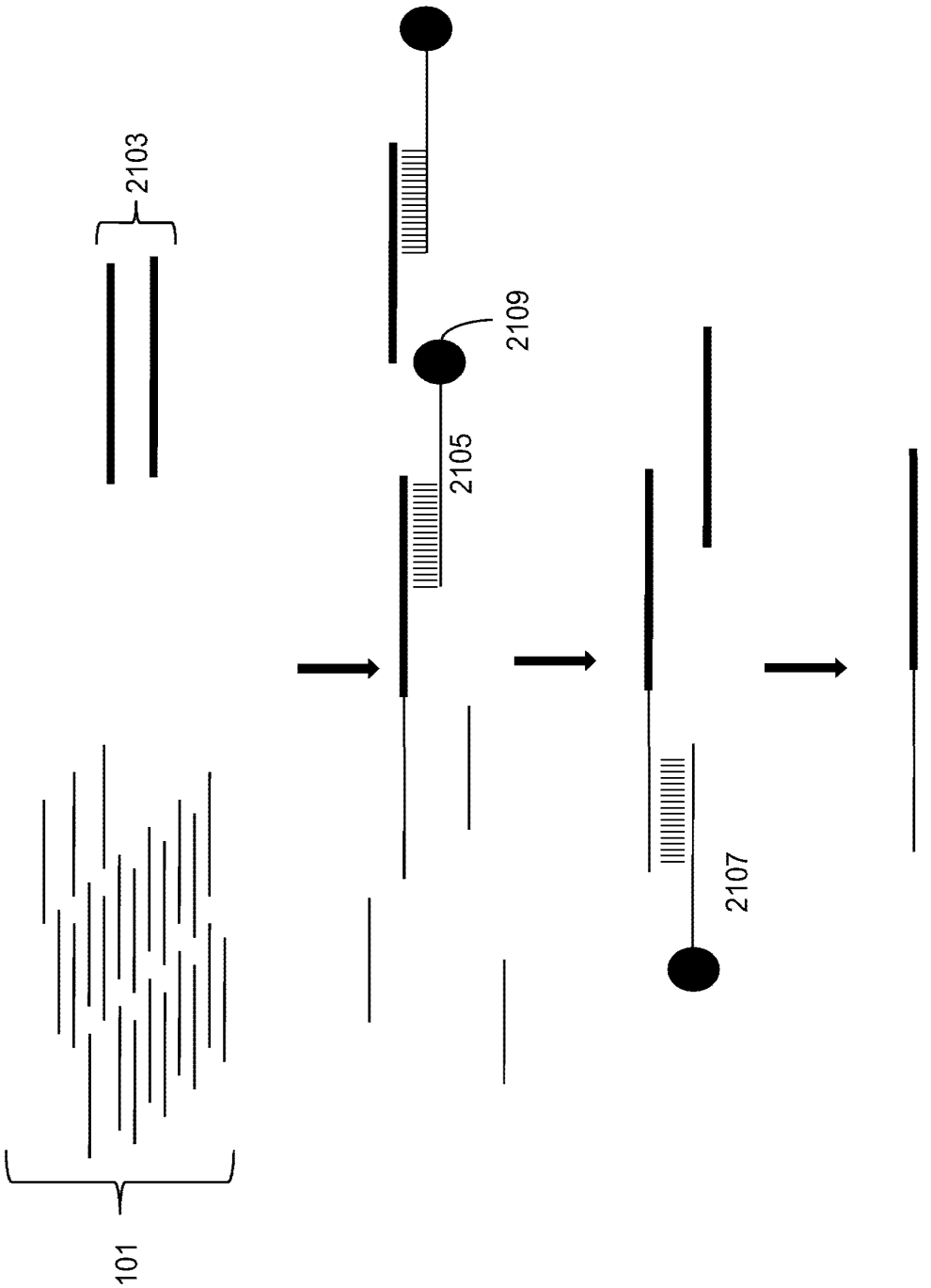


FIG. 19

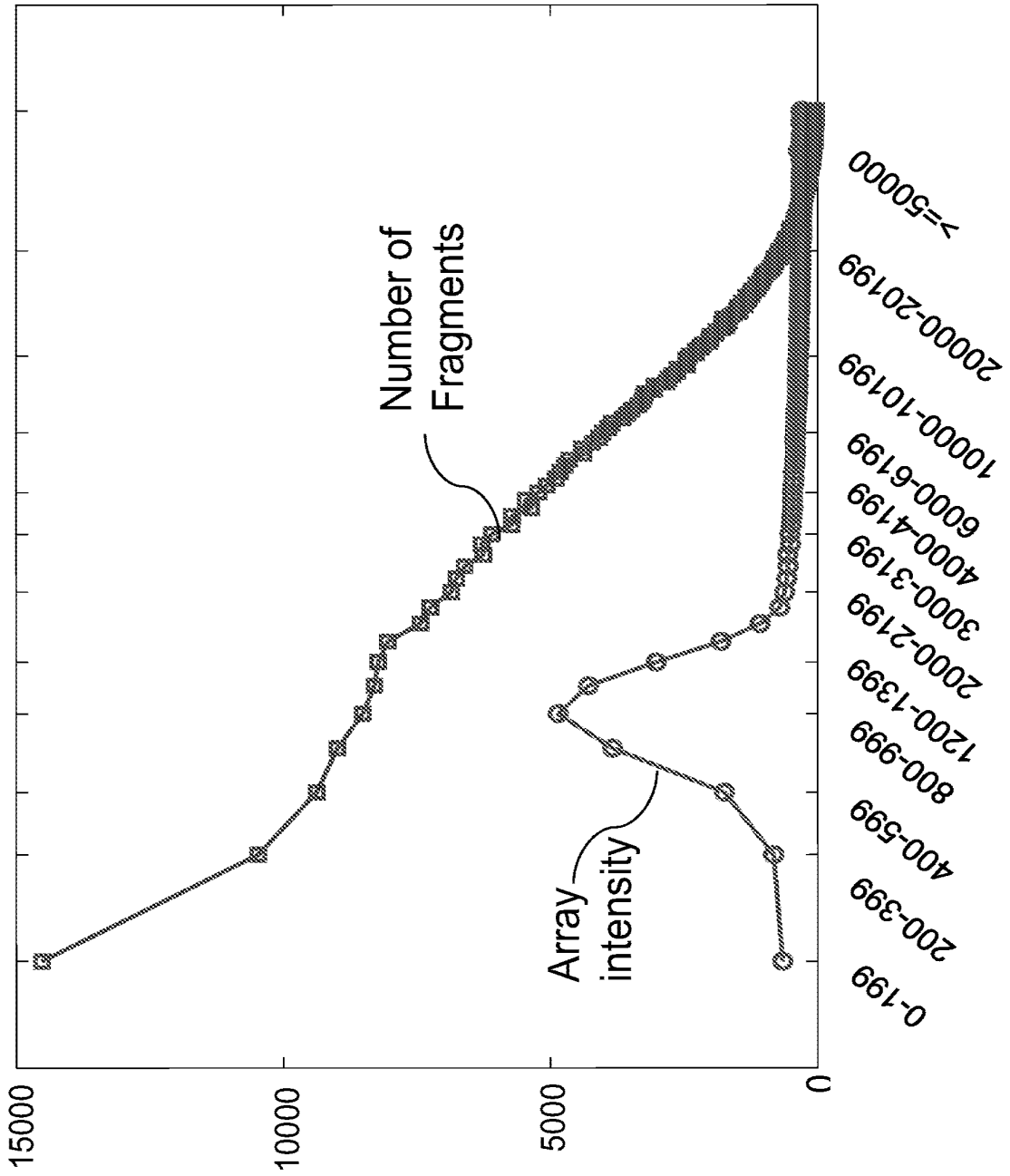

Number of Fragments
Array intensity
15000
10000
5000
0
0-199
200-399
400-599
800-999
1200-1399
2000-2199
3000-3199
4000-4199
6000-6199
10000-10199
20000-20199
>=50000

FIG. 20

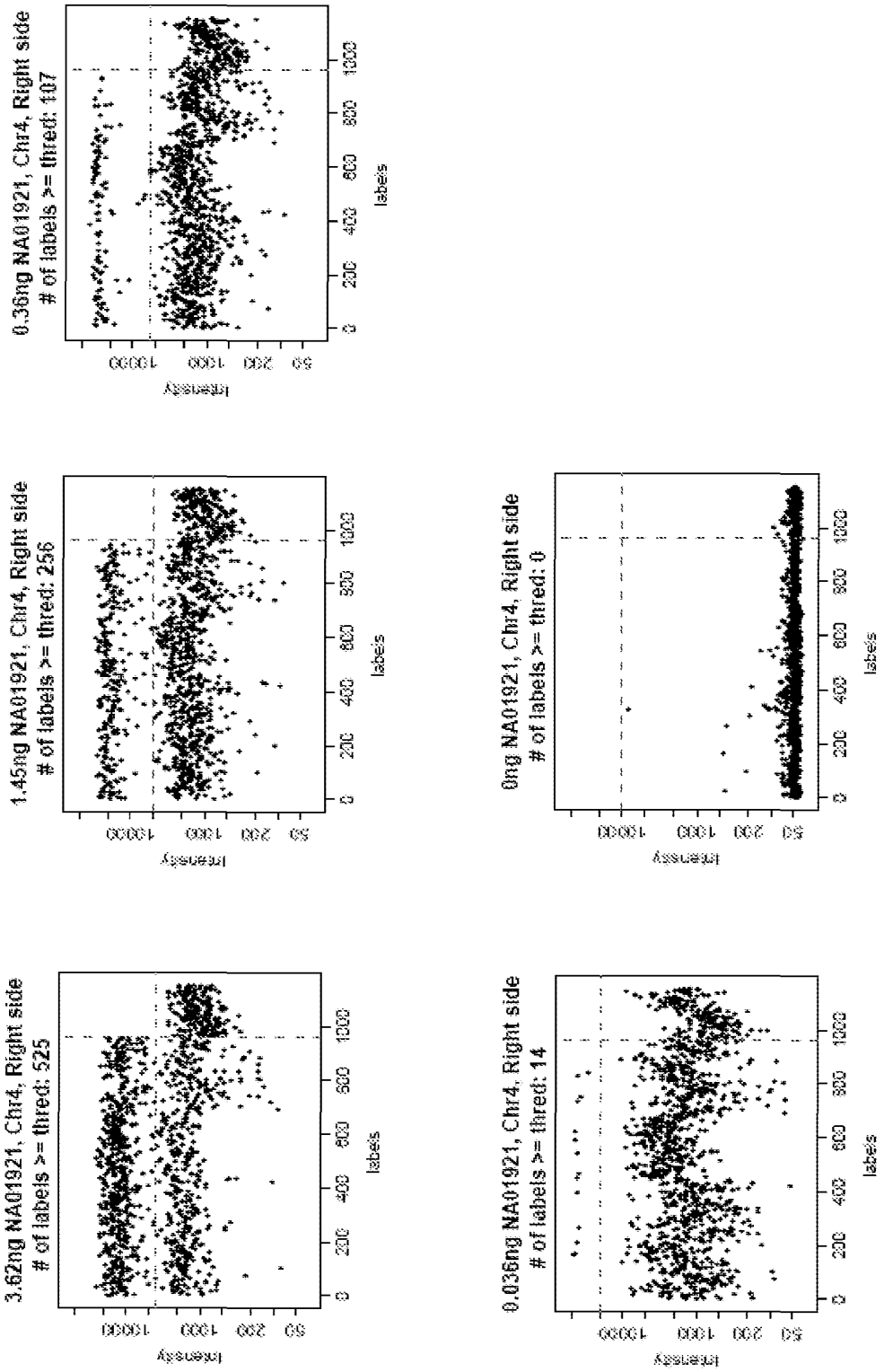

0.36ng NA01921, Chr4, Right side
# of labels >= thred: 107
1.45ng NA01921, Chr4, Right side
# of labels >= thred: 256
0ng NA01921, Chr4, Right side
# of labels >= thred: 0
3.62ng NA01921, Chr4, Right side
# of labels >= thred: 525
0.036ng NA01921, Chr4, Right side
# of labels >= thred: 14
Intensity
labels

FIG. 21

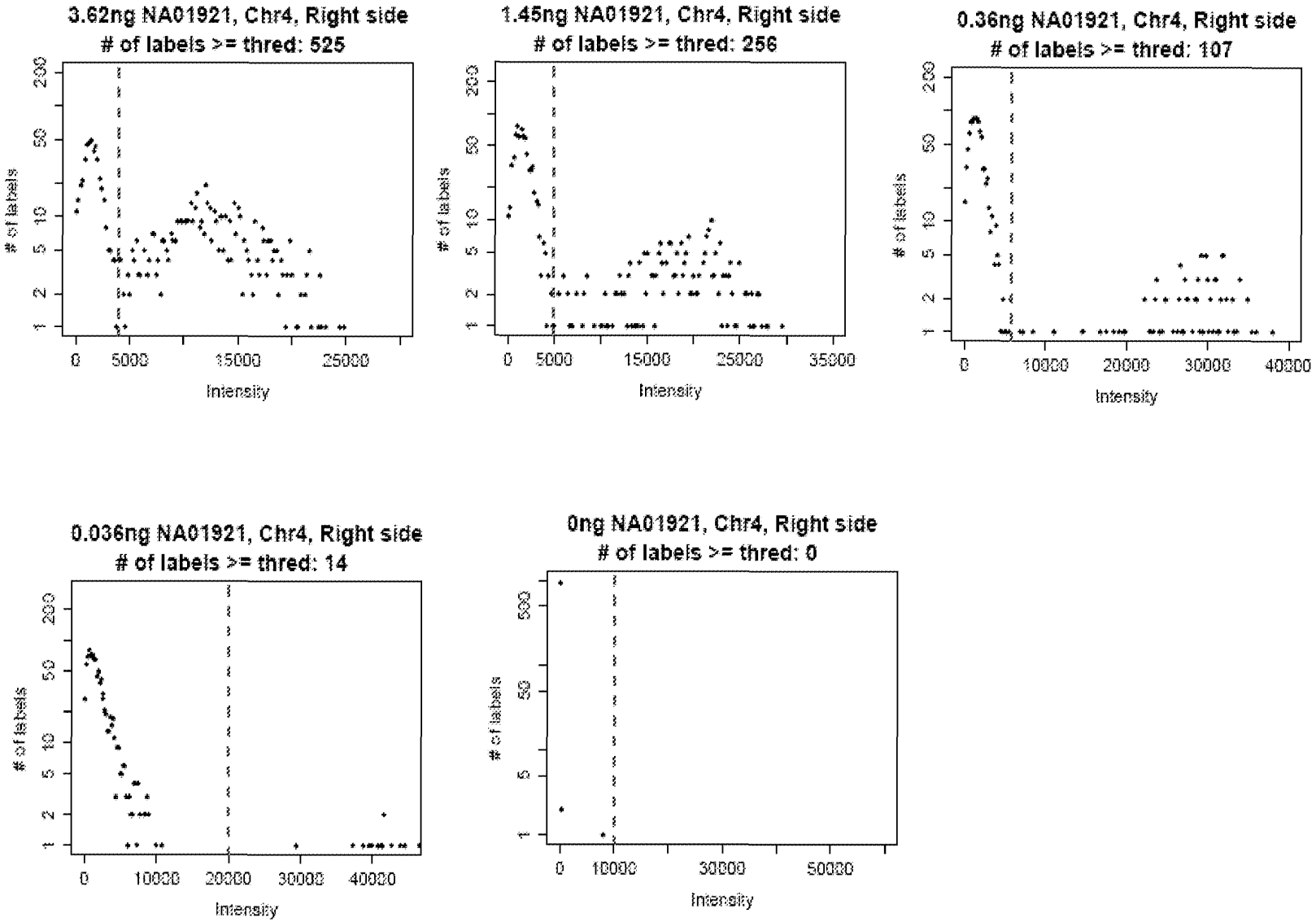

3.62ng NA01921, Chr4, Right side
# of labels >= thred: 525
1.45ng NA01921, Chr4, Right side
# of labels >= thred: 256
0.36ng NA01921, Chr4, Right side
# of labels >= thred: 107
0.036ng NA01921, Chr4, Right side
# of labels >= thred: 14
0ng NA01921, Chr4, Right side
# of labels >= thred: 0
# of labels
Intensity

FIG. 22

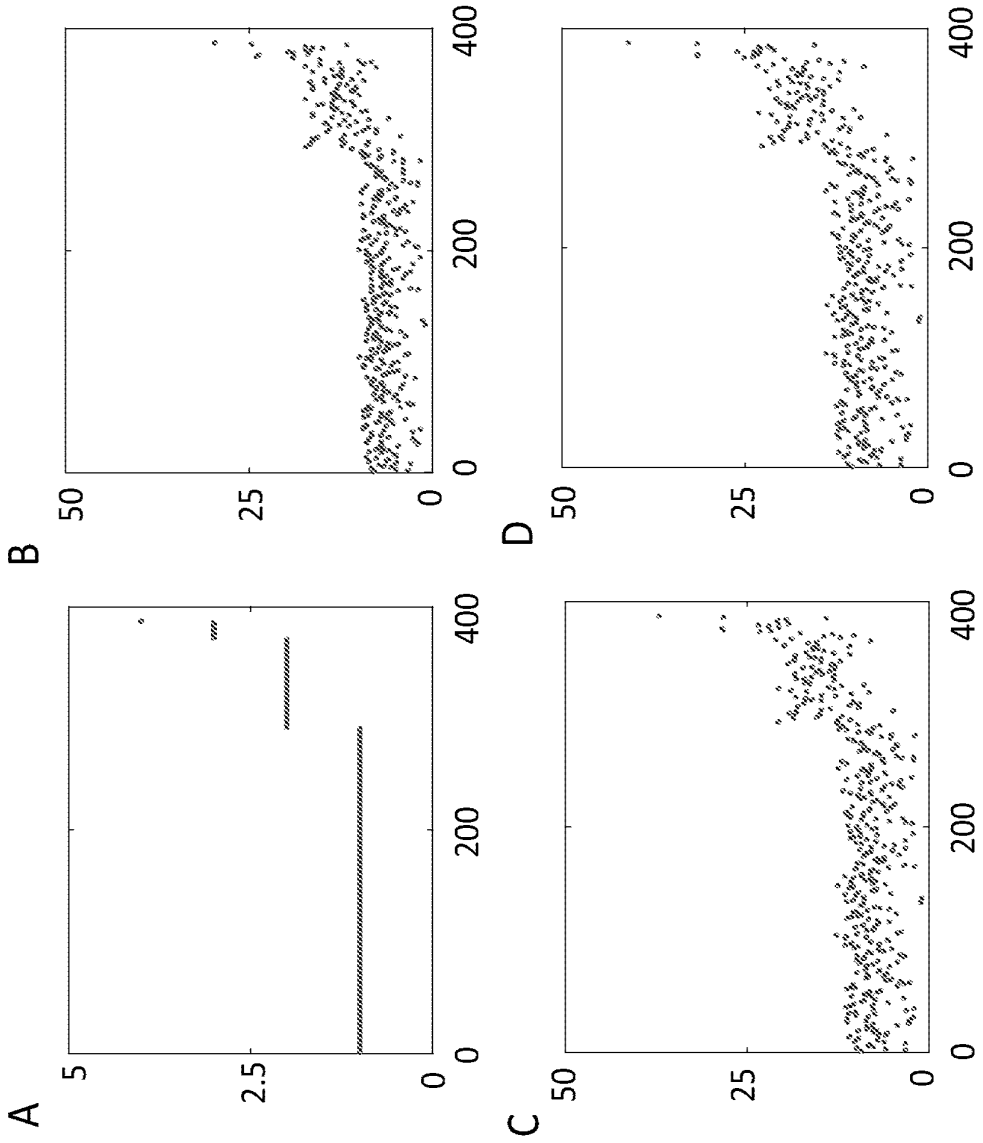


FIG. 23

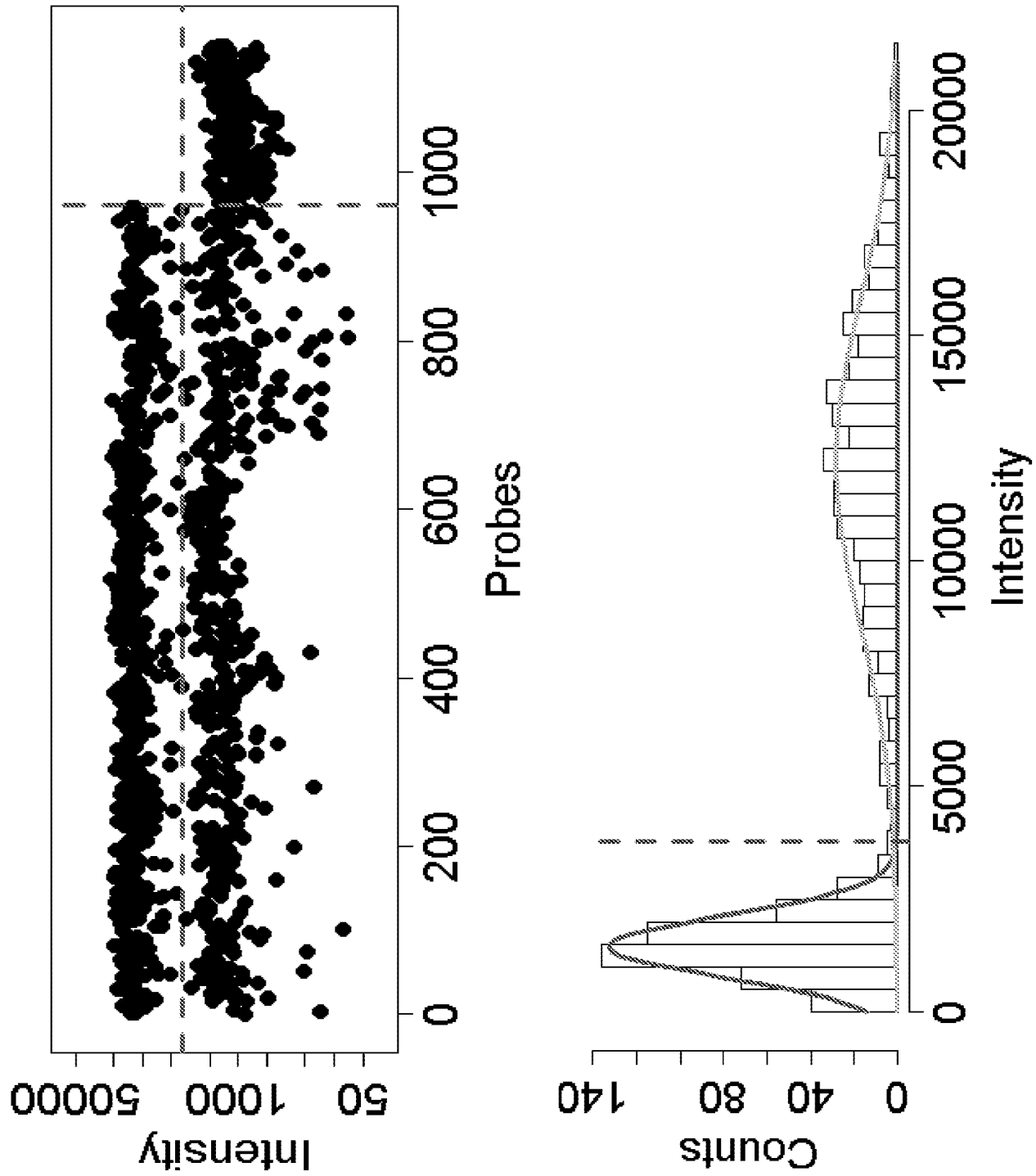

Intensity
50
1000
50000
Probes
0
200
400
600
800
1000
Counts
0
40
80
140
Intensity
0
5000
10000
15000
20000

FIG. 24

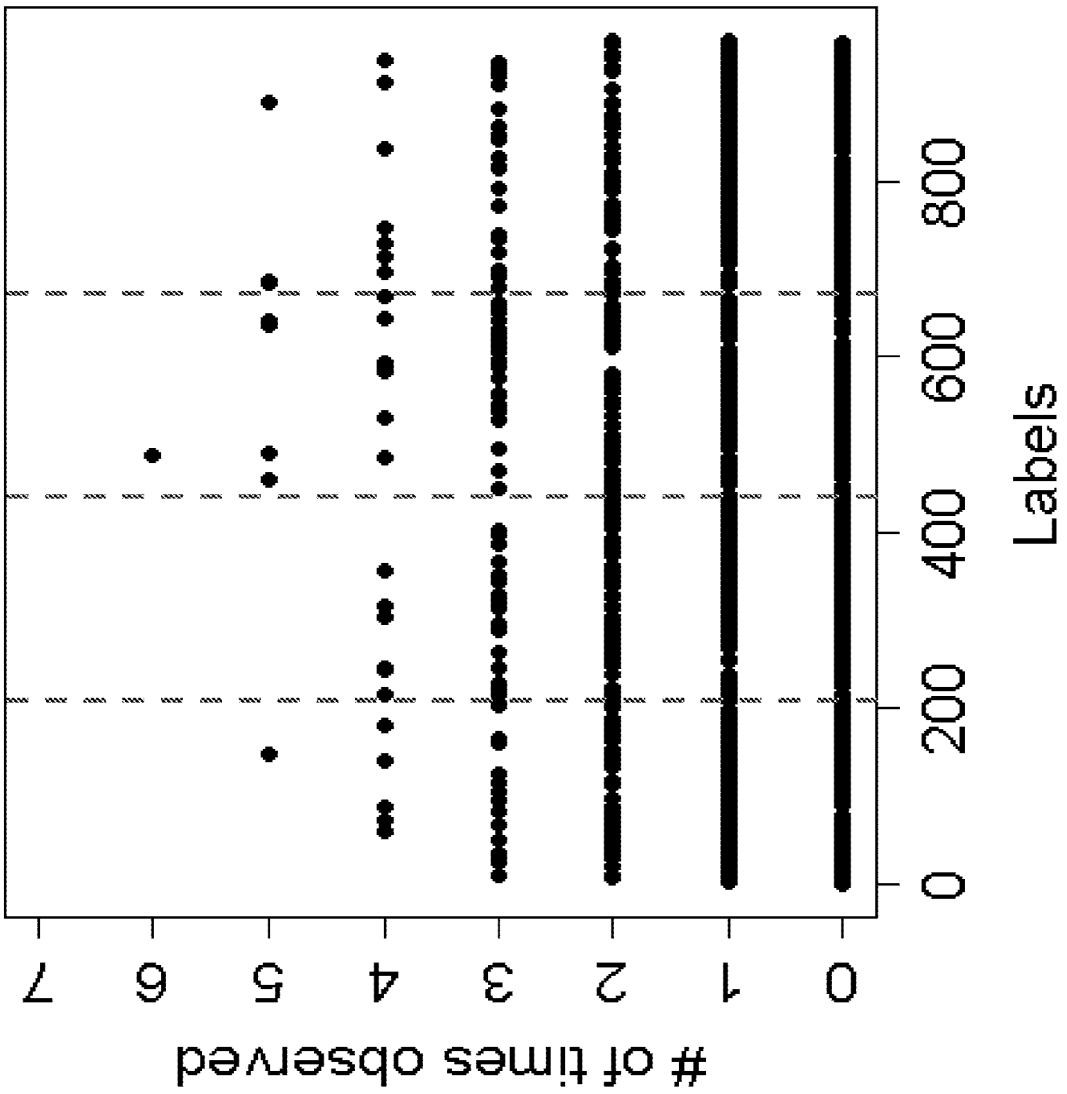


Fig. 25

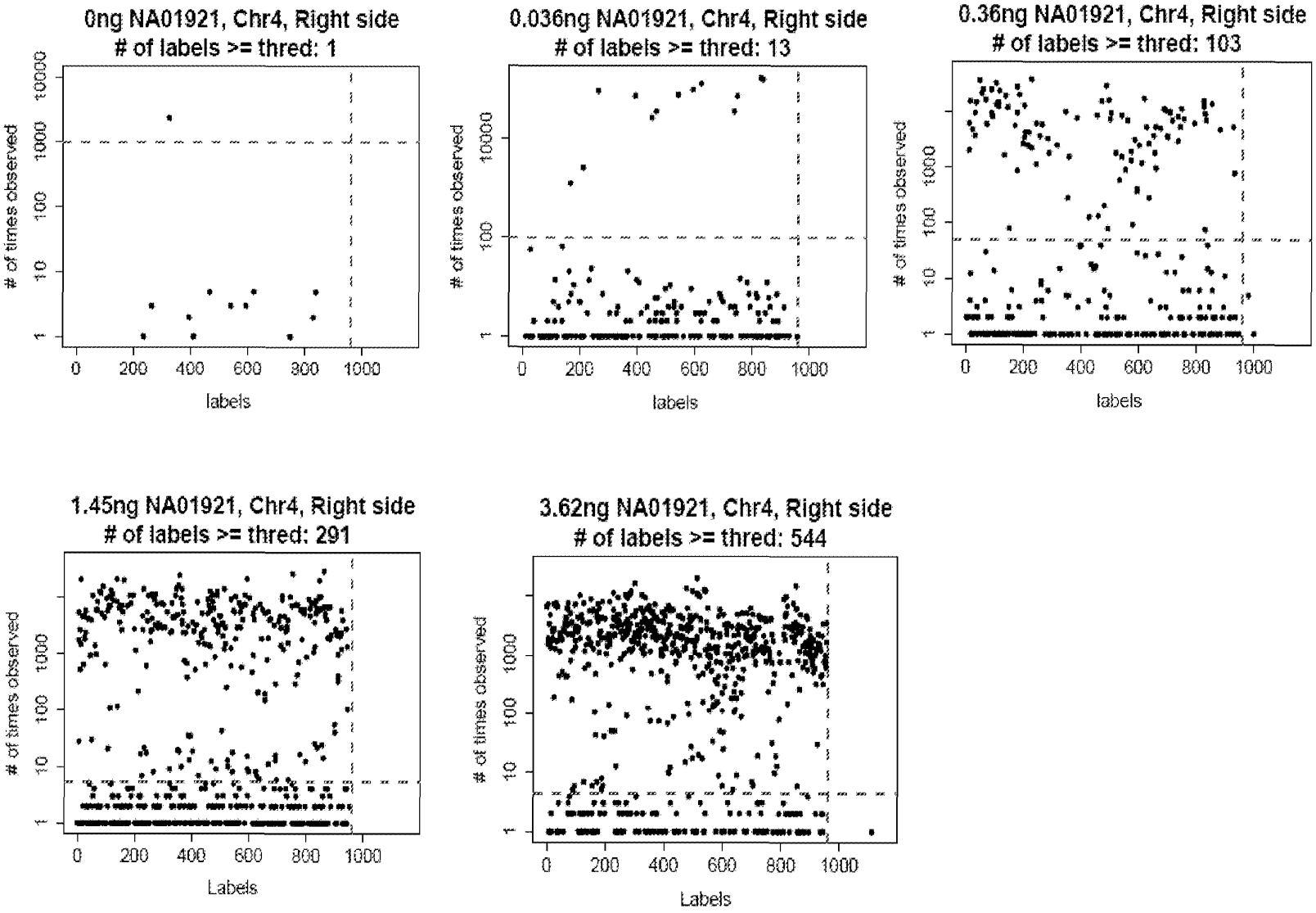

0ng NA01921, Chr4, Right side
# of labels >= thred: 1
0.036ng NA01921, Chr4, Right side
# of labels >= thred: 13
0.36ng NA01921, Chr4, Right side
# of labels >= thred: 103
1.45ng NA01921, Chr4, Right side
# of labels >= thred: 291
3.62ng NA01921, Chr4, Right side
# of labels >= thred: 544
# of times observed
labels
Labels

Fig. 26

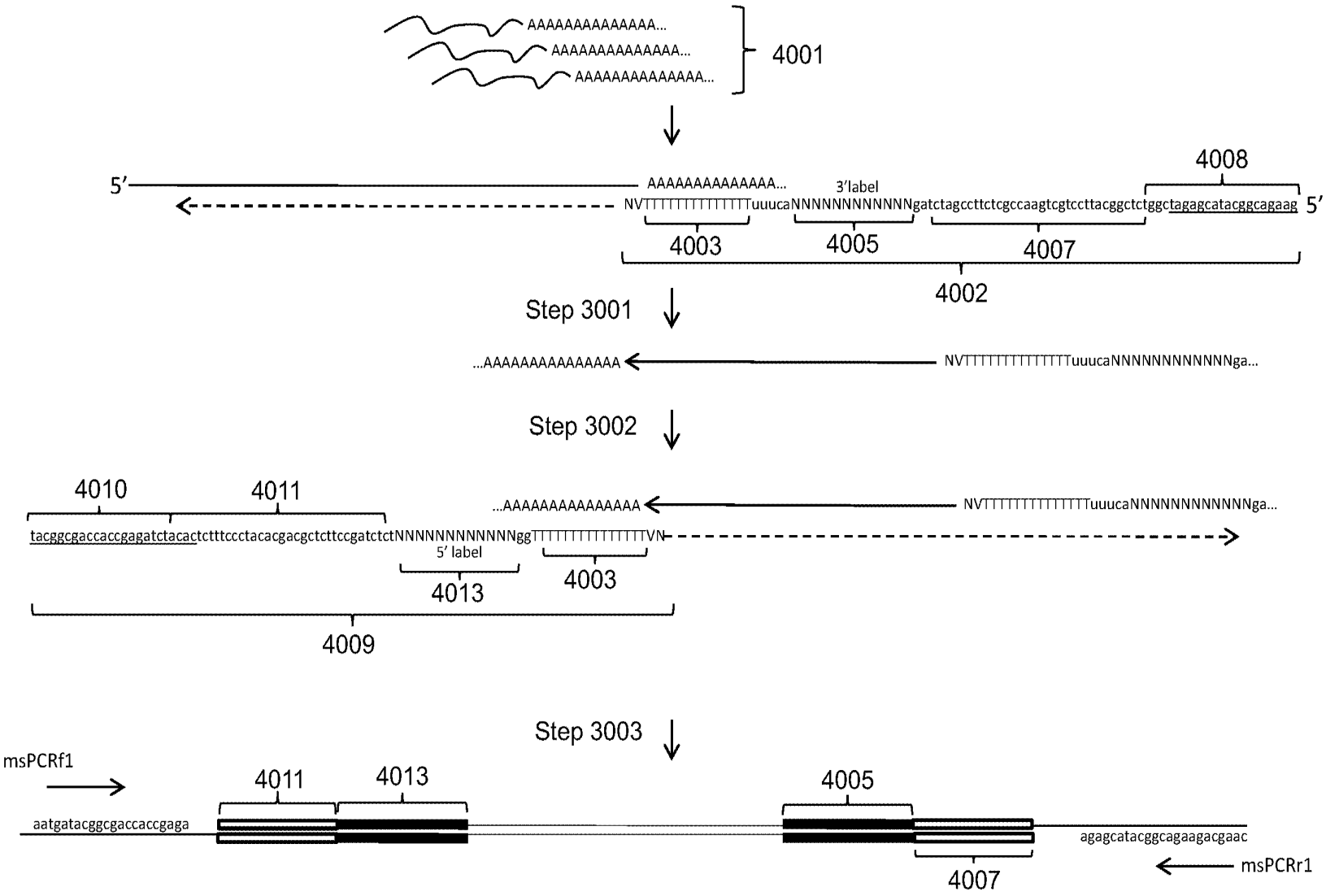

AAAAAAAAAAAAAA...
AAAAAAAAAAAAAA...
AAAAAAAAAAAAAA...
4001
5'
AAAAAAAAAAAAAA...
3'label
NVTTTTTTTTTTTTTTuuucaNNNNNNNNNNNNgatctagccttctcgccaagtcgtccttacggctctggctagagcatacggcagaag
5'
4008
4003
4005
4007
4002
Step 3001
...AAAAAAAAAAAAAAA
NVTTTTTTTTTTTTTTuuucaNNNNNNNNNNNNga...
Step 3002
4010
4011
...AAAAAAAAAAAAAAA
NVTTTTTTTTTTTTTTuuucaNNNNNNNNNNNNga...
tacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctctNNNNNNNNNNNNggTTTTTTTTTTTTTTVN
5' label
4013
4003
4009
Step 3003
msPCRf1
4011
4013
4005
aatgatacggcgaccaccgaga
agagcatacggcagaagacgaac
4007
msPCRr1

Fig. 27

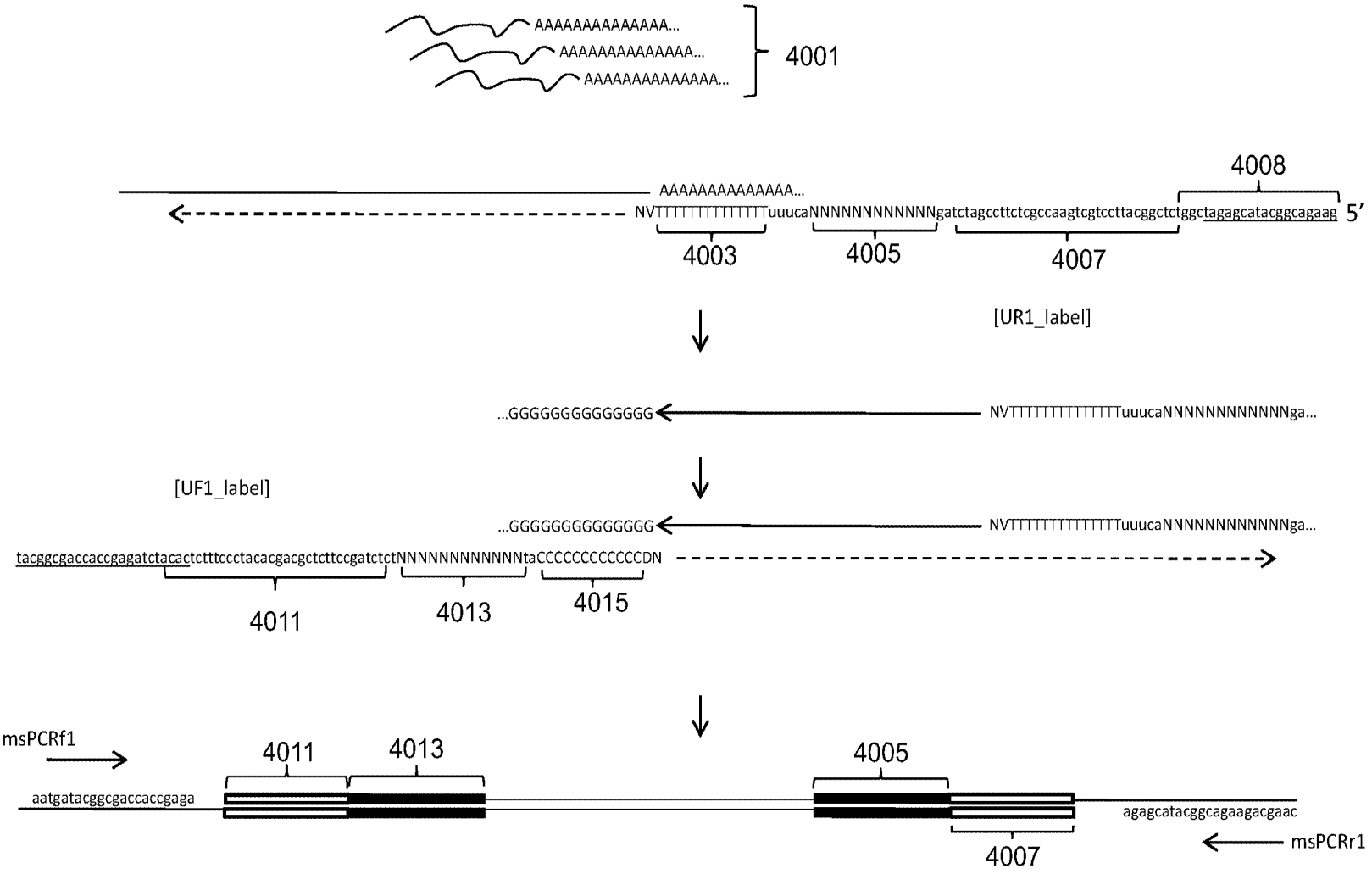

AAAAAAAAAAAAAA...
AAAAAAAAAAAAAA...
AAAAAAAAAAAAAA...
4001
4008
AAAAAAAAAAAAAA...
NVTTTTTTTTTTTTTuuucaNNNNNNNNNNNNgatctagccttctcgccaagtcgtccttacggctctggctagagcatacggcagaag 5'
4003
4005
4007
[UR1_label]
...GGGGGGGGGGGGGG
NVTTTTTTTTTTTTTuuucaNNNNNNNNNNNNga...
[UF1_label]
...GGGGGGGGGGGGGG
NVTTTTTTTTTTTTTuuucaNNNNNNNNNNNNga...
tacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctctNNNNNNNNNNNNtaCCCCCCCCCCCCDN
4011
4013
4015
msPCRf1
4011
4013
4005
aatgatacggcgaccaccgaga
agagcatacggcagaagacgaac
msPCRr1
4007

Fig. 28A

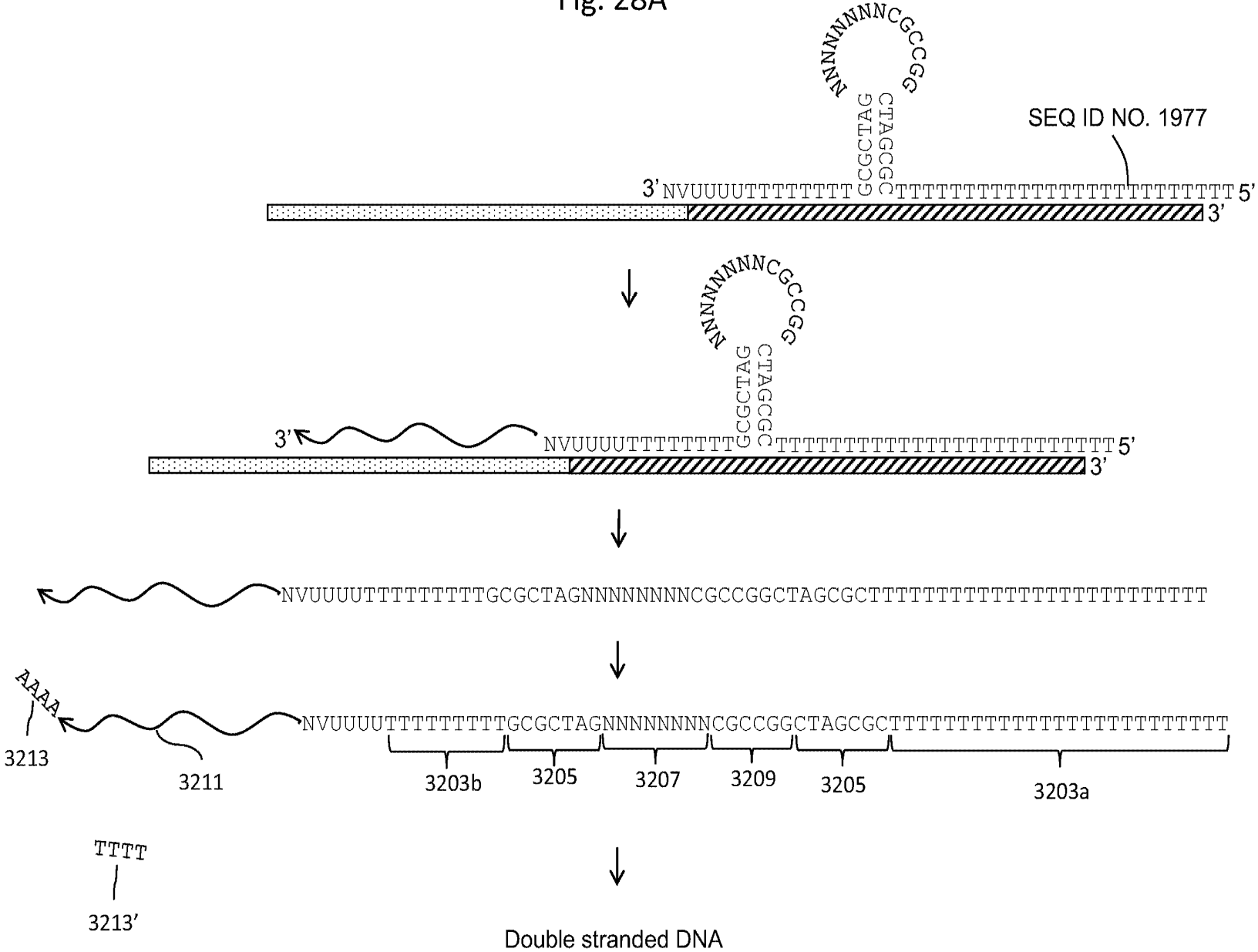
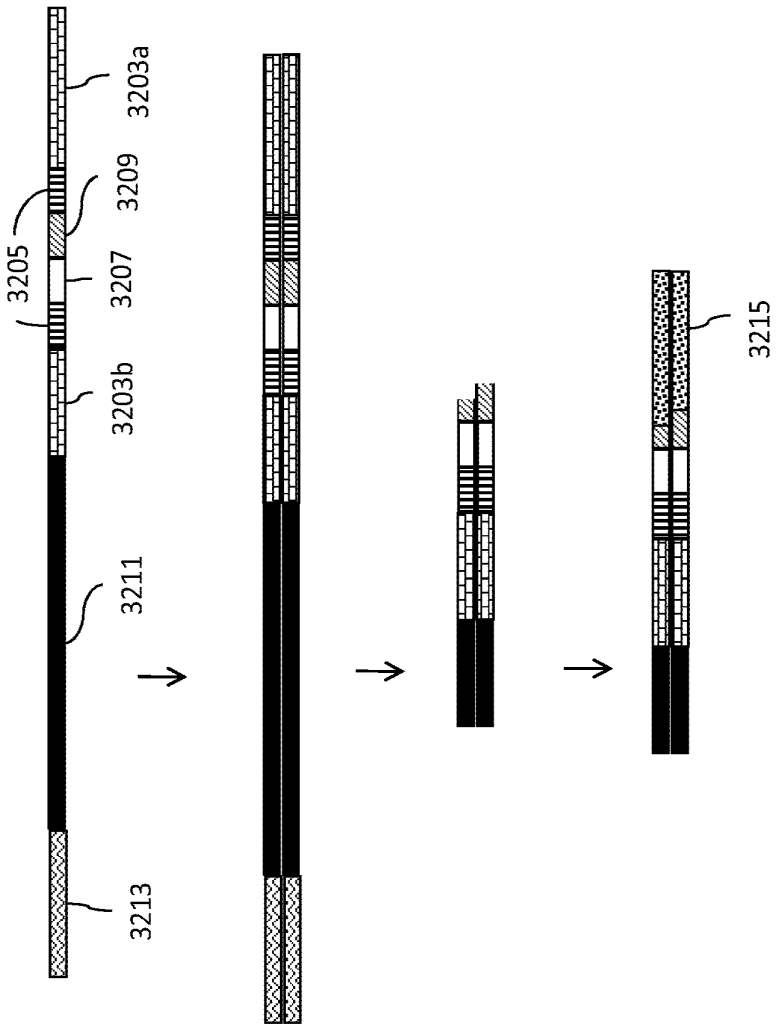

NNNNNNNNCGCCGG
GCGCTAG
CTAGCGC
SEQ ID NO. 1977
3' NVUUUUTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTT 5'
3'
NNNNNNNNCGCCGG
GCGCTAG
CTAGCGC
3' NVUUUUTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTT 5'
3'
NVUUUUTTTTTTTTTGCGCTAGNNNNNNNNCGCCGGCTAGCGCTTTTTTTTTTTTTTTTTTTTTTTTT
AAAA
NVUUUUTTTTTTTTTGCGCTAGNNNNNNNNCGCCGGCTAGCGCTTTTTTTTTTTTTTTTTTTTTTTTT
3213
3211
3203b
3205
3207
3209
3205
3203a
TTTT
3213'
Double stranded DNA

Fig. 28B



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/065291

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

EPO-Internal , MEDLINE, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/152928 A2 (GENXPRO GMBH; ROTTER BJOERN [DE] GENXPRO GMBH [DE] ; ROTTER BJOERN [DE]) 23 December 2009 (2009-12-23) the whole document --- HUBERT HUG ET AL: "Measurement of the number of molecules of a single mRNA species in a complex mRNA preparation .", JOURNAL OF THEORETICAL BIOLOGY, vol . 221 , no. 4 , 1 April 2003 (2003-04-01) , pages 615-624, XP55017448, ISSN : 0022-5193 the whole document --- -/ -	1-13, 18-25 , 35-38,51
X		1 ,35,38, 51

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* 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) orwhich 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
24 May 2012	06/06/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Botz, Jurgen

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/065291

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D'ANTONI C M ET AL: "Rapi d quanti tati ve analysi s using a singl e molecule counting approach" , ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol . 352 , no. 1, 1 May 2005 (2006-05-01) , pages 97-109 , XP024942384, ISSN : 0003-2697, D0I : 10. 1015/J .AB.2006.01 .031 [retri eved on 2006-05-01] the whole document	1-72
E	W0 2012/042374 A2 (TAI PALE ANSSI JUSSI NI KOLAI [SE] ; LINNARSS0N STEN [SE]) 5 Apri l 2012 (2012-04-05) the whole document	1-72
A	LARSON D R ET AL: "A single molecule view of gene expression" , TRENDS I N CELL BIOLOGY, ELSEVI ER SCI ENCE LTD, XX, vol . 19, no. 11, 1 November 2009 (2009-11-01) , pages 630-637, XP026716912 , ISSN : 0962-8924, D0I : 10. 1016/J .TCB.2009.08.008 [retri eved on 2009-10-08] the whole document	1-72
X. P	Gl enn K Fu ET AL: "Counti ng i ndi vidual DNA molecules by the stochasti c attachment of di verse label s", PNAS, 31 May 2011 (2011-05-31) , pages 9026-9031 , XP55017200, D0I : 10. 1073/pnas . l017621108/-/DCSuppl emental Retri eved from the Internet: URL: http ://www. pnas . org/content/108/22/902 6. ful 1.pdf#page=l&view=Fi t H [retri eved on 2012-01-23] the whole document	1-72
A	V0GELSTEIN BERT ET AL: "Digi tal PCR", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCI ENCES, NATIONAL ACADEMY OF SCI ENCES, vol . 96, no. 16, 3 August 1999 (1999-08-03) , pages 9236-9241 , XP002185144, ISSN : 0027-8424, DOI : 10. 1073/PNAS. 96. 16.9236 the whole document	1-72

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2011/065291

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐ on paper

   ☐ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/065291

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009152928 A2	23-12-2009	DE 102008025656 A1	03-12-2009
		WO 2009152928 A2	23-12-2009
WO 2012042374 A2	05-04-2012	NONE	

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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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	10752951	B2	2020-08-25	Salk et al.	Entire Document

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	1	20130096011	A1	2013-04-18	Rava et al.	Entire Document

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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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16945124
	Filing Date	2020-07-31
	First Named Inventor	AmirAli TALASAZ
	Art Unit	1637
	Examiner Name	Kenneth R. HORLICK
	Attorney Docket Number	42534-708.306

	1	JIANG et al. "Basics in Bioinformatics Lecture Notes of the Graduate Summer School on Bioinformatics of China" Springer Heidelberg New York Dordrecht London (2013)	
	2	Opposition Form and Statement to EP3378952 filed November 4, 2020 by Dirk Buhler	
	3	Opposition Form and Statement to EP3378952 filed November 5, 2020 by Foundation Medicine Inc.	
	4	Opposition Form and Statement to EP3378952 filed November 5, 2020 by Grunecker	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-12-09
Name/Print	Timothy A. Hott	Registration Number	67740

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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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.

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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.

Attorney Docket No. 42534-708.306/GH0004US-CON5
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	AmirAli TALASAZ et al.	Confirmation No.:	2506
Serial No.:	16/945,124	Art Unit:	1637
Filing Date:	July 31, 2020	Examiner:	Kenneth R. HORLICK
Title:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

A. ☐ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☒ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☒ a fee of $260.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $260.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure

statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $260.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 60-2231(Docket No. GH0004US-CON5).

Respectfully submitted,

Dated: December 9, 2020

By: /Timothy A. Hott/
Timothy A. Hott, Reg. No. 67740

Customer No. 115823
GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063

Electronic Acknowledgement Receipt

EFS ID:	41342253
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	09-DEC-2020
Filing Date:	31-JUL-2020
Time Stamp:	18:46:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$170
RAM confirmation Number	E2020B9I46011061
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	38039 98a84bc49ec1f6a5fcae49513fb0ae9c6b3f4cdb	no	3
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30392 29de9b18c28d0dcfa6c0420067114be0e614fb6b	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	68431		

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.

Electronic Acknowledgement Receipt

EFS ID:	41342205
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	09-DEC-2020
Filing Date:	31-JUL-2020
Time Stamp:	18:39:41
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$260
RAM confirmation Number	E2020B9I40030984
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2020-12-09_GH0004US-CON5_OA1Response.pdf	135068 8b8eee275c83ec8de72f13bea030fc36b3c718a1	yes	9

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Specification	2	2
Claims	3	7
Applicant Arguments/Remarks Made in an Amendment	8	9

Warnings:

Information:

2	Letter specifying the conditions for filing under 37CFR1.97	2020-12-09_GH0004US-CON5_IDSTrans.pdf	144724 b27e5e637ad8122e7af77d0192fa2fcebe37bcab	no	4

Warnings:

Information:

3	Information Disclosure Statement (IDS) Form (SB08)	2020-12-09_GH0004US-CON5_SB08.pdf	1053483 d6853c4464dda2d7773795a31add590d86bef425	no	4

Warnings:

Information:

4	Foreign Reference	WO_2012148477_A1_Fodor.pdf	1675144 bc7c1bb119f773c5f4c08d4d1ed6e2ea17be5448	no	135

Warnings:

Information:

5	Non Patent Literature	JIANG_BasicsOfBioinformatics_2013.pdf	2138325 0c4d97251474cc7d5bb46ae6a46611b56087d230	no	10
Warnings:					
Information:					
6	Other Reference-Patent/App/Search documents	OppositionFormStmt_EP3378952_Buhler.pdf	1511881 e03ce779677aa149f0caefd70e04e2bd9401feb3	no	34
Warnings:					
Information:					
7	Other Reference-Patent/App/Search documents	OppositionFormStmt_EP3378952_FMI.pdf	3461881 0b3f17ef8109e1e5a0e83f1174298b04fe634d3d	no	46
Warnings:					
Information:					
8	Other Reference-Patent/App/Search documents	OppositionFormStmt_EP3378952_Grunecker.pdf	10836077 1f2fdf74ef833f2e8bca7c5632497858453f7427	no	51
Warnings:					
Information:					
9	Fee Worksheet (SB06)	fee-info.pdf	30654 289bbd0a02ecf4c041195068e9f4a48ad2380143	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	20987237		

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

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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.

REMARKS

After entry of the above-referenced claim amendments, claims 1-30 are pending. Claims 1-5, 7-9, 11, 14-15, 17, 20, 22-23, and 25-30 are amended herein for clarity, antecedent basis, and/or claim dependency purposes. Support for these amendments may be found throughout the application as filed. No new matter is being introduced.

The specification has been amended to reflect updated priority information in para [0001]. No new matter is added by this amendment.

Applicant also submits that the present claims are derived from subject matter disclosed and/or claimed in at least priority application PCT/US2014/072383 ('383). It appears that US2019/0338358 and the resulting issued patent US10,752,951 to Salk et al. contain claims that are substantially copied from those of Applicant's '383 application.

I. Objection

Claim 22 was objected to as lacking a period. Applicant has amended claim 22 to correct this omission. No new matter is being introduced.

II. Rejection Under 35 U.S.C. §112(b)

Claim 20 was rejected under 35 U.S.C. §112(b) as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the Applicant regards as the invention. Claim 20 has been amended to replace the term "plurality" with "population" to provide for proper antecedent basis.

III. Double Patenting Rejection

Claims 1-30 were rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claim 2 of U.S. Patent No. 9,920,366.

Claims 1-30 were provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 31, 34-42, 44-49, 52-56, and 61-68 of co-pending U.S. Application No. 16/714,579, 16/672,267, 16/601,168, and 15/892,178.

Without conceding in the basis of the rejection and solely to advance prosecution, Applicant submits herewith terminal disclaimers over the aforementioned patent and

applications. Applicant notes that previously copending Application No. 16/601,168 issued as U.S. Patent No. 10,801,063 on October 13, 2020.

Accordingly, for at least the reasons provided above, Applicant respectfully requests these rejections be withdrawn.

CONCLUSION

This communication fully addresses the rejections raised in the Office Action mailed on September 9, 2020. Applicant believes that the present application is now in condition for allowance and respectfully requests that the Examiner expedite the prosecution of this application to allowance. The Commissioner is authorized to charge any underpayment, or credit any overpayment, to Deposit Account No. 60-2231 (Attorney Docket No. GH0004US-CON5).

Respectfully submitted,
GUARDANT HEALTH, INC.

Date: December 9, 2020 By: /Timothy A. Hott/

Timothy A. Hott
Registration No.: 67740

GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063
Customer No. 115823

AMENDMENTS TO THE SPECIFICATION

Please amend paragraph [0001] as follows:

[0001] This application is a continuation of U.S. Application No. 16/601,168, filed October 14, 2019 (now U.S. Patent 10,801,063, issued October 13, 2020), which is a continuation of U.S. Application No. 15/892,178, filed February 8, 2018, which is a continuation of U.S. Application No. 14/861,989, filed September 22, 2015 (now U.S. Patent 9,920,366, issued March 20, 2018), which is a continuation application of International Application No. PCT/US2014/072383, filed December 24, 2014, which application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/921,456, filed December 28, 2013, and U.S. Provisional Application No. 61/948,509, filed March 5, 2014, each of which is entirely incorporated herein by reference.

Electronic Patent Application Fee Transmittal

Application Number:	16945124
Filing Date:	31-Jul-2020
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Filer:	Timothy A Hott/Michelle Chan
Attorney Docket Number:	42534-708.306

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
STATUTORY OR TERMINAL DISCLAIMER	1814	1	170	170
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**170**

Electronic Patent Application Fee Transmittal

Application Number:	16945124
Filing Date:	31-Jul-2020
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
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Attorney Docket Number:	42534-708.306

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**260**

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. 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.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/945,124	Filing Date 07/31/2020	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ 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).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	12/09/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 30	Minus	** 30	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/TINA J BARDEN/
*** 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 number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. 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 12 minutes 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.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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
/K.R.H/	1	10752951	B2	2020-08-25	Salk et al.	Entire Document

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

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
/K.R.H/	1	20130096011	A1	2013-04-18	Rava et al.	Entire Document

If you wish to add additional U.S. Published Application citation information please click the Add button. Add

FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
/K.R.H/	1	2012148477	WO	A1	2012-11-01	FODOR et al.	Entire Document	

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

/K.R.H/	1	JIANG et al. "Basics in Bioinformatics Lecture Notes of the Graduate Summer School on Bioinformatics of China" Springer Heidelberg New York Dordrecht London (2013)	
/K.R.H/	2	Opposition Form and Statement to EP3378952 filed November 4, 2020 by Dirk Buhler	
/K.R.H/	3	Opposition Form and Statement to EP3378952 filed November 5, 2020 by Foundation Medicine Inc.	
/K.R.H/	4	Opposition Form and Statement to EP3378952 filed November 5, 2020 by Grunecker	

If you wish to add additional non-patent literature document citation information please click the Add button [Add]

EXAMINER SIGNATURE

Examiner Signature	/KENNETH R HORLICK/	Date Considered	12/15/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2020-12-09
Name/Print	Timothy A. Hott	Registration Number	67740

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.**

Privacy Act Statement

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.

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BIB DATA SHEET

CONFIRMATION NO. 2506

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
16/945,124	07/31/2020 RULE	435	1637	42534-708.306

APPLICANTS

GUARDANT HEALTH, INC., Redwood City, CA;

INVENTORS

AmirAli TALASAZ, Atherton, CA;
Helmy ELTOUKHY, Atherton, CA;
Stefanie Ann Ward MORTIMER, Morgan Hill, CA;

**** CONTINUING DATA ***************************

This application is a CON of 16/601,168 10/14/2019 PAT 10801063
which is a CON of 15/892,178 02/08/2018
which is a CON of 14/861,989 09/22/2015 PAT 9920366
which is a CON of PCT/US2014/072383 12/24/2014
which claims benefit of 61/948,509 03/05/2014
and claims benefit of 61/921,456 12/28/2013

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

08/07/2020

Foreign Priority claimed	❑ Yes ☑ No	❑ Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
35 USC 119(a-d) conditions met	❑ Yes ☑ No		CA	11	30	2
Verified and Acknowledged	/KENNETH R HORLICK/ Examiner's Signature	Initials				

ADDRESS

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304
UNITED STATES

TITLE

METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

FILING FEE RECEIVED		
2720	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	❑ All Fees ❑ 1.16 Fees (Filing) ❑ 1.17 Fees (Processing Ext. of time) ❑ 1.18 Fees (Issue) ❑ Other _____ ❑ Credit

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

115823 7590 12/21/2020

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER
HORLICK, KENNETH R

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
12/21/2020	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):

Patents@guardanthealth.com
patentdocket@wsgr.com

Office Action Summary	Application No. 16/945,124	Applicant(s) TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	AIA (FITF) 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 3 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 12/09/20.

☐ 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) 1-30 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) 1-6 and 8-30 is/are rejected.

8) ☑ Claim(s) 7 is/are objected to.

9) ☐ Claim(s) _____ 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 12/9/20.

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

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

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

2. The terminal disclaimer filed on 12/09/20 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of **US '366 or '063, or of any patent issuing from US Application Nos. '579, '178, or '267** has been reviewed and is accepted. The terminal disclaimer has been recorded.

NEW GROUND OF REJECTION NECESSITATED BY THE IDS FILED 12/09/20

3. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

4. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(2) the claimed invention was described in a patent issued under section 151, or in an application for patent published or deemed published under section 122(b), in which the patent or application, as the case may be, names another inventor and was effectively filed before the effective filing date of the claimed invention.

Claims 1-2, 9, 11-12, 14-15, 18-19, 21, 23, and 25-29 are rejected under 35 U.S.C. 102(a)(2) as being anticipated by Salk et al. (US 10,752,951; effective filing date at least March 15, 2013; newly cited by Applicant).

It is readily apparent that the noted instant claims are substantially identical to claims 1-28 of Salk et al. Thus, the claimed methods cannot be distinguished from the patented methods of Salk et al.

5. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

Claims 3-6, 8, 10, 13, 16-17, 20, 22, 24, and 30 are rejected under 35 U.S.C. 103 as being unpatentable over Salk et al.

While the further limitations of these dependent claims are not explicitly disclosed in the claims or specification of Salk et al., they clearly merely relate to routine optimization of known-important reaction parameters, which as well established in U.S. patent practice does not support unobviousness. Thus, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the application was filed to modify the method of Salk et al. in the manner of these claims.

ALLOWABLE SUBJECT MATTER

6. Claim 7 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims. It is noted that in the Final Written Decision of IPR2019-00652, the Patent Trial and Appeal Board found, with respect to related U.S. Patent No. 9,834,822, that the Salk et al. specification (specifically in U.S. Patent No. 9,752,188) did not teach or suggest wherein 'cfDNA molecules that map to a mappable base position of a reference sequence are tagged with a number of different molecular barcodes ranging from at least 2 to fewer than a number the cfDNA molecules that map to the mappable base position.'

CONCLUSION

7. No claims are allowable.

8. Applicant's submission of an information disclosure statement under 37 CFR 1.97(c) with the fee set forth in 37 CFR 1.17(p) on 12/09/20 prompted the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 609.04(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing

date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

9. Any inquiry concerning this communication or earlier communications from the examiner should be directed to KENNETH R HORLICK whose telephone number is (571)272-0784. The examiner can normally be reached on Mon. - Thurs. 8:30 - 6:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

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 https://ppair-my.uspto.gov/pair/PrivatePair. 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.

12/15/20

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/945,124	TALASAZ et al.
	Examiner	Art Unit
	KENNETH R HORLICK	1637

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
inventor name search	09/02/2020	KH
reviewed parent applications and references therein	09/02/2020	KH
updated parent searches in USPAT and PGPUB	09/02/2020	KH
updated parent searches in USPAT and PGPUB	12/16/2020	KH

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637	

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

115823 7590 03/02/2021

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
HORLICK, KENNETH R	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
03/02/2021	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):

Patents@guardanthealth.com
patentdocket@wsgr.com

Applicant-Initiated Interview Summary	**Application No.** 16/945,124	**Applicant(s)** TALASAZ et al.		
	Examiner KENNETH R HORLICK	**Art Unit** 1637	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
KENNETH R HORLICK	Primary Examiner	Telephonic
TIMOTHY HOTT	Attorney of Record	

Date of Interview: 25 February 2021

Issues Discussed:

35 U.S.C. 102

Discussed the 102 rejection in view of Salk et al. ('951) and the Final Written Decision in IPR2019-00652
.

35 U.S.C. 103

Discussed the 103 rejection in view of Salk et al. ('951) and the Final Written Decision in IPR2019-00652
.

/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): AmirAli TALASAZ et al.	Confirmation No.: 2506
Serial Number: 16/945,124	Customer No.: 115823
Filing Date: July 31, 2020	Group Art Unit: 1637
Title: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	Examiner: Kenneth R. HORLICK

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

RESPONSE TO FINAL OFFICE ACTION AND REQUEST FOR CONTINUED EXAMINATION

Sir:

This communication is in response to the Final Office Action mailed on December 21, 2020. Applicant submits herewith a Request for Continued Examination along with the requisite fee. Applicant also submits herewith a petition for a 2-month extension of time, setting the period for reply by May 21, 2021; therefore, this response is timely filed. Applicant respectfully requests reconsideration of the above-referenced application in view of the following remarks:

Amendments to the Claims begin on page 2 of this paper.
Summary of the Interview appears on page 7 of this paper.
Remarks begin on page 8 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings in the above-referenced patent application. The foregoing amendments are without prejudice and do not constitute an admission regarding the patentability of the amended subject matter and should not so be construed. Applicant reserves the right to pursue the subject matter of the canceled claims in this or any other appropriate patent application.

Listing of Claims:

1. (Currently amended): A method, comprising:

(a) providing a population of cell-free deoxyribonucleic acid (cfDNA) molecules having first and second complementary strands;

(b) tagging a plurality of the cfDNA molecules in the population with duplex tags comprising molecular barcodes from a set of molecular barcodes to produce tagged parent polynucleotides, wherein the duplex tags are attached to both ends of a molecule of the plurality of the cfDNA molecules;

(c) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(d) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(e) reducing ~~and/~~or tracking redundancy ~~in~~ of a plurality of sequence reads from the set of sequence reads using at least sequencing information from the molecular barcodes of the duplex tags to determine distinct cfDNA molecules from among the tagged parent polynucleotides, wherein the distinct cfDNA molecules are determined based on (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, ~~and/~~or (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, wherein reducing or tracking the redundancy of the plurality of sequence reads comprises mapping at least a subset of the plurality of sequence reads to a reference sequence.

2. (Previously presented): The method of claim 1, wherein the population of cfDNA molecules is obtained or derived from a subject having cancer.

3. (Previously presented): The method of claim 1, wherein the population of cfDNA molecules comprises 1 nanogram (ng) to 100 ng of cfDNA molecules.

4. (Currently amended): The method of claim 1, wherein the molecular barcodes are ligated to the plurality of the cfDNA molecules using more than a 10X ~~molar~~ excess of duplex tags as compared to the population of cfDNA molecules, wherein at least 20% of the cfDNA molecules from the population are tagged with the duplex tags.

5. (Currently amended): The method of claim 1, wherein the molecular barcodes are ligated to the plurality of the cfDNA molecules using more than an 80X ~~molar~~ excess of duplex tags as compared to the population of cfDNA molecules.

6. (Original): The method of claim 5, wherein at least 40% of the cfDNA molecules from the population are tagged with the duplex tags.

7. (Previously presented): The method of claim 1, wherein the tagging comprises non-uniquely tagging the plurality of the cfDNA molecules, wherein the cfDNA molecules that map to a mappable base position of a reference sequence are tagged with a number of different molecular barcodes ranging from at least 2 to fewer than a number of the cfDNA molecules that map to the mappable base position.

8. (Previously presented): The method of claim 1, wherein the set of molecular barcodes comprises 5 to 10,000 different molecular barcode sequences, and wherein the molecular barcodes in the set of molecular barcodes have a length of 5 to 20 base pairs.

9. (Previously presented): The method of claim 1, further comprising, prior to the sequencing, enriching at least a subset of the amplified progeny polynucleotides for target regions of interest to produce enriched progeny polynucleotides.

10. (Original): The method of claim 9, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1,

CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

11. (Previously presented): The method of claim 10, further comprising, prior to the sequencing, amplifying a plurality of the enriched progeny polynucleotides.

12. (Original): The method of claim 1, wherein the duplex tags are part of sequencing adapters.

13. (Canceled)

14. (Currently amended): The method of claim 1, wherein ~~the~~ reducing ~~and/~~or tracking the redundancy ~~in~~ of the plurality of sequence reads comprises grouping the paired reads or the unpaired reads into families based at least in part on (i) the molecular barcodes of the paired reads or the unpaired reads and (ii) sequence information at the start and stop positions of the paired reads or the unpaired reads~~mapping at least a subset of the plurality of sequence reads to a reference sequence~~.

15. (Currently amended): The method of claim ~~1~~41, further comprising:

(f) determining quantitative measures of (i) paired reads ~~and/~~or (ii) unpaired reads that map to a genomic locus of the reference sequence.

16. (Currently amended): The method of claim 15, further comprising:

(g) estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides that map to the genomic locus based on the quantitative measures of the paired reads and the unpaired reads.

17. (Currently amended): The method of claim 16, further comprising:

(h) detecting copy number variation in the population of cfDNA molecules by determining a normalized quantitative measure determined in (g) at each of one or more genomic loci.

18. (Currently amended): A method, comprising:

(a) tagging a population of double-stranded cell-free deoxyribonucleic acid (cfDNA) molecules obtained or derived from a sample of a subject with a set of tags comprising molecular barcodes to ~~generate~~ produce tagged parent polynucleotides;

(b) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(c) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(d) sorting a plurality of sequence reads from the set of sequence reads into (i) families comprising paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides, and (ii) families comprising unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides.

19. (Original): The method of claim 18, wherein the sample is blood, plasma, or serum.

20. (Previously presented): The method of claim 18, wherein the population of double-stranded cfDNA molecules comprises 1 nanogram (ng) to 100 ng of double-stranded cfDNA molecules.

21. (Currently amended): The method of claim 18, wherein the tagging ~~is performed by~~ comprises ligating the molecular barcodes to double-stranded cfDNA molecules.

22. (Previously presented): The method of claim 18, wherein the set of tags comprises 2 to 10,000 different molecular barcode sequences.

23. (Previously presented): The method of claim 18, further comprising, prior to the sequencing, enriching at least a subset of the amplified progeny polynucleotides for target regions of interest to produce enriched progeny polynucleotides.

24. (Original): The method of claim 23, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

25. (Currently amended): The method of claim 18, further comprising, prior to (d), reducing ~~and/~~or tracking redundancy ~~in~~ of a plurality of sequence reads from the set of sequencing reads.

26. (Currently amended): The method of claim 25, wherein ~~the~~ reducing ~~and/~~or tracking the redundancy ~~in~~ of the plurality of sequence reads comprises mapping at least a subset of the plurality of sequence reads to a reference sequence.

27. (Currently amended): The method of claim 26, wherein ~~the~~ reducing ~~and/~~or tracking the redundancy ~~in~~ of the plurality of sequence reads comprises determining a base call at one or more genomic loci for a plurality of sequence reads that map to the one or more genomic loci on the reference sequence.

28. (Currently amended): The method of claim 26, wherein ~~the~~ reducing ~~and/~~or tracking the redundancy ~~in~~ of the plurality of sequence reads comprises collapsing the plurality of sequence reads to ~~generate~~ produce consensus sequences representative of original double-stranded cfDNA molecules from among the tagged parent polynucleotides.

29. (Previously presented): The method of claim 26, further comprising determining quantitative measures of at least two of (i) paired reads, (ii) unpaired reads, (iii) a read depth of the paired reads, and (iv) a read depth of the unpaired reads.

30. (Currently amended): The method of claim 29, further comprising estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides that map to a genomic locus of the reference sequence based on the quantitative measures of at least two of (i) paired reads, (ii) unpaired reads, (iii) a read depth of the paired reads, and (iv) a read depth of the unpaired reads at the genomic locus.

31. (New) The method of claim 1, wherein the distinct cfDNA molecules in (e) are determined based on (i) the paired reads and (ii) the unpaired reads.

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(54) **Title:** ENZYMATIC LIGATION OF NUCLEIC ACIDS

(57) **Abstract:** Methods, assays, compositions and kits for the ligation of short polynucleotides are presented herein. The short poly-nucleotides are optionally no more than **7** nucleotides in length, and can be as short as 3 or 4 nucleotides in length. The ligation is optionally performed by CV ligase.

ENZYMATIC LIGATION OF NUCLEIC ACIDS

[0001] This application claims priority to the following U.S. provisional applications, each of which is incorporated by reference in its entirety: App. No. 61/474,205 filed April 11, 2011; App. No. 61/474,168 filed April 11, 2011; App. No. 61/433,502 filed January 17, 2011; and App. No. 61/433,488 filed January 17, 2011.

Background

[0002] DNA ligases can join polynucleotides together, for example by catalyzing the formation of a phosphodiester bond at single- or double-stranded breaks on duplex DNA. Ligases can be sensitive to the degree of hybridization between opposing nucleic acid strands in a duplex. For example, successful ligation can occur less frequently (or not at all) where a strand to be ligated to an adjacent strand is in a duplex and is not complementary to its opposing strand in the duplex. In some cases a single nucleotide mismatch between strands in a duplex can significantly impair or prevent ligation. The capacity of ligases for discrimination based on hybridization, including single nucleotide discrimination, has led to the development of ligase-mediated detection techniques *(e.g.,* Landegren, U., Bioessays, 15(11):761-765 (1993), and Barany, PNAS USA, 88(1):189-193 (1991)). Ligase-based linear signal amplification known as LDR *(i.e.,* ligase detection reaction), combined with PCR *(i.e.,* polymerase chain reaction)-based gene specific target amplification, has been proven to be a useful tool in cancer and disease gene mutation detection. PCR/LDR techniques typically rely on two properties of a DNA ligase: (i) specificity, and (ii) thermostability.

Summary

[0003] This application relates to ligation reagents and methods. Among other things, methods of and reagents for ligating nucleic acids to other nucleic acids are provided, including ligation of two polynucleotides. Either or both polynucleotides can be single-stranded or double-stranded. One or both polynucleotides can be a short oligonucleotide. In some embodiments oligonucleotides and/or polynucleotides of different lengths can be ligated to each other. The ligation can be enzymatic, and ligations can be template-

dependent or template-independent. The nucleic acids that are ligated or involved in the ligation reaction can be labeled or unlabeled and immobilized or in solution.

[0004] Some embodiments involve template-independent ligation of double-stranded or single-stranded polynucleotides (e.g., oligonucleotides). The single-stranded polynucleotides are optionally not hybridized to another polynucleotide. In some embodiments, an oligonucleotide can be hybridized to or otherwise associated with all or a portion of an overhang or other single-stranded region of a duplex nucleic acid and ligated to a free end of a strand of the duplex or to another oligonucleotide that is hybridized or otherwise associated with an overhang or other single-stranded portion of the duplex.

[0005] Some embodiments involve ligation of single-stranded polynucleotides (e.g., oligonucleotides). Optionally, the single-stranded polynucleotides are hybridized hybridized adjacent or near each other on another single polynucleotide. In some embodiments, an oligonucleotide can be hybridized to or otherwise associated with all or a portion of an overhang or other single-stranded region of a duplex nucleic acid and ligated to a free end of a strand of the duplex or to another oligonucleotide that is hybridized or otherwise associated with an overhang or other single-stranded portion of the duplex.

[0006] In some aspects, methods and reagents are provided for hybridizing or otherwise associating a first oligonucleotide and a second oligonucleotide to a third oligonucleotide or to a polynucleotide such that the termini of the first and second oligonucleotides are adjacent to or near each other. Such hybridization or association can occur sequentially, simultaneously, or substantially simultaneously. The terminus of the first oligonucleotide can be ligated to the adjacent or nearby terminus of the second oligonucleotide.

[0007] Nucleotide base mismatches between a first and/or second oligonucleotide and a third oligonucleotide or polynucleotide can affect the efficiency of ligation. For example, mismatches at the terminal position of either or both a first and second oligonucleotides can affect ligation efficiency, reducing the probability of successful ligation or precluded ligation entirely. Mismatches at other or at multiple positions can also affect ligation efficiency, reducing the probability of successful ligation or precluding ligation entirely.

[0008] Also provided are methods and reagents for performing multiple ligations sequentially, in parallel, or both sequentially and in parallel.

[0009] Optionally, one or more of the primer, probe or template is labeled. For example the probe can be labeled. Else the primer or template is labeled.

[0010] In some embodiments, methods of ligation are provided that provide information about the sequence of a nucleic acid. For example, in some aspects a ligation can be performed in the presence of multiple oligonucleotides that are at least partially complementary to a target region on a template. Oligonucleotide probes can be used that hybridize or otherwise associate with a template adjacent to or near a terminus of a primer or probe that is hybridized to or otherwise associated with a template. Multiple oligonucleotide probes, each at least partially complementary to a region of a template can be used to determine sequence information in a template-dependent manner as is known in the art. For example, oligonucleotide ligation is used to determine nucleic acid sequence information in the SOLiD System (Life Technologies-Applied Biosystems, Carlsbad, CA). According to some embodiments, sequence information is determined ligating oligonucleotide probes to an oligonucleotide primer in a template sequence-dependent manner, for example by using a SFL. The oligonucleotide probes can be a set of multiple probes having different sequences and distinguishing labels, and the primer and probes can have lengths of not more than 8, 7, 6, 5, 4, 3 or 2 nucleotides. The labels can provide information about the sequence of the probe.

[0011] In some embodiments, ligation can be performed by a "small footprint ligase" (herein "SFL") that can ligate short polynucleotides. SFLs can be used in each of the embodiments of ligations discussed above and in the remainder of this disclosure, as well as in other embodiments of ligations known to person of skill in the art. For example, in some embodiments an SFL can ligate the termini of a first oligonucleotide and a second oligonucleotide. The first oligonucleotide can be a primer and the second oligonucleotide can be a probe, each hybridizing or otherwise associating with a portion of a third oligonucleotide or a polynucleotide.

[0012] In some embodiments, the SFL can ligate oligonucleotides that are 8, 7, 6, 5, 4, 3 or 2 nucleotides in length to a polynucleotide. Ligation of such oligonucleotides can be to oligonucleotides of the same length or of different length or to a polynucleotide. For example, an oligonucleotide of 2 or 3 nucleotides in length can be ligated to an oligonucleotide of 2, 3, 4, 5, 6, 7, 8 or more nucleotides in length or to longer oligonucleotides or to a polynucleotide.

[0013] Also provided are kit comprising a small footprint ligase ("SFL") or functional variant or fragment or derivative thereof. Exemplary SFLs are identified herein, and their

sequences provided. Optionally, the kit can also include one or more oligonucleotide probes less than 12 nucleotides in length, (e.g., not more than 8, 6, 5, 4, 3 or 2 nucleotides in length). Optionally, the kit includes CV ligase and one or more oligonucleotides probes less than 6 nucleotides in length.

Brief Description of the Drawings

[0014] **Figure 1.** (A) Extended P1 covalent bead ligation substrate. Shown is a ligation substrate designed to mimic the conditions used in SOLiD™ sequencing. Figure 1(A) represents DNA covalently linked to a bead (such as a magnetic bead, e.g., of 0.1 to 1 μιη diameter). DNA on this bead was enriched by PCR using the 40 nucleotide P1 sequence. To the region GCGGATGTACGGTACAGCAG, a 20-mer complementary "primer" is annealed that has a 5' PO_4 which reacts with the 3'O H group of the SOLiD probe, as well as a 3' fluorescent label for detection using capillary electrophoresis. To the overhang sequence CGAATAGA, complementary probes can hybridize to demonstrate the ligation reaction. (B) P1 covalent bead ligation substrate. Shown is a similar ligation substrate to that in Figure 1(A), however the reaction in this case will occur much closer to the bead surface. Probes are hybridized to the overhang region AGTCGGTGAT, where the underlined residues are opposite the inosine triplet of probes having the structure Dye-5' III (s)-xy-NNN 3', as described in further detail herein.

[0015] **Figure 2:** Sequences of exemplary ligases. The GenBank ID and source organism are provided where applicable. Three artificial ligases are also provided. DLX differs from Hin DNA Ligase at 1 amino acid —designated as the underlined emboldened letter. DLXd differs from Hin DNA Ligase at 2 amino acids —designated as the underlined emboldened letters. DLXd2 is 22 amino acids shorter than Hin DNA Ligase and differs from Hin DNA Ligase at 2 amino acids, designated as the underlined emboldened letters.

[0016] **Figure 3:** Ligation of short oligonucleotides with various ligases. Ligation of short oligonucleotides with (A) CV ligase, (B) DLXd ligase and (C) MnM ligase. Forward ligation reactions were performed under the following conditions : 2.0 μM ligase, 2-5 μM short oligo, 2.0 nM primer/template (tethered to magnetic beads) and proceeded for 20 minutes at 15°C. The ligation efficiency was calculated as the as the

ratio of peak areas determined by CE where a FAM labeled primer was used. Efficiency = ligated/(ligated + unligated).

[0017] **Figure 4:** Ligation of short oligonucleotides with various ligases. Ligation of short oligonucleotides with (A) CV ligase, (B) DLXd ligase and (C) MnM ligase. Reverse ligation reactions were performed under the following conditions : 2.0 μM ligase, 2-5 μM short oligo, 2.0 nM primer/template (tethered to magnetic beads) and proceeded for 20 minutes at 15°C. The ligation efficiency was calculated as the as the ratio of peak areas determined by CE where a FAM labeled primer was used. Efficiency = ligated/(ligated + unligated).

[0018] **Figure 5:** Ligation of 2-mers. Ligation reaction in the forward direction was performed under the following conditions : 2.0 μM DLXd, 123 μM dinucleotide (5'-CG-3'), 2.0 nM primer/template (tethered to magnetic beads) and proceeded for 20 minutes at 15°C. The ligation efficiency was calculated as the as the ratio of peak areas determined by CE where a FAM labeled primer was used. Efficiency = ligated/(ligated + unligated).

Definitions

[0019] "Degenerate", with respect to a position in a polynucleotide that is one of a population of polynucleotides, means that the identity of the base of the nucleoside occupying that position varies among different members of the population. A population of polynucleotides in this context is optionally a mixture of polynucleotides within a single continuous phase (e.g., a fluid). The "position" can be designated by a numerical value assigned to one or more nucleotides in a polynucleotide, generally with respect to the 5' or 3' end. For example, the terminal nucleotide at the 3' end of an extension probe may be assigned position 1. Thus in a pool of extension probes of structure 3'-XXXNXXXX-5', the N is at position 4. A position is said to be k-fold degenerate if it can be occupied by nucleosides having any of k different identities. For example, a position that can be occupied by nucleosides comprising either of 4 different bases is 4-fold degenerate.

[0020] Along similar lines, it should be understood that a statement that a result has occurred (e.g., ligation, binding) is intended to indicate that the result has occurred at a significant or substantial level or an enhanced level compared to when it has not occurred. For example. Ligation is said to have not occurred if it is not significant, insubstantial or

greatly reduced (e.g., reduced by at least 80%, 90 %, 95% or 99% compared to when ligation does occur (e.g., under the conditions described in the last paragraph).

[0021] The terms "microparticle," "beads" "microbeads", etc., refer to particles (optionally but not necessarily spherical in shape) having a smallest cross-sectional length (e.g., diameter) of 50 microns or less, preferably 10 microns or less, 3 microns or less, approximately 1 micron or less, approximately 0.5 microns or less, e.g., approximately 0.1, 0.2, 0.3, or 0.4 microns, or smaller (e.g., under 1 nanometer, about 1-10 nanometer, about 10-100 nanometers, or about 100-500 nanometers). Microparticles (e.g., Dynabeads from from Dynal, Oslo, Norway) may be made of a variety of inorganic or organic materials including, but not limited to, glass (e.g., controlled pore glass), silica, zirconia, cross-linked polystyrene, polyacrylate, polymethymethacrylate, titanium dioxide, latex, polystyrene, etc. Magnetization can facilitate collection and concentration of the microparticle-attached reagents (e.g., polynucleotides or ligases) after amplification, and facilitates additional steps (e.g., washes, reagent removal, etc.). In certain embodiments of the invention a population of microparticles having different shapes sizes and/or colors can be used. The microparticles can optionally be encoded, e.g., with quantum dots such that each microparticle can be individually or uniquely identified.

[0022] The term "sequence" refers to sequence information about a polynucleotide or polypeptide or any portion of the polynucleotide or polypeptide that is two or more units (nucleotides or amino acids) long. The term can also be used as a reference to the polynucleotide or polypeptide molecule itself or a relevant portion thereof. Polynucleotide sequence information relates to the succession of nucleotide bases on the polynucleotide, and in a polypeptide relates to the succession of amino acid side chains in the polypeptide or portion thereof. For example, if the polynucleotide contains bases Adenine, Guanine, Cytosine, Thymine, or Uracil, the polynucleotide sequence can be represented by a corresponding succession of letters A, G, C, T, or U), e.g., a DNA or RNA molecule. Sequences shown herein are presented in a 5'-→3' orientation unless otherwise indicated.

[0023] "Perfectly matched duplex" in reference to probes and template polynucleotides means that one forms a double stranded structure with the other such that each nucleoside in the double stranded structure undergoes Watson-Crick basepairing with a nucleoside on the other. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that

may be employed to reduce the degeneracy of the probes, whether or not such pairing involves formation of hydrogen bonds.

[0024] The term "polymorphism" is given its ordinary meaning in the art and refers to a difference in genome sequence among individuals of the same species. A "single nucleotide polymorphism" (SNP) refers to a polymorphism at a single position.

[0025] "Probes", "oligonucleotides" or "primers" are intended to be interchangeable terms herein, so that any one of these can be taken as a reference to another. These are polynucleotides not necessarily limited to any length. Where so wished, these can be less than 100 nucleotides long, sometimes less than 30 nucleotides long, e.g., less than 20 nucleotides, optionally less than 12 nucleotides, for example less than eight nucleotides in length. In some cases, these are 2, 3, 4, 5, 6, 7, 8, or more nucleotides in length. In some cases, these are 3 or 4 nucleotides in length.

[0026] A "polynucleotide," also called a "nucleic acid," is a linear polymer of two or more nucleotides joined by covalent internucleosidic linkages, or variant or functional fragment thereof. A sequence of letters, such as "ATGCCTG," is intended to represent a polynucleotide sequence in the 5'→3' order from left to right unless otherwise specified. In naturally occuring examples of these, the internucleoside linkage is typically a phosphodiester bond. However, other examples optionally comprise other internucleoside linkages, such as phosphorothiolate linkages and may or may not comprise a phosphate group. In other cases, the polynucleotide can contain non-nucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids (PNAs)) and polymorpholino and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA.

[0027] As used herein, "polynucleotide," "oligonucleotide", "probe", "primer", "template", "nucleic acid" and the like can be taken to refer to a populations or pools of individual molecules that are substantially identical across their entire length or across a relevant portion of interest. For example, the term "template" can indicate a plurality of template molecules that are substantially identical, etc. In the case of polynucleotides that are degenerate at one or more positions, it will be appreciated that the degenerate polynucleotide comprises a plurality of polynucleotide molecules, which have sequences that are substantially identical only at the nondegenerate position(s) and differ in sequence at the degenerate positions. Thus, reference to "a" polynucleotide (e.g., "a" primer, probe, oligonucleotide, template, etc.) can be taken to mean a population of

polynucleotide molecules that are substantially identical over at least a portion of interest, such that the plural nature of the population need not be explicitly indicated, but can if so desired. These terms are also intended to provide adequate support for a claim that explicitly specifies a single polynucleotide molecule itself. It will be understood that members of a population need not be 100% identical, e.g., a certain number of "errors" may occur during the course of synthesis. Preferably at least 90%, at least 95%, at least 99%, or more of the members of a population are substantially identical. Preferably the percent identity of at least 95% or more preferably at least 99% of the members of the population to a reference nucleic acid molecule is at least 98%, 99%, 99.9% or greater. Percent identity may be computed by comparing two optimally aligned sequences, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions, and multiplying the result by 100 to yield the percentage of sequence identity. It will be appreciated that in certain instances a nucleic acid molecule such as a template, probe, primer, etc., may be a portion of a larger nucleic acid molecule that also contains a portion that does not serve a template, probe, or primer function. In that case individual members of a population need not be substantially identical with respect to that portion.

[0028] The nucleotides of a polynucleotide can have any combination of bases, including those mentioned herein, for example uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. Optionally, the polynucleotide is a DNA having the nucleotide bases A, C, T and/or G. Optionally, the polynucleotide is an RNA having the nucleotide bases A, C, T and/or U.

[0029] Polynucleotides include double- and single-stranded DNA, as well as double- and single-stranded RNA, DNA:RNA hybrids, peptide-nucleic acids (PNAs) and hybrids between PNAs and DNA or RNA, and also include known types of modifications, for example, labels which are known in the art, methylation, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), and with positively charged linkages (e.g., aminoalklyphosphoramidates, aminoalkylphosphotriesters), those containing pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen,

etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide or oligonucleotide. Polynucleotides can optionally be attached to one or more non-nucleotide moieties such as labels and other small molecules, large molecules such proteins, lipids, sugars, and solid or semi-solid supports, for example through either the 5' or 3' end. Labels include any moiety that is detectable using a detection method of choice, and thus renders the attached nucleotide or polynucleotide similarly detectable using a detection method of choice. Optionally, the label emits electromagnetic radiation that is optically detectable or visible. In some cases, the nucleotide or polynucleotide is not attached to a label, and the presence of the nucleotide or polynucleotide is directly detected, and/or the generation of byproducts of ligation such as PPi or NMN is detected. Optionally, the presence of the nucleotide, polynucleotide or byproduct is sensed by a chemical field-effect transistor, e.g., where the charge on the gate electrode is generated by a chemical process. Optionally, the chemical field-effect transistor is an ion-sensitive field-effect transistor.

[0030] Where two or more reagents are labeled, the labels are preferably distinguishable from each other with the detection method of choice. For example, the labels can be spectrally resolvable, i.e., distinguishable on the basis of their spectral characteristics, particularly fluorescence emission wavelength, under conditions of operation. In other instances, the label may comprise a signal-generating compound (SGC). A SGC is optionally a substance that it itself detectable in an assay of choice, or capable of reacting to form a chemical or physical entity (i.e., a reaction product) that is detectable in an assay of choice. Representative examples of reaction products include precipitates, fluorescent signals, compounds having a color, and the like. Representative SGC include e.g., bioluminescent compounds (e.g., luciferase), fluorophores (e.g., below), bioluminescent and chemiluminescent compounds, radioisotopes (e.g., ^{131}I, ^{125}I, ^{14}C, ^{3}H, ^{35}S, ^{32}P and the like), enzymes (e.g., below), binding proteins (e.g., biotin, avidin, streptavidin and the like), magnetic particles, chemically reactive compounds (e.g., colored stains), labeled oligonucleotides; molecular probes (e.g., CY3, Research Organics, Inc.), and the like. Representative fluorophores include fluorescein isothiocyanate, succinyl fluorescein, rhodamine B, lissamine, 9,10-diphenlyanthracene, perylene, rubrene, pyrene and fluorescent derivatives thereof such as isocyanate, isothiocyanate, acid chloride or sulfonyl chloride, umbelliferone, rare earth chelates of lanthanides such as Europium (Eu) and the like. Signal generating compounds also

include SGC whose products are detectable by fluorescent and chemiluminescent wavelengths, e.g., sequencing dyes, luciferase, fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series; compounds such as luminol, isoluminol, acridinium salts, and the like; bioluminescent compounds such as luciferin; fluorescent proteins (e.g., GFP or variants thereof); and the like. The subject SGC are optionally detectable using a visual or optical method; preferably, with a method amenable to automation such as a spectrophotometric method, a fluorescence method, a chemiluminescent method, an electrical nanometric method involving e.g., a change in conductance, impedance, resistance and the like and a magnetic field method. Some SGC's are optionally detectable with the naked eye or with a signal detection apparatus when at an appropriate concentration.

[0031] A "nucleotide" refers to a nucleotide, nucleosideor analog thereof. Optionally, the nucleotide is an N- or C-glycoside of a purine or pyrimidine base. (e.g., deoxyribonucleoside containing 2-deoxy-**D**-ribose or ribonucleoside containing **D**-ribose). Examples of other analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides.

[0032] Nucleotide bases or nucleobases usually have a substituted or unsubstituted parent aromatic ring or rings. In certain embodiments, the aromatic ring or rings contain at least one nitrogen atom. In certain embodiments, the nucleotide base is capable of forming Watson-Crick and/or Hoogsteen hydrogen bonds with an appropriately complementary nucleotide base. Exemplary nucleotide bases and analogs thereof include, but are not limited to, purines such as 2-aminopurine, 2,6-diaminopurine, adenine (A), ethenoadenine, N6-A2-isopentenyladenine (6iA), N6-A2-isopentenyl-2-methylthioadenine (2ms6iA), N6-methyladenine, guanine (G), isoguanine, N2-dimethylguanine (dmG), 7-methylguanine (7 mG), 2-thiopyrimidine, 6-thioguanine (6sG) hypoxanthine and 06-methylguanine; 7-deaza-purines such as 7-deazaadenine (7-deaza-A) and 7-deazaguanine (7-deaza-G); pyrimidines such as cytosine (C), 5-propynylcytosine, isocytosine, thymine (T), 4-thiothymine (4sT), 5,6-dihydrothymine, 04-methylthymine, uracil (U), 4-thiouracil (4sU) and 5,6-dihydrouracil (dihydrouracil; **D);** indoles such as nitroindole and 4-methylindole; pyrroles such as nitropyrrole; nebularine; base (Y); etc. In certain embodiments, nucleotide bases are universal nucleotide bases. Additional exemplary nucleotide bases can be found, e.g., in Fasman, 1989, Practical Handbook of Biochemistry and Molecular Biology, pp. 385-394, CRC

Press, Boca Raton, Fla., and the references cited therein. A nucleoside is usually a compound having a nucleotide base covalently linked to the C-1′ carbon of a pentose sugar. In certain embodiments, the linkage is via a heteroaromatic ring nitrogen. Typical pentose sugars include, but are not limited to, those pentoses in which one or more of the carbon atoms are each independently substituted with one or more of the same or different —R, —OR, —NRR or halogen groups, where each R is independently hydrogen, (C1-C6) alkyl or (C5-C14) aryl. The pentose sugar may be saturated or unsaturated. Exemplary pentose sugars and analogs thereof include, but are not limited to, ribose, 2'-deoxyribose, 2'-(Cl-C6)alkoxyribose, 2'-(C5-C14)aryloxyribose, 2',3'-dideoxyribose, 2',3'-didehydroribose, 2'-deoxy-3'-haloribose, 2'-deoxy-3'-fluororibose, 2'-deoxy-3'-chlororibose, 2'-deoxy-3'-aminoribose, 2'-deoxy-3'-(Cl-C6)alkylribose, 2'-deoxy -3'-(Cl-C6)alkoxyribose and 2'-deoxy-3'-(C5-C14)aryloxyribose. One or more of the pentose carbons of a nucleoside may be substituted with a phosphate ester, as disclosed in US Pat. No 7255994. In certain embodiments, the nucleosides are those in which the nucleotide base is a purine, a 7-deazapurine, a pyrimidine, a universal nucleotide base, a specific nucleotide base, or an analog thereof. Nucleotide analogs include derivatives in which the pentose sugar and/or the nucleotide base and/or one or more of the phosphate esters of a nucleoside may be replaced with its respective analog. Exemplary pentose sugar analogs and nucleotide base analog are described above. Exemplary phosphate ester analogs include, but are not limited to, alkylphosphonates, methylphosphonates, phosphoramidates, phosphotriesters, phosphorothioates, phosphorodithioates, phosphoroselenoates, phosphorodiselenoates, phosphoroanilothioates, phosphoroanilidates, phosphoroamidates, boronophosphates, etc., and may include associated counterions. Other nucleotide analogs are nucleotide analog monomers which can be polymerized into polynucleotide analogs in which the DNA/RNA phosphate ester and/or sugar phosphate ester backbone is replaced with a different type of linkage. Exemplary polynucleotide analogs include, but are not limited to, peptide nucleic acids, in which the sugar phosphate backbone of the polynucleotide is replaced by a peptide backbone.

[0033] The internucleoside linkages can be a phosphodiester linkage, although other linkages (e.g., scissile linkages which can be substantially cleaved under conditions in which phosphodiester linkages are not substantially cleaved) can be used. For example, a linkage that contains an AP endonuclease sensitive site, for example an abasic residue, a residue containing a damaged base that is a substrate for removal by a DNA glycosylase,

or another residue or linkage that is a substrate for cleavage by an AP endonuclease, or a disaccharide nucleoside.

[0034] The adjectival term "hybridized" optionally refers to two polynucleotides which are bonded to each other by two or more sequentially adjacent base pairings. The term "hybridization" refers to the process by which polynucleotides become hybridized to each other. Two single-stranded polynucleotides can be regarded as "complementary" if when hybridized together the longer polynucleotide forms a single-stranded overhang and the shorter polynucleotide can be efficiently ligated to a third adjacent polynucleotide that forms a perfectly-matched duplex with the single-stranded overhang. Where the single-stranded overhang is less than eight nucleotides, it can be arbitrarily lengthened to eight nucleotides by adding a random combination of nucleotides to the overhang.

[0035] Similarly, nucleotide residues can be regarded as complementary if when both are base-paired with each other within two hybridized polynucleotides, either nucleotide can be ligated in a template-driven ligation reaction when situated as the terminal nucleotide in its polynucleotide. Nucleotides that are efficiently incorporated by DNA polymerases opposite each other during DNA replication under physiological conditions are also considered complementary. In an embodiment, complementary nucleotides can form base pairs with each other, such as the A-T/U and G-C base pairs formed through specific Watson-Crick type hydrogen bonding between the nucleobases of nucleotides and/or polynucleotides positions antiparallel to each other. The complementarity of other artificial base pairs can be based on other types of hydrogen bonding and/or hydrophobicity of bases and/or shape complementarity between bases.

[0036] In appropriate instances, polynucleotides can be regarded as complementary when they can undergo cumulative base pairing at two or more individual corresponding positions in antiparallel orientation, as in a hybridized duplex. Optionally there can be "complete" or "total" complementarity between a first and second polynucleotide sequence where each nucleotide in the first polynucleotide sequence can undergo a stabilizing base pairing interaction with a nucleotide in the corresponding antiparallel position on the second polynucleotide. "Partial" complementarity describes polynucleotide sequences in which at least 20%, but less than 100%, of the residues of one polynucleotide are complementary to residues in the other polynucleotide. A "mismatch" is present at any position in which the two opposed nucleotides are not complementary. In some ligation assays, a polynucleotide can undergo substantial template-dependent ligation even when it has one or more mismatches to its hybridized

template. Optionally, the polynucleotide has no more than 4, 3, or 2 mismatches, e.g., 0 or 1 mismatch, with its template. In some assays, the polynucleotide will not undergo substantial template-dependent ligation unless it is at least 60% complementary, e.g., at least about 70%, 80%, 85%, 90%, 95%, 99% or 100% complementary to its template.

[0037] As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, plasma, serum, spinal fluid, semen, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of in vitro cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components).

[0038] Sequence identity (also called homology) refers to similarity in sequence of two or more sequences (e.g., nucleotide or polypeptide sequences). In the context of two or more homologous sequences, the percent identity or homology of the sequences or subsequences thereof indicates the percentage of all monomeric units (e.g., nucleotides or amino acids) that are the same (i.e., about 70% identity, preferably 75%, 80%, 85%, 90%, 95% or 99% identity). The percent identity can be over a specified region, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection. Sequences are said to be "substantially identical" when there is at least 90% identity at the amino acid level or at the nucleotide level. Preferably, the identity exists over a region that is at least about 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20, 25, 50, or 100 residues in length, or across the entire length of at least one compared sequence. A preferred algorithm for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al, Nuc. Acids Res. 25:3389-3402 (1977). Other methods include the algorithms of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), andNeedleman & Wunsch, J. Mol. Biol. 48:443 (1970), etc. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions.

[0039] In the claims, any active verb (or its gerund) is intended to indicate the corresponding actual or attempted action, even if no actual action occurs. For example, the verb "hybridize" and gerund form "hybridizing" and the like refer to actual

hybridization or to attempted hybridization by contacting nucleic acid sequences under conditions suitable for hybridization, even if no actual hybridization occurs. Similarly, "detecting" and "detection" when used in the claims refer to actual detection or to attempted detection, even if no target is actually detected.

[0040] "Nonspecific hybridization" is used to refer to any unintended or insignificant hybridization, for example hybridization to an unintended polynucleotide sequence other than the intended target polynucleotide sequence. The uninentended polynucleotide sequence can be on the same or different polynucleotide from the intended target. In some cases, the only intended hybridization can be from Watson-Crick base pairing between two polynucleotides. Other kinds of intended base pairings can include base pairing between corresponding analogs of such nucleotides or between iso-cytidine and iso-guanine. In some cases where hybridization is only intended between complementary bases, any bonding between non-complementary bases is considered to be non-specific hybridization.

[0041] In reference to ligation of two polynucleotides, the "proximal" terminus of either polynucleotide is the terminus that is intended to be ligated to the other polynucleotide. This is generally the terminus that is contacted by the active site of the ligase, or the terminus that is eventually ligated to the other polynucleotide, while the opposite terminus is the "distal" terminus. The terminal nucleotide residue at the proximal terminus can be termed the proximal nucleotide, and the proximal nucleotide position optionally designated as position 1, or -1 depending on which side of the ligation site we are referring to, the penultimate nucleotide position as position 2 or -2, etc. With reference to two adjacently-hybridized polynucleotides, the proximal terminus is generally the terminus of one polynucleotide that is closer to the other polynucleotide. In some non-limiting instances of template-dependent ligation, the proximal termini of both polynucleotides are hybridized adjacently to each other.

[0042] "Support", as used herein, refers to a structure or matrix on or in which ligation reagents, e.g., nucleic acid molecules, microparticles, and the like may be immobilized so that they are significantly or entirely prevented from diffusing freely or moving with respect to one another. The reagents can for example be placed in contact with the support, and optionally covalently or noncovalently attached or partially/completely embedded

[0043] A "universal base", as used herein, is a base that is complementary to more than one other base. Fully universal bases can pair with any of the bases typically found

in naturally occurring nucleic acids. The base need not be equally capable of pairing with each of the naturally occurring bases. Alternatively, the universal base may pair only or selectively with two or more bases but not all bases. Optionally the universal base pairs only or selectively with purines, or alternatively with pyrimidines. If so desired, two or more universal bases can be included at a particular position in a probe. A number of universal bases are known in the art including, but not limited to, inosine, hypoxanthine, 3-nitropyrrole, 4- nitroindole, 5-nitroindole, 4-nitrobenzimidazole, 5-nitroindazole, 8-aza-7-deazaadenine, 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one, 2-amino-6-methoxyaminopurine, etc. Hypoxanthine is one preferred fully univeral base. Nucleosides comprising hypoxanthine include, but are not limited to, inosine, isoinosine, 2'-deoxy inosine, and 7-deaza-2'-deoxyinosine, 2-aza-2'deoxyinosine.

[0044] "Purified" generally refers to isolation of a substance (compound, polynucleotide, protein, polypeptide, polypeptide composition) such that the substance comprises a significant percent, such as a higher proportion than it is naturally found (e.g., greater than 2%, greater than 5%, greater than 10%, greater than 20%, greater than 50%, or more, sometimes more than 90%, 95% or 99%) of the sample in which it resides. In certain embodiments, a substantially purified component comprises at least 50%, 80%-85%, or 90-95% of the sample. Techniques for purifying polynucleotides and polypeptides of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density. "Isolated" refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is altered "by the hand of man" from its natural state. For example, an isolated polynucleotide could be part of a vector or a composition of matter, or could be contained within a cell, and still be "isolated" because that vector, composition of matter, or particular cell is not the original or naturally-occurring environment of the polynucleotide.

[0045] Exemplary ligases comprise a polypeptide. The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues, or any variant or functional fragment thereof. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers.

[0046] The term "amino acid" includes naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar

to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, .gamma.-carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0047] Variants or derivatives of a given nucleotide sequence or polypeptide sequence are optionally conservatively modified variants. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences.

[0048] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention. (see e.g., Creighton, Proteins (1984)).

Sequence identity (also called homology) refers to similarity in sequence of two or more sequences (e.g., nucleotide or polypeptide sequences). In the context of two or more homologous sequences, the percent identity or homology of the sequences or subsequences thereof indicates the percentage of all monomeric units (e.g., nucleotides or amino acids) that are the same (i.e., about 70% identity, preferably 75%, 80%, 85%, 90%, 95% or 99% identity). The percent identity can be over a specified region, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual

inspection. Sequences are said to be "substantially identical" when there is at least 90% identity at the amino acid level or at the nucleotide level. This definition also refers to the complement of a test sequence. Preferably, the identity exists over a region that is at least about 25, 50, or 100 residues in length, or across the entire length of at least one compared sequence. A preferred algorithm for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al, Nuc. Acids Res. 25:3389-3402 (1977). Other methods include the algorithms of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), and Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), etc. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements specifically hybridize to each other under stringent conditions, such as those described herein. Nucleic acids that do not specifically hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical.

[0049] Two polynucleotides selectively (or specifically) hybridize to each other if they bind significantly or detectably to each other under stringent hybridization conditions when present in a complex polynucleotide mixture such as total cellular or library DNA. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Optionally, stringent conditions are selected to be about 5-10° C. lower than the thermal melting point for the specific sequence at a defined ionic strength pH. Stringent conditions are optionally in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5.times.SSC, and 1% SDS, incubating at 42° C , or, 5.times.SSC, 1% SDS, incubating at 65° C , with wash in 0.2.times.SSC, and 0.1% SDS at 65° C.

Detailed Description

[0050] Among other novel and surprising information presented herein, the novel and surprising enzymatic ligation of short polynucleotides is presented herein.

1) LIGATIONS

[0051] Ligation herein refers to the enzymatic formation of a covalent bond between the termini of two or more or polynucleotides strands. "Ligation" involves the formation of a covalent bond or linkage between the 5' and 3' termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, optionally in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation is preferably achieved enzymatically. The nature of the bond or linkage can vary widely. Non-limiting exemplary ligations are carried out enzymatically to form a phosphodiester linkage between a 5' terminal nucleotide of a polynucleotide strand with a 3' terminal nucleotide of a polynucleotide.

[0052] The ligation can be one or more of the following types of ligation described herein. A first type of enzymatic ligation involves the formation of a covalent bond between a first terminus of a first polynucleotide strand and a second different terminus of a second polynucleotide strand. The first and second polynucleotide termini can be on different polynucleotide strands, or can both be on the same polynucleotide strand (resulting in circularization). Optionally, the first and second polynucleotide strands are not both hybridized to a third polynucleotide. Optionally, the termini of the first and second polynucleotide strand are joined irrespective of their sequences (e.g., blunt-end ligation, or non-homologous end joining). In another variation, two double-stranded polynucleotides with protruding single-stranded portions that are complementary to each other can be ligated (e.g., cohesive-end ligation). A third type of ligation (template-dependent ligation) is described further below.

[0053] In any one of the methods described herein, the polynucleotide strands can be in single-stranded format or are hybridized to complementary strands in double-stranded format. In blunt-end ligation, both polynucleotide strands to be ligated are hybridized to two different complementary strands such that no overhang exists.

[0054] Methods are provided to ligate two polynucleotides. An exemplary ligation method achieves ligation between a first terminus of a first polynucleotide sequence and a second terminus of a second polynucleotide sequence. For ease of reference, the first polynucleotide sequence is called the "initializing probe" or "primer," the second polynucleotide called the "extension probe" or "probe." A third polynucleotide sequence that is optionally present (e.g., in template-dependent ligation) is termed the template or target sequence. Any one or more of the primer (initializing probe), probe (extension

probe) and/or template sequences can be located on the same polynucleotide strand, or on different polynucleotide strands. In a one-strand system, the primer, probe and template are all on the same polynucleotide strand. In an example of a two-strand system, either the probe or primer (but not both) are on the same strand as the template (e.g., hybridization between the template sequence and the primer or probe sequences forms a stem-loop structure or hairpin). In another example of a two-strand system, the probe or primer are both on the same strand. In a three-strand system, the template, primer and probe are all on separate polynucleotide strands. Any ligation method described herein can be performed in a one-strand, two-strand or three-strand system.

[0055] Optionally, ligation converts a linear polynucleotide strand into a circular polynucleotide strand (e.g., in a one-stranded to two-stranded system). Optionally, ligation reduces the number of polynucleotide strands by one (e.g., in a two-stranded or three-stranded system).

[0056] Ligation optionally creates a bond between a terminal nucleotide of the probe with the terminal nucleotide of the primer. Optionally, the proximal terminus of the primer and/or probe is ligated. The terminal nucleotide of the primer can be the 5' terminal nucleotide and the terminal nucleotide of the extension probe can be the 3' terminal nucleotide. Alternatively, the terminal nucleotide of the primer can be the 3' terminal nucleotide and the terminal nucleotide of the extension probe can be the 5' terminal nucleotide. The 5' terminus of a polynucleotide for example has the fifth carbon in the sugar-ring of the deoxyribose or ribose at its terminus, optionally with a phosphate group attached to it, where the phosphate group is capable of forming a phosphodiester bond with a 3' terminal nucleotide. The 3' terminal nucleotide optionally has a 3'-hydroxyl group that is capable of forming a phosphodiester bond with a 5' terminal nucleotide. Ligation optionally results in the formation of a phophodiester bond.

[0057] Any of the polynucleotides no matter how designated (e.g., as "probes" or "primers" or "templates") can be of any sequence, any length, in any form and from any source. The polynucleotides can comprise a naturally-occurring sequence or be highly homologous to a naturally-occurring sequence, and/or derived from a naturally occurring sequence. The naturally occurring sequence can be any portion of a gene, a regulatory sequence, genomic DNA or fragment, cDNA, RNA including mRNA and rRNA, or others. The polynucleotides can optionally comprise any artificial sequence as well. The polynucleotide can be derived or obtained from a sample such as a diagnostic sample. The polynucleotide can be a secondary product of a reaction - for example a ligation

product from a ligation reaction or assay such as those described herein, an extended probe from a PCR reaction, or PCR amplification product, ("amplicon"), the product of an invasive cleavage reaction, etc. The polynucleotide can have a 5' phosphate, or can alternatively lack a 5' phosphate.

[0058] The ligation product of any one reaction can optionally be subjected to further ligation and/or non-ligation reactions in turn. For example, the ligation product can be used as the primer (initializing probe) or extension probe and/or template in a subsequent ligation. Also for example, it can be used as a template and/or primer for a polymerase extension reaction, such as in PCR. The probe, primer, template and/or ligation product optionally can be subjected to any one or more modifications before or after ligation. For example, the probe, primer, template and/or ligation product can be cleaved enzymatically or chemically (for example at scissile linkages), treated with exo- or endonucleases, kinases, phosphatases, etc. The ends of a double-stranded product can be blunt-ended or filled in, capped, or adenylated, etc.

[0059] Optionally, the probe is not more than 20 consecutive nucleotides long, for example not more than 15, 12, 10, 8, 7 consecutive nucleotides, preferably not more than 6, 5, 4 3, or 2 consecutive nucleotides. Optionally, the probe is at least 2, 3, 4, 5, 6 or 7 nucleotides long. In some instances the probe is from any of the specified minimum lengths (e.g., 2, 3, 4, 5, 6 or 7 nucleotides long) and is not more than 20, 15, 12, 10, 8, 7 nucleotides, preferably not more than 6, 5, 4 3, or 2 nucleotides. In some examples, the probe is a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, 7-mer, 8-mer, 9-mer, 10-mer, 11-mer, 12-mer, 13-mer, 14-mer, 15-mer or 20-mer, or any combination of such oligonucleotides.

[0060] Optionally, the primer is not more than 20 consecutive nucleotides long, for example not more than 15, 12, 10, 8, 7 consecutive nucleotides, preferably not more than 6, 5, 4, 3, or 2 consecutive nucleotides. In some examples, the primer is one or more 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, 7-mer, 8-mer, 9-mer, 10-mer, 11-mer, 12-mer, 13-mer, 14-mer, 15-mer or 20-mer, or any combination of such oligonucleotides.

[0061] It should be understood that the probe or primer (or template, if present) can be "mixed" or "composite," i.e., comprising a mixture of one or more polynucleotides of different sequences.

[0062] Optionally, the ligation is performed using CV ligase in combination with one or more probes that is at least 3 nucleotides long and not more than 6 or 5 nucleotides long. Optionally, the one or more probes are at least 3 nucleotides long and not more than 4 nucleotides long. In other examples the one or more probes are at least 4

nucleotides long and not more than 5 nucleotides long. Alternatively all of the one or more probes probe can be 3-mers. Otherwise all of the one or more probes probe can be 4-mers.

[0063] When the ligation is performed using DLX, DLXd, DLXd2 ligase, then optionally ligation is performed with one or more probes that is at least 2 or 3 nucleotides long and not more than 6 or 5 nucleotides long. Optionally, the one or more probes are at least 3 nucleotides long and not more than 4 nucleotides long. In other examples the one or more probes are at least 4 nucleotides long and not more than 5 nucleotides long. Alternatively all of the one or more probes probe can be 3-mers. Otherwise all of the one or more probes probe can be 4-mers.

[0064] The ligation can be a template-dependent ligation. In template-dependent ligation, ligation between a primer sequence and a probe sequence occurs upon hybridization of at least a portion of either or both sequences to a template sequence. In some instances, both probes must hybridize to the template for significant ligation to occur. In a typical example, template-dependent ligation cannot take place unless both polynucleotides are hybridized to the template sequence. The portion of the primer or probe that is hybridized to the target sequence is generally at least two nucleotides long. The hybridized portion is optionally not more than 20 consecutive nucleotides long, for example not more than 15, 12, 10, 8, 7 consecutive nucleotides, preferably not more than 6, 5, 4, 3, or 2 consecutive nucleotides. The hybridized portion is optionally a terminal portion of the first or second polynucleotide (e.g., a portion that includes the 5' or 3' terminal nucleotide). For example, the hybridized portion can consist of the terminal 2, 3, 4, 5, 6, 7, 8, 10, 15 or 20 nucleotides of the 5' or 3' end.

[0065] Optionally, ligation occurs when no mismatch is present within one or more hybridized portions. In other cases, ligation occurs when one, two or three mismatches can be present within one or more hybridized portions. In some cases ligation does not occur when the terminal nucleotide and/or second-most terminal nucleotide and/or third-most terminal nucleotide is mismatched. As mentioned, the terminal nucleotides can be the 5'- or 3'- terminal nucleotides of the polynucleotide.

[0066] Optionally the template, if present, is not more than 11 nucleotides in length, for example not more 10, 9, 8, 7, 6, 5, or 4 nucleotides. Optionally the template is one or more N-mers, where N is 4, 5, 6, 7, 8, 9, 10 or 11.

[0067] Optionally, template-dependent ligation of a nucleic acid comprises: a) providing a first oligonucleotide having less than 6 nucleotides; b) providing a second

oligonucleotide; c) bringing the 3' termini of one of the first and second oligonucleotides into proximity with the 5' termini of the other oligonucleotide; and d) ligating the first and the second oligonucleotides. Optionally, the first oligonucleotide has a length of 5 nucleotides. Optionally, the first oligonucleotide has a length of 4 nucleotides. Optionally, the first oligonucleotide has a length of 3 nucleotides. Optionally, the ligation is performed using a small-footprint ligase (SFL). Optionally, the second oligonucleotide includes a sequence complementary to a portion of a template nucleic acid. Optionally, the second oligonucleotide is hybridized to the template nucleic acid at the region of complementarity. Optionally, the first oligonucleotide has a sequence complementary to the template nucleic acid. Optionally, the first oligonucleotide is hybridized to the template nucleic acid at the region of complementarity, and wherein a terminus of the first oligonucleotide is adjacent to a terminus of the second oligonucleotide.

[0068] In some variations, (e.g., "nick ligation" or "template-dependent" ligation), both primer and probe must hybridize adjacently to each other on the template for ligation to occur. Optionally, the probe and primer are adjacently hybridized and can be ligated only when a terminal nucleotide of the primer is hybridized to a first nucleotide of the template and a terminal nucleotide of the extension probe is hybridized to a second nucleotide of the template, where the first and second nucleotides on the template are not separated by an intervening nucleotide of the template. In other embodiments, intervening nucleotides may be present between the first and second nucleotides on the template (optionally a few nucleotides, e.g., not more than 1, 2, 3, 5, 10 or 15 nucleotides). In such embodiments, a "gap-filling" step can be performed to extend the 3' terminus of the probe or probe before it can be ligated to the 5' terminus of the other.

[0069] Optionally, at least one of the probe, the template (if the ligation is template-dependent) and/or the primer is immobilized while another of these three is labeled. For example in ligation sequencing the template and/or primer can be immobilized and the probe can be labeled.

[0070] A probe can for example be N nucleotide residues in length, where N is from 2 to 10, e.g., 2, 3, or 4. N can also be less than 6, for example if the proximal terminus of the probe is its 3' terminus.

[0071] Optionally, ligation is a "forward" ligation (i.e., ligation of the 3' terminus of the probe to the 5' terminus of the primer). Alternatively, "reverse" ligation can be achieved, where the 5' terminus of the probe is ligated to the 3' terminus of the primer.

[0072] A probe can also be of length N, and can comprise a proximal portion that is perfectly hybridized to the template and is L nucleotides long, where the probe's L+1th nucleotide is mismatched with the template. L can be, for example, from 2 to 8, and furthermore can be less than 6 if the proximal terminus of the probe is its 3' terminus. In other embodiments, L can be 2, 3, 4, 5, 6, 7 or 8.

[0073] The ligation can be repeated at least once, for any desired number of cycles. Optionally, any ligation product of a previous ligation reaction is used as the primer of a next ligation. Optionally, all ligations extend the ligation product in the same direction. For example, all ligation reactions can be forward ligations or all can be reverse ligations. In other embodiments, some ligations can be forward and some reverse. Optionally any unligated primer is rendered unligatable before initiating the next ligation. If so desired (e.g., in ligation sequencing), the method further comprises detecting whether the probe has ligated to the primer before repeating the next ligation reaction.

[0074] Where so desired, any ligation product of the previous ligation reaction can be used as the template of the next ligation reaction, e.g., in a ligase chain reaction.

[0075] Optionally, the 5' end of the probe less than 6 nucleotides long is ligated to the 3' end of the primer by a SFL such as CV ligase. For example the probe is 2, 3 or 4 nucleotides long. If so desired, the primer is also a short oligonucleotides. For example, the primer can be less than 6 nucleotides, e.g., 3 or 4 nucleotides long.

[0076] The ligation should produce a significant or detectable amount of ligation product. Optionally, the efficiency of ligation is at least 5%, sometimes at 10%, 20%, 30%, 50%, 60%, 70%, 75%, 80%, 85%, 90% or 95%. The efficiency of ligation (in percentage terms) can in some embodiment be regarded as the percent portion of ligation reagent that is ligated to form ligation product at the end of the ligation reaction. Efficiency is optionally determined after a ligation reaction has reach equilibrium such that increasing the ligation time will not result in a substantial increase of ligation product formed. Generally, a ligation reaction can be said to have reached equilibrium after 20 minutes. For example, a ligation reaction in which 90% of the primer or probe is ligated can be said to have proceeded at 90% efficiency. The ligation reagent used to measure ligation efficiency is optionally whichever reagent that is in lower concentration than the others. Optionally, the other reagents and conditions are non-limiting to the ligation efficiency (e.g., other reagents are present in excess or at a concentration that is at least equal to or higher than the limiting reagent).

[0077] In some embodiments, the SFL can ligate a short probe that is shorter than N nucleotides at least X% as efficiently as the SFL can ligate the corresponding N-mer. N is optionally 4, 5, 6, 7, 8, 10, 12, 15 or 20. In some embodiments N is 6 or 7. X is optionally at least 30%, 50%, 60%, 70%, 80% or 90%. In some embodiments of template-dependent ligation, the length of short probe is Y residues, and the Y proximal residues of the corresponding longer N-mer are identical to the probe, and the distal N-Y residues of the corresponding longer N-mer are perfectly complementary to the template. Y is for example 2, 3, 4, 5 or 6.

[0078] In some embodiments, the SFL can ligate a short probe that is shorter than N nucleotides when the short probe is conjugated to a dye approximately as efficiently as the SFL can ligate the unconjugated probe. N is optionally 4, 5, 6, 7, 8, 10, 12, 15 or 20. In some embodiments N is 6 or 7. In other embodiments, the SFL can ligate the short probe conjugated to a dye at least 30%, 50%, 60%, 70%, 80% or 90% as efficiently as the SFL can ligate the unconjugated probe. Exemplary dyes include Cy5.

[0079] It is understood that ligation efficiency (whether expressed in absolute or relative terms) may increase or decrease depending on the exact reaction conditions used. Optionally, the ligation efficiency is measured when the primer or probe is at a concentration of about 10^{-9} to 10^{-4} M, for example at about 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M. In certain applications, for example sequencing applications, a working concentration often used in the range of 10^{-8} to 10^{-5}, e.g., 10^{-7}or 10^{-6} M of probe. Where a mixture of probes are used, the probe concentration is the concentration of only those probes that are capable of being ligated in that particular ligation reaction (e.g., probe(s) complementary to the template). Thus other probes that are not capable of participating in the ligation reaction are optionally not considered when calculating the concentration of the probe of interest. Optionally, the concentration of probe is between 1 picomolar and 1 millimolar, for example about 0.01-100 μM, e.g., 1-10 μM, e.g., 1-5 μM. In the case of 2-mers, the concentration is optionally increased to 10-1000 μM, for example about 100 μM. Optionally, the concentration of ligase is between 1 picomolar and 1 millimolar, e.g., 1-2 micromolar.. Optionally, the ligation assay is performed at 15-35°C, for example 15 °C, 20 °C, 25 °C or 30 °C. Where two or more reagents are involved and the concentration of one particular reagent is specified, the other reagents are optionally in excess and/or not at a concentration that is limiting for the ligation.

[0080] Ligation can be performed under in vitro conditions that have been experimentally determined to be suitable or optimal for ligase activity. Preferably, the

reaction conditions of choice are (i) substantially similar to in vivo or physiological conditions in which a naturally-occurring form of the ligase being used is active, or (ii) have been experimentally determined to result in a ligation efficiency that is comparable to or better than the efficiency obtained using conditions of type (i). If exemplary in vitro ligation conditions are specified herein for a particular SFL, then substantially similar reaction conditions are generally appropriate for that particular ligase. In other embodiments, the conditions are such that the reference ligation assay produces significant or detectable ligation within 30 minutes, within 10 minutes, within 1 minute, or within ten seconds. Another non-limiting example of a significant or detectable efficiency of ligation generates in the range of 100 pM of ligation product, optionally about 1000 pM or 10,000 pM.

[0081] Optionally, relative efficiency can be expressed as relative percent efficiency, which can be calculated as A/B x 100, where A is the percent of test reagent (e.g., probe) ligated in a test assay and B is the percent of the reference reagent (e.g., reference probe) that is ligated in a reference assay. Where the relative efficiency of ligation is specified in comparative or relative terms by comparison to a reference ligation assay, it is implicit that all other reagents and conditions (e.g., temperature, concentration of all reagents, pH, concentration of requisite ions such as Mg++ and Mn++, concentration of requisite cofactors such as NAD and/or ATP, salts, buffers, molar concentrations of all reagents, including enzyme, template, probe, primer, oligonucleotides, etc) are otherwise kept identical. For example, a proviso that a SFL can ligate a short (e.g., less than 6 nucleotides) probe at least X% as efficiently as the SFL can ligate a corresponding octanucleotide, can be taken to mean that the two different ligation assays all reagents except for the probes (e.g., primer, template, enzymes and any other reagents) and all reaction conditions (e.g., temperature, reagent concentrations, concentrations of any other reagents, etc) are kept the same for practical purposes.

[0082] Optionally, the ligase has a better ligation efficiency than T4 DNA ligase, for example in any method described herein. In an embodiment, the ligation efficiency is higher than that of T4 DNA ligase for the same mix of probe(s), primer(s) and template(s), in any ligation method and chosen conditions, including any described herein. The ligation efficiency is for example at least 5% higher than T4 ligase, optionally at least 10%, 15% or 20% higher. The increase in efficiency should be statistically reliable and significant, e.g., with a confidence interval of at least 95%, 99%, 99.9%, 99.99%, or 99.999999%. In an example, the SFL shows higher efficiency than T4

DNA ligase when ligating a probe of 8 nucleotides or less to a primer in the forward or reverse direction. The ligase, template, probe, primer and/or ligation assay conditions are for example any described herein.

[0083] The ligase optionally has good ligation fidelity. The ligation fidelity can be assessed as the percentage of incorrect ligation events for a given combination of ligase, template, primer and probe. An incorrect ligation event is one in which the probe or the primer is not perfectly complementary to the template, or the ligation product is not perfectly complementary to the template. In an embodiment, the ligation fidelity is higher than that of T4 DNA ligase for the same mix of probe(s), primer(s) and template(s), in any ligation method and chosen conditions, including any described herein. The ligation fidelity is for example at least 5% higher than T4 ligase, optionally at least 10%, 15% or 20% higher. The increase in fidelity should be statistically reliable and significant, e.g., with a confidence interval of at least 95%, 99%, 99.9%, 99.99%, or 99.999999%. In an example, the SFL shows higher fidelity than T4 DNA ligase when ligating a probe of 8 nucleotides or less to a primer in the forward or reverse direction. The ligase, template, probe, primer and/or ligation assay conditions are for example any described herein.

2) SMALL FOOTPRINT LIGASES

[0084] Optionally, the enzymatic ligation of polynucleotides is achieved by a small footprint ligase (SFL). For example, any ligation method provided herein can use a ligase shown in Table 1A, IB or 1C. A SFL is a ligase that can ligate short polynucleotides. As used herein, the term "ligase" is intended to include any fragment or variant or derivative of that ligase. The fragment or variant or derivative optionally possesses one or more functional activities of a ligase. A SFL optionally comprises a polypeptide having any one or more of the following activities: (1) nucleophilic attack on ATP or NAD^+ resulting in release of PPi or NMN and formation of a covalent ligase-adenylate intermediate; (2) transferring the adenylate to the 5'-end of the 5'-phosphate-terminated DNA strand to form DNA-adenylate (e.g., the 5'-phosphate oxygen of the DNA strand attacks the phosphorus of ligase-adenylate); and (3) formation of a covalent bond joining the polynucleotide termini and liberation of AMP (e.g., by the attack by the 3'-OH on DNA-adenylate). Optionally, the SFL can mediate any one or more of the following bond transformations: from phosphoanhydride (ATP) to phosphoramidate (ligase-

adenylate); from phosphoramidate (ligase-adenylate) to phosphoanhydride (DNA-adenylate); or from phosphoanhydride (DNA-adenylate) to phosphodiester (sealed DNA). Thus, exemplary SFLs can comprise a polypeptide sequence that is homologous to or a variant of a known SFL sequence or any portion thereof. Exemplary SFLs optionally have amino acid sequence identity of at least 70%, optionally at least 85%, optionally at least 90, 95%, 97% or 99%, with a known ligase or known SFL.

[0085] Representative examples of SFLs include CV ligase, DLX, DLXd, DLXd2 and MnM ligase. A preferred SFL is Chlorella Virus ligase. Some exemplary ligases are identified and their GI or accession numbers are provided in Table 1A below:

Table 1A

PRK08224		
Organism	**Protein name**	**Accession**
B.Acidobacteria		
Candidatus Koribacter versatilis Ellin345Candidatus Koribacter (1 proteins)	ATP-Dependent DNA Ligase	YP_592504
Candidatus Solibacter usitatus Ellin6076Candidatus Solibacter (1 proteins)	ATP-Dependent DNA Ligase	YP_826317
CActinobacteria		
Acidothermus cellulolyticus 11BAcidothermus (1 proteins)	ATP-Dependent DNA Ligase	YP_873134
Actinosynnema mirum DSM 43827Actinosynnema (1 proteins)	ATP-Dependent DNA Ligase	YP_003099374
Arthrobacter aurescens TC1Arthrobacter (3 proteins)	ATP-Dependent DNA Ligase	YP_949544
Arthrobacter chlorophenolicus A6Arthrobacter (3 proteins)	ATP-Dependent DNA Ligase	YP_002489901
Arthrobacter sp. FB24Arthrobacter (3 proteins)	ATP-Dependent DNA Ligase	YP_833558
Beutenbergia cavernae DSM 12333Beutenbergia (1 proteins)	ATP-Dependent DNA Ligase	YP_002880505
Catenulispora acidiphila DSM 44928Catenulispora (2 proteins)	ATP-Dependent DNA Ligase	YP_003116519

Catenulispora acidiphila DSM 44928Catenulispora (2 proteins)	ATP-Dependent DNA Ligase	YP_003 116565
Frankia alni ACN14aFrankia (2 proteins)	ATP-Dependent DNA Ligase	YP_712338
Frankia sp. EANlpecFrankia (2 proteins)	ATP-Dependent DNA Ligase	YP_00 1509433
Kineococcus radiotolerans SRS30216Kineococcus (1 proteins)	ATP-Dependent DNA Ligase	YP_00136 0406
Kytococcus sedentarius DSM 20547Kytococcus (1 proteins)	ATP-Dependent DNA Ligase	YP_003 149340
Mycobacterium abscessus ATCC 19977Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001701033
Mycobacterium avium 1(MMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_879648
Mycobacterium avium subsp. paratuberculosis K-1 (Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	NP_959275
Mycobacterium gilvum PYR-GCKMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_00 1132524
Mycobacterium gilvum PYR-GCKMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001132543
Mycobacterium marinum MMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001 853525
Mycobacterium smegmatis str. MC2 155Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_890520
Mycobacterium smegmatis str. MC2 155Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_890521
Mycobacterium sp. JLSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001073538
Mycobacterium sp. JLSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001073546
Mycobacterium sp. JLSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001073574
Mycobacterium ulcerans Agy99Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_907815
Mycobacterium vanbaalenii PYR-	ATP-Dependent DNA	YP_956315

1Mycobacterium (26 proteins)	Ligase	
Mycobacterium vanbaalenii PYR-1Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_956321
Mycobacterium tuberculosis H37RaMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001285120
Mycobacterium tuberculosis H37RvMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	NP_2 18248
Mycobacterium bovis AF2122/97Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	NP_857396
Mycobacterium bovis BCG str. Pasteur 1173P2Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_979871
Mycobacterium bovis BCG str. Tokyo 172Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_002646832
Mycobacterium tuberculosis CDC1551Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	NP_338389
Mycobacterium tuberculosis F1 1Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_001289690
Mycobacterium tuberculosis KZN 1435Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_003033779
Mycobacterium sp. KMSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_940984
Mycobacterium sp. MCSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_642076
Mycobacterium sp. KMSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_941006
Mycobacterium sp. MCSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_642099
Nakamurella multipartita DSM 44233Nakamurella (1 proteins)	ATP-Dependent DNA Ligase	YP_003200226
Nocardia farcinica IFM 10152Nocardia (1 proteins)	ATP-Dependent DNA Ligase	YP_1 18771
Nocardioides sp. JS614Nocardioides (1 proteins)	ATP-Dependent DNA Ligase	YP_922117
Rhodococcus erythropolis PR4Rhodococcus (3 proteins)	ATP-Dependent DNA Ligase	YP_002768423
Rhodococcus jostii RHA1 Rhodococcus (3	ATP-Dependent DNA	YP_705046

proteins)	Ligase	
Rhodococcus opacus B4Rhodococcus (3 proteins)	ATP-Dependent DNA Ligase	YP_002782360
Saccharopolyspora erythraea NRRL 2338Saccharopolyspora (1 proteins)	ATP-Dependent DNA Ligase	YP_001104098
Salinispora arenicola CNS-205 Salinispora (2 proteins)	ATP-Dependent DNA Ligase	YP_001536378
Salinispora tropica CNB-440Salinispora (2 proteins)	ATP-Dependent DNA Ligase	YP_001 158390
Streptomyces avermitilis MA-4680Streptomyces (3 proteins)	ATP-Dependent DNA Ligase	NP_822873
Streptomyces coelicolor A3(2)Streptomyces (3 proteins)	ATP-Dependent DNA Ligase	NP_630780
Streptomyces griseus subsp. griseus NBRC 13350Streptomyces (3 proteins)	ATP-Dependent DNA Ligase	YP_00 1822536
F.Chlamydiae/Verrucomicrobia		
Opitutus terrae PB90-lOpitutus (1 proteins)	ATP-Dependent DNA Ligase	YP_001821013
N.Alphaproteobacteria		
Bradyrhizobium japonicum USDA llOBradyrhizobium (3 proteins)	ATP-Dependent DNA Ligase	NP_771853
Bradyrhizobium sp. BTAilBradyrhizobium (3 proteins)	ATP-Dependent DNA Ligase	YP_00123 6299
Bradyrhizobium sp. ORS278Bradyrhizobium (3 proteins)	ATP-Dependent DNA Ligase	YP_00 1202307
Chelativorans sp. BNClChelativorans (1 proteins)	ATP-Dependent DNA Ligase	YP_675242
Mesorhizobium loti MAFF303099Mesorhizobium (2 proteins)	ATP-Dependent DNA Ligase	NP_108245
Mesorhizobium loti MAFF303099 (plasmid)Mesorhizobium (2 proteins)	ATP-Dependent DNA Ligase	NP_109531
Methylocella silvestris BL2Methylocella (1 proteins)	ATP-Dependent DNA Ligase	YP_002363964
Nitrobacter hamburgensis X14Nitrobacter (1	ATP-Dependent DNA	YP_579055

proteins)	Ligase	
Phenylobacterium zucineum HLKIPhenylobacterium (1 proteins)	ATP-Dependent DNA Ligase	YP_002131547
Rhizobium leguminosarum bv. trifolii WSM2304 (plasmid)Rhizobium (2 proteins)	ATP-Dependent DNA Ligase	YP_002279148
Rhizobium sp. NGR234 (plasmid)Rhizobium (2 proteins)	ATP-Dependent DNA Ligase	YP_002823307
	ATP-Dependent DNA Ligase	
Sinorhizobium medicae WSM419 (plasmid)Sinorhizobium (2 proteins)	ATP-Dependent DNA Ligase	YP_001312861
Sinorhizobium meliloti 1021 (plasmid)Sinorhizobium (2 proteins)	ATP-Dependent DNA Ligase	NP_436551
P.Deltaproteobacteria		
Anaeromyxobacter dehalogenans 2CP-1Anaeromyxobacter (5 proteins)	ATP-Dependent DNA Ligase	YP_002491286
Anaeromyxobacter dehalogenans 2CP-CAnaeromyxobacter (5 proteins)	ATP-Dependent DNA Ligase	YP_464028
Anaeromyxobacter sp. Fwl09-5Anaeromyxobacter (5 proteins)	ATP-Dependent DNA Ligase	YP_001378773
Anaeromyxobacter sp. Fwl09-5Anaeromyxobacter (5 proteins)	ATP-Dependent DNA Ligase	YP_001381200
Anaeromyxobacter sp. KAnaeromyxobacter (5 proteins)	ATP-Dependent DNA Ligase	YP_002 133229
PRK09125		
Organism	**Protein name**	**Accession**
O.Betaproteobacteria		
Acidovorax sp. JS42Acidovorax (1 proteins)	DNA ligase	YP_986978
Aromatoleum aromaticum EbNlAromatoleum (1 proteins)	DNA ligase	YP_161050
Azoarcus sp. BH72Azoarcus (1 proteins)	DNA ligase	YP_934633
Candidatus Accumulibacter phosphatis clade IIA str. UW-1 Candidatus Accumulibacter (1 proteins)	DNA ligase	YP_003 169249
Dechloromonas aromatica RCBDechloromonas (1 proteins)	DNA ligase	YP_2 84461

Diaphorobacter sp. TPSYDiaphorobacter (1 proteins)	DNA ligase	YP_002553689
Herminiimonas arsenicoxydansHerminiimonas (1 proteins)	DNA ligase	YP_00 1100009
Leptothrix cholodnii SP-6Leptothrix (1 proteins)	DNA ligase	YP_00 179 1742
Methylibium petroleiphilum PMlMethylibium (1 proteins)	DNA ligase	YP_001020556
Neisseria gonorrhoeae FA 1090Neisseria (7 proteins)	DNA ligase	YP_209054
Neisseria gonorrhoeae NCCPl 1945Neisseria (7 proteins)	DNA ligase	YP_002002827
Neisseria meningitidis 053442Neisseria (7 proteins)	DNA ligase	YP_001598310
Neisseria meningitidis FAM18Neisseria (7 proteins)	DNA ligase	YP_975951
Neisseria meningitidis MC58Neisseria (7 proteins)	DNA ligase	NP_275038
Neisseria meningitidis Z2491Neisseria (7 proteins)	DNA ligase	YP_002341892
Neisseria meningitidis alphal4Neisseria (7 proteins)	DNA ligase	YP_003082363
Polaromonas naphthalenivorans CJ2Polaromonas (2 proteins)	DNA ligase	YP_9 82249
Polaromonas sp. JS666Polaromonas (2 proteins)	DNA ligase	YP_549233
Rhodoferax ferrireducens T 118Rhodoferax (1 proteins)	DNA ligase	YP_522700
Thauera sp. MZlTThauera (1 proteins)	DNA ligase	YP_002353773
Thiobacillus denitrificans ATCC 25259Thiobacillus (1 proteins)	DNA ligase	YP_3 14570
Variovorax paradoxus SI lOVariovorax (1 proteins)	DNA ligase	YP_002944627
Verminephrobacter eiseniae EF01-2Verminephrobacter (1 proteins)	DNA ligase	YP_998235

P.Deltaproteobacteria		
Desulfobacterium autotrophicum HRM2Desulfobacterium (1 proteins)	LigA2	YP_002604477
Myxococcus xanthus DK 1622Myxococcus (1 proteins)	DNA ligase	YP_628883
Q.Epsilonproteobacteria		
Arcobacter butzleri RM4018Arcobacter (1 proteins)	ATP-dependent DNA ligase	YP_oo1489632
Campylobacter concisus 13826Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_001467307
Campylobacter curvus 525.92Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_001407695
Campylobacter fetus subsp. fetus 82-40Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_892536
Campylobacter hominis ATCC BAA-381Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_001406356
Campylobacter jejuni RM 122 1Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_17981 1
Campylobacter jejuni subsp. doylei 269.97Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_001398949
Campylobacter jejuni subsp. jejuni 81-176Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_001001312
Campylobacter jejuni subsp. jejuni 81116Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_00 1483 144
Campylobacter jejuni subsp. jejuni NCTC 11168Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_002345037
Campylobacter lari RM2100Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_002574655
Sulfurimonas denitrificans DSM 1251Sulfurimonas (1 proteins)	DNA ligase	YP_393098
R.Gammaproteobacteria		
Actinobacillus succinogenes 130ZActinobacillus (1 proteins)	ATP-dependent DNA ligase	YP_001344487
Aggregatibacter actinomycetemcomitans D11S-1Aggregatibacter (2 proteins)	ATP-dependent DNA ligase	YP_003256304
Aggregatibacter aphrophilus	ATP-dependent DNA ligase	YP_003007537

NJ8700Aggregatibacter (2 proteins)		
Alcanivorax borkumensis SK2Alcanivorax (1 proteins)	ATP-dependent DNA ligase	YP_694422
Aliivibrio salmonicida LFI1238Aliivibrio (3 proteins)	ATP-dependent DNA ligase	YP_002262821
Vibrio fischeri ESI 14Aliivibrio (3 proteins)	ATP-dependent DNA ligase	YP_204833
Vibrio fischeri MJ11Aliivibrio (3 proteins)	ATP-dependent DNA ligase	YP_002156265
Alteromonas macleodii 'Deep ecotype'Alteromonas (1 proteins)	ATP-dependent DNA ligase	YP_002127707
Mannheimia succiniciproducens MBEL55EBasfia (1 proteins)	ATP-dependent DNA ligase	YP_088131
Colwellia psychrerythraea 34HColwellia (1 proteins)	ATP-dependent DNA ligase	YP_271053
Haemophilus influenzae 86-028NPHaemophilus (3 proteins)	ATP-dependent DNA ligase	YP_248841
Haemophilus influenzae PittEEHaemophilus (3 proteins)	ATP-dependent DNA ligase	YP_001290961
Haemophilus influenzae PittGGHaemophilus (3 proteins)	ATP-dependent DNA ligase	YP_001293088
Haemophilus somnus 129PTHistophilus (2 proteins)	ATP-dependent DNA ligase	YP_719536
Haemophilus somnus 2336Histophilus (2 proteins)	ATP-dependent DNA ligase	YP_001783642
Idiomarina loihiensis L2TRIdiomarina (1 proteins)	ATP-dependent DNA ligase	YP_156435
Marinobacter aquaeolei VT8Marinobacter (1 proteins)	ATP-dependent DNA ligase	YP_960951
Marinomonas sp. MWYL1Marinomonas (1 proteins)	ATP-dependent DNA ligase	YP_001341226
Photobacterium profundum SS9Photobacterium (1 proteins)	ATP-dependent DNA ligase	YP_132765
Pseudoalteromonas atlantica T6cPseudoalteromonas (2 proteins)	ATP-dependent DNA ligase	YP_659659
Pseudoalteromonas haloplanktis TAC125Pseudoalteromonas (2 proteins)	ATP-dependent DNA ligase	YPJ40675

Psychromonas ingrahamii 37Psychromonas (1 proteins)	ATP-dependent DNA ligase	YP_942593
Shewanella amazonensis SB2B Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_927870
Shewanella baltica OS155Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_oo1050227
Shewanella baltica OS 185 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_00 13 66044
Shewanella baltica OS 195 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_001554317
Shewanella baltica OS223 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_002358357
Shewanella frigidimarina NCIMB 400Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_750174
Shewanella halifaxensis HAW-EB4Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_001673966
Shewanella loihica PV-4Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_00 10937 13
Shewanella oneidensis MR- 1Shewanella (18 proteins)	ATP-dependent DNA ligase	NP_7 17802
Shewanella pealeana ATCC 700345 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_00 1502366
Shewanella piezotolerans WP3 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_0023 12387
Shewanella sediminis HAW-EB3 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_00 1474374
Shewanella sp. ANA-3 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_870036
Shewanella sp. MR-4 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_734321
Shewanella sp. MR-7 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_738313
Shewanella woodyi ATCC 51908Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_00 1760369
Shewanella putrefaciens CN-32Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_00 1183272
Shewanella sp. W3- 18-1 Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_963655

Thiomicrospira crunogena XCL-2Thiomicrospira (1 proteins)	ATP-dependent DNA ligase	YP_391938
Vibrio cholerae MJ-1236Vibrio (9 proteins)	ATP-dependent DNA ligase	YP_002878565
Vibrio parahaemolyticus RIMD 22 1063 3Vibrio (9 proteins)	DNA ligase	NP_797856
Vibrio splendidus LGP32Vibrio (9 proteins)	DNA ligase	YP_002417130
Vibrio vulnificus CMCP6Vibrio (9 proteins)	DNA ligase	NP_761477
Vibrio vulnificus YJ016Vibrio (9 proteins)	DNA ligase	NP_934427
Vibrio cholerae M66-2Vibrio (9 proteins)	DNA ligase	YP_002810248
Vibrio cholerae 0 1 biovar El Tor str. N 16961 Vibrio (9 proteins)	DNA ligase	NP_231 182
Vibrio cholerae 0 395Vibrio (9 proteins)	DNA ligase	YP_oo 12 17094
Vibrio cholerae 0 395Vibrio (9 proteins)	DNA ligase	YP_0028 19900
PHA0454		
Organism	**Protein name**	**Accession**
b.Viruses		
Enterobacteria phage 13aT7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_002003941
Enterobacteria phage BA14T7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_002003458
Enterobacteria phage EcoDSlT7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_002003747
Enterobacteria phage KlFT7-like viruses (16 proteins)	ATP-dependent DNA ligase	YPJ38096
Enterobacteria phage T3T7-like viruses (16 proteins)	ATP-dependent DNA ligase	NP_523305
Enterobacteria phage T7T7-like viruses (16 proteins)	ATP-dependent DNA ligase	NP_041963
Klebsiella phage K1 1T7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_002003797
Kluyvera phage KvplT7-like viruses (16 proteins)	DNA ligase	YP_002308390
Morganella phage MmPlT7-like viruses (16	ATP-dependent DNA ligase	YP_002048633

proteins)		
Pseudomonas phage gh-lT7-like viruses (16 proteins)	ATP-dependent DNA ligase	NP_8 13751
Salmonella phage phiSG-JL2T7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_00 1949754
Vibriophage VP4T7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_249578
Yersinia pestis phage phiAl 122T7-like viruses (16 proteins)	ATP-dependent DNA ligase	NP_848267
Yersinia phage BerlinT7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_9 18989
Yersinia phage Yepe2T7-like viruses (16 proteins)	ATP-dependent DNA ligase	YP_002003318
Yersinia phage phiYe03-12T7-like viruses (16 proteins)	ATP-dependent DNA ligase	NP_052075
Enterobacteria phage LKAlphiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	YP_00 1522868
Enterobacteria phage phiKMVphiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	NP_877456
Pseudomonas phage LKD16phiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	YP_00 1522807
Pseudomonas phage LUZ19phiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	YP_00 167 1961
Pseudomonas phage PT2phiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	YP_002117800
Pseudomonas phage PT5phiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	YP_002117741
Pseudomonas phage phikF77phiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	YP_002727838
CLSZ2445448		
Organism	**Protein name**	**Accession**
a.Eukaryota		
Paramecium tetraurelia strain d4-2Paramecium (5 proteins)	DNA ligase	XP_00 1347270
Paramecium tetraurelia strain d4-2Paramecium (5 proteins)	hypothetical protein	XP_00 1422985

Paramecium tetraurelia strain d4-2Paramecium (5 proteins)	hypothetical protein	XP_00143 1968
Paramecium tetraurelia strain d4-2Paramecium (5 proteins)	hypothetical protein	XP_001435874
Paramecium tetraurelia strain d4-2Paramecium (5 proteins)	hypothetical protein	XP_001460273
Tetrahymena thermophilaTetrahymena (1 proteins)	ATP dependent DNA ligase	XP_00101 1861
CLSP2344013		
Organism	**Protein name**	**Accession**
b.Viruses		
Enterobacteria phage Felix Olunclassified Myoviridae (4 proteins)	Putative DNA ligase	NP_944942
Enterobacteria phage WV8unclassified Myoviridae (4 proteins)	hypothetical protein	YP_002922879
Erwinia phage phiEa2 l-4unclassified Myoviridae (4 proteins)	putative DNA ligase	YP_002456101
Escherichia phage rv5unclassified Myoviridae (4 proteins)	ATP-dependent DNA ligase	YP_002003586
PRK07636		
Organism	**Protein name**	**Accession**
J.Firmicutes		
Bacillus clausii KSM-K16Bacillus	ATP-dependent DNA ligase	YP_1 76304
Bacillus subtilis subsp. subtilis str. 168Bacillus	ATP-dependent DNA ligase	NP_3 89932
Bacillus licheniformis ATCC 14580Bacillus	ATP-dependent DNA ligase	YP_078721
Bacillus licheniformis ATCC 14580Bacillus	ATP-dependent DNA ligase	YP_091 132
Geobacillus sp. Y412MC10Geobacillus	ATP dependent DNA ligase	YP_003240778
Paenibacillus sp. JDR-2Paenibacillus	ATP dependent DNA ligase	YP_003009892
CLSK2551528		
Organism	**Protein name**	**Accession**
J.Firmicutes		

Geobacillus sp. Y412MC10Geobacillus (1 proteins)	ATP dependent DNA ligase	YP_003245332
Paenibacillus sp. JDR-2Paenibacillus (1 proteins)	ATP dependent DNA ligase	YP_003013681
CLSK2532515		
Organism	**Protein name**	**Accession**
B.Acidobacteria		
Candidatus Solibacter usitatus Ellin6076Candidatus Solibacter (1 proteins)	ATP dependent DNA ligase	YP_829024
E.Bacteroides/Chlorobi		
Flavobacteriaceae bacterium 3519-10unclassified Flavobacteriaceae (1 proteins)	ATP-dependent DNA ligase	YP_003095681
CLSK2470953		
Organism	**Protein name**	**Accession**
CActinobacteria		
Arthrobacter chlorophenolicus A6 (plasmid)Arthrobacter (2 proteins)	ATP dependent DNA ligase	YP_002478051
Arthrobacter chlorophenolicus A6 (plasmid)Arthrobacter (2 proteins)	ATP dependent DNA ligase	YP_002478427
Nocardioides sp. JS614Nocardioides (1 proteins)	ATP dependent DNA ligase	YP_923463
CLSK2469924		
Organism	**Protein name**	**Accession**
J.Firmicutes		
Alicyclobacillus acidocaldarius subsp. acidocaldarius DSM 446Alicyclobacillus (1 proteins)	ATP dependent DNA ligase	YP_003185050
Brevibacillus brevis NBRC 100599Brevibacillus (1 proteins)	putative ATP-dependent DNA ligase	YP_002773127
CLSK2340991		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Phenylobacterium zucineum HLK1 (plasmid)Phenylobacterium (2 proteins)	ATP dependent DNA ligase	YP_002128561
Phenylobacterium zucineum HLK1	ATP-dependent DNA ligase	YP_002128631

(plasmid)Phenylobacterium (2 proteins)		
CLSK2333706		
Organism	**Protein name**	**Accession**
J.Firmicutes		
Candidatus Desulforudis audaxviator MP104CCandidatus Desulforudis (1 proteins)	ATP dependent DNA ligase	YP_001716762
Natranaerobius thermophilus JW/NM-WN-LFNatranaerobius (1 proteins)	ATP dependent DNA ligase	YP_001916325
CLSK2303611		
Organism	**Protein name**	**Accession**
CActinobacteria		
Streptomyces coelicolor A3(2)Streptomyces (2 proteins)	ATP-dependent DNA ligase	NP_631399
Streptomyces griseus subsp. griseus NBRC 13350Streptomyces (2 proteins)	putative ATP-dependent DNA ligase	YP_00 182 8202
CLSK962101		
Organism	**Protein name**	**Accession**
CActinobacteria		
Nocardioides sp. JS614Nocardioides (1 proteins)	ATP dependent DNA ligase	YP_922436
Salinispora arenicola CNS-205Salinispora (2 proteins)	DNA polymerase LigD ligase region	YP_001539124
Salinispora tropica CNB-440Salinispora (2 proteins)	ATP dependent DNA ligase	YP_001160776
CLSK915249		
Organism	**Protein name**	**Accession**
CActinobacteria See CLSK2303611 above		
Streptomyces avermitilis MA-4680 (plasmid)Streptomyces (2 proteins)	putative ATP-dependint DNA ligase	NP_828839
Streptomyces sp. HK1 (plasmid)Streptomyces (2 proteins)	putative ATP-dependent DNA ligase	YP_001661618
CLSK899085		
Organism	**Protein name**	**Accession**
O.Betaproteobacteria		

Burkholderia cenocepacia HI2424 (plasmid)Burkholderia (3 proteins)	ATP dependent DNA ligase	YP_840498
Burkholderia cenocepacia J23 15 (plasmid)Burkholderia (3 proteins)	putative ligase	YP_002235530
Burkholderia multivorans ATCC 17616 (plasmid)Burkholderia (3 proteins)	DNA polymerase LigD ligase subunit	YP_001573706
R.Gammaproteobacteria		
Pseudomonas putida (plasmid)Pseudomonas (1 proteins)	putative ligase	NP_542805
CLSK862724		
Organism	**Protein name**	**Accession**
A.Archaea		
Archaeoglobus fulgidus DSM 4304Archaeoglobus (1 proteins)	DNA ligase, putative	NP_070553
J.Firmicutes		
Desulfotomaculum reducens MI-1Desulfotomaculum (1 proteins)	ATP dependent DNA ligase	YP_001 113345
Moorella thermoacetica ATCC 39073Moorella (1 proteins)	ATP dependent DNA ligase, central	YP_430340
Pelotomaculum thermopropionicum SIPelotomaculum (1 proteins)	ATP-dependent DNA ligase	YP_0012 11793
Thermoanaerobacter pseudethanolicus ATCC 33223Thermoanaerobacter (2 proteins)	ATP dependent DNA ligase	YP_00 1664477
Thermoanaerobacter sp. X514Thermoanaerobacter (2 proteins)	ATP dependent DNA ligase	YP_00 16625 89
CLSK820690		
Organism	**Protein name**	**Accession**
A.Archaea		
uncultured methanogenic archaeon RC-Ienvironmental samples (1 proteins)	ATP-dependent DNA ligase	YP_686457
CActinobacteria		
Mycobacterium avium 104Mycobacterium (2 proteins)	DNA polymerase LigD ligase subunit	YP_882332
Mycobacterium avium subsp. paratuberculosis	hypothetical protein	NP_960263

K-l (Mycobacterium (2 proteins)		
Saccharopolyspora erythraea NRRL 2338Saccharopolyspora (1 proteins)	DNA ligase, ATP-dependent	YP_001 107793
N.Alphaproteobacteria		
Bradyrhizobium japonicum USDA 1lOBradyrhizobium (2 proteins)	DNA ligase	NP_774671
Bradyrhizobium sp. BTAilBradyrhizobium (2 proteins)	putative ATP-dependent DNA ligase	YP_001243518
CLSK808255		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Sinorhizobium medicae WSM419Sinorhizobium (2 proteins)	DNA polymerase LigD ligase region	YP_001326990
Sinorhizobium meliloti 1021 (plasmid)Sinorhizobium (2 proteins)	putative ATP-dependent DNA ligase protein	NP_437750
CLSK806855		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Agrobacterium tumefaciens str. C58 (plasmid)Agrobacterium (3 proteins)	ATP-dependent DNA ligase	NPJ95985
Agrobacterium tumefaciens str. C58 (plasmid)Agrobacterium (3 proteins)	ATP-dependent DNA ligase	NPJ96032
Agrobacterium tumefaciens str. C58 (plasmid)Agrobacterium (3 proteins)	ATP-dependent DNA ligase	NP_3 96609
Rhizobium etli CFN 42 (plasmid)Rhizobium (10 proteins)	putative DNA ligase (ATP) protein	YP_472413
Rhizobium etli CIAT 652Rhizobium (10 proteins)	probable ATP-dependent DNA ligase protein	YP_00 19773 17
Rhizobium etli CIAT 652 (plasmid)Rhizobium (10 proteins)	putative ATP-dependent DNA ligase protein	YP_001985803
Rhizobium leguminosarum bv. trifolii WSM1325 (plasmid)Rhizobium (10 proteins)	DNA polymerase LigD, ligase domain protein	YP_002973496
Rhizobium leguminosarum bv. trifolii WSM1325 (plasmid)Rhizobium (10 proteins)	DNA polymerase LigD, ligase domain protein	YP_002984974

Rhizobium leguminosarum bv. trifolii WSM1325 (plasmid)Rhizobium (10 proteins)	DNA polymerase LigD, ligase domain protein	YP_002984992
Rhizobium leguminosarum bv. trifolii WSM2304Rhizobium (10 proteins)	DNA polymerase LigD, ligase domain protein	YP_002281897
Rhizobium leguminosarum bv. trifolii WSM2304 (plasmid)Rhizobium (10 proteins)	DNA polymerase LigD, ligase domain protein	YP_002278005
Rhizobium leguminosarum bv. viciae 384 1 (plasmid)Rhizobium (10 proteins)	putative DNA ligase	YP_764723
Rhizobium leguminosarum bv. viciae 3841 (plasmid)Rhizobium (10 proteins)	putative DNA ligase	YP_771 149
Sinorhizobium meliloti (plasmid)Sinorhizobium (2 proteins)	putative ATP-dependent DNA ligase	YP_00 1965531
Sinorhizobium meliloti 1021 (plasmid)Sinorhizobium (2 proteins)	ATP-dependent DNA ligase	NP_435470
CLSK762775		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Rhodococcus jostii RHA1 (plasmid)Rhodococcus (2 proteins)	ATP-dependent DNA ligase	YP_708952
Rhodococcus opacus B4 (plasmid)Rhodococcus (2 proteins)	putative ATP-dependent DNA ligase	YP_002776886
CLSK761995		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Nitrobacter hamburgensis X14Nitrobacter (1 proteins)	ATP dependent DNA ligase	YP_579015
Rhodopseudomonas palustris BisB5Rhodopseudomonas (2 proteins)	ATP dependent DNA ligase	YP_569297
Rhodopseudomonas palustris TIE-1Rhodopseudomonas (2 proteins)	DNA polymerase LigD, ligase domain protein	YP_00 199 1309
CLSK523944		
Organism	**Protein name**	**Accession**
R.Gammaproteobacteria		
Pseudomonas fluorescens (plasmid)Pseudomonas (3 proteins)	putative ATP-dependent DNA ligase	YP_002887417
Pseudomonas putida (plasmid)Pseudomonas (3 proteins)	putative ligase fragment	NP_863069

Pseudomonas sp. ND6 (plasmid)Pseudomonas (3 proteins)	ATP-dependent DNA ligase	NP_943185
CLSK390680		
Organism	**Protein name**	**Accession**
R.Gammaproteobacteria		
Mesorhizobium loti MAFF303099Mesorhizobium (3 proteins)	ATP-dependent DNA ligase	NP_108227
Mesorhizobium loti MAFF303099Mesorhizobium (3 proteins)	hypothetical protein	NPJ08282
Mesorhizobium loti MAFF303099 (plasmid)Mesorhizobium (3 proteins)	DNA ligase-like protein	NP_109396

A subset of ligases of interest is in Table IB below.

Table IB

PRK08224		
B.Acidobacteria		
Bacteria; Fibrobacteres/Acidobacteria group; Acidobacteria; unclassifed Acidobacteria; Candidatus Koribacter; Candidatus Koribacter versatiiis		
Candidatus Solibacter usitatus Ellin6076Candidatus Solibacter (1 proteins)	ATP-Dependent DNA Ligase	YP_826317
CActinobacteria		
Bacteria; Actinobacteria; Actinobacteria (class); Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium; Mycobacterium marinum		
Mycobacterium gilvum PYR-GCKMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_00 1132524
Mycobacterium vanbaalenii PYR-1Mycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_956315
Mycobacterium sp. MCSMycobacterium (26 proteins)	ATP-Dependent DNA Ligase	YP_642076
F.Chlamydiae/Verrucomicrobia		
Bacteria; Chlamydiae/Verrucomicrobia group; Verrucomicrobia; Opitutae; Opitutales; Opitutaceae; Opitutus; Opitutus terrae		

Opitutus terrae PB90-lOpitutus (1 proteins)	ATP-Dependent DNA Ligase	YP_001821013
PRK09125		
Organism	**Protein name**	**Accession**
O.Betaproteobacteria		
Neisseria meningitidis Z2491Neisseria (7 proteins)	DNA ligase	YP_002341892
Thiobacillus denitrificans ATCC 25259Thiobacillus (1 proteins)	DNA ligase	YP_314570
Variovorax paradoxus S110Variovorax (1 proteins)	DNA ligase	YP_002944627
Verminephrobacter eiseniae EF01-2Verminephrobacter (1 proteins)	DNA ligase	YP_998235
	-	-
P.Deltaproteobacteria		
Desulfobacterium autotrophicum HRM2Desulfobacterium (1 proteins)	LigA2	YP_002604477
Myxococcus xanthus DK 1622Myxococcus (1 proteins)	DNA ligase	YP_628883
	-	-
Q.Epsilonproteobacteria		
Campylobacter jejuni subsp. jejuni NCTC 11168Campylobacter (10 proteins)	ATP-dependent DNA ligase	YP_002345037
Sulfurimonas denitrificans DSM 1251Sulfurimonas (1 proteins)	DNA ligase	YP_393098
	-	-
R.Gammaproteobacteria		
Aggregatibacter aphrophilus NJ8700Aggregatibacter (2 proteins)	ATP-dependent DNA ligase	YP_003007537
Haemophilus influenzae PittEEHaemophilus (3 proteins)	ATP-dependent DNA ligase	YP_001290961
Shewanella baltica OS195Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_001554317
Shewanella loihica PV-4Shewanella (18 proteins)	ATP-dependent DNA ligase	YP_001093713
Vibrio cholerae M66-2Vibrio (9 proteins)	DNA ligase	YP_0028 10248
	-	-
PHA0454		
Organism	**Protein name**	**Accession**
b.Viruses		
Viruses; dsDNA viruses, no RNA stage; Caudovirales; Podoviridae; Autographivirinae; phiKMV-iike viruses		
Pseudomonas phage LKD 16phiKMV-like viruses (7 proteins)	ATP-dependent DNA ligase	YP_001522807
CLSZ2445448		

Organism	Protein name	Accession
a.Eukaryota		
Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Oligohymenophorea; Peniculida; Parameciidae; Paramecium; Paramecium tetraurelia		
Paramecium tetraurelia strain d4-2Paramecium (5 proteins)	DNA ligase	XP_00 1347270
PRK07636		
Organism	**Protein name**	**Accession**
J.Firmicutes		
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus clausii		
Bacillus subtilis subsp. subtilis str. 168Bacillus	ATP-dependent DNA ligase	NP_389932
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Geobacillus		
Geobacillus sp. Y412MC10Geobacillus	ATP dependent DNA ligase	YP_003240778
CLSK2551528		
Organism	**Protein name**	**Accession**
J.Firmicutes		
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Geobacillus		
Geobacillus sp. Y412MC10Geobacillus (1 proteins)	ATP dependent DNA ligase	YP_003245332
	-	-
CLSK2470953		
Organism	**Protein name**	**Accession**
CActinobacteria		
Bacteria; Actinobacteria; Actinobacteria (class); Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter; Arthrobacter chlorophenolicus		
Arthrobacter chlorophenolicus A6 (plasmid)Arthrobacter (2 proteins)	ATP dependent DNA ligase	YP_002478427
CLSK2469924		
Organism	**Protein name**	**Accession**
J.Firmicutes		
Bacteria; Firmicutes; Bacilli; Bacillales; Alicyclobacillaceae; Alicyclobacillus; Alicyclobacillus acidocaldarius; Alicyclobacillus acidocaldarius subsp. acidocaldarius		
Alicyclobacillus acidocaldarius subsp. acidocaldarius DSM 446Alicyclobacillus	ATP dependent DNA ligase	YP_003 185050
CLSK2340991		

Organism	Protein name	Accession
N.Alphaproteobacteria		
Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium; Phenylobacterium zucineum		
Phenylobacterium zucineum HLK1 (plasmid)Phenylobacterium (2 proteins)	ATP-dependent DNA ligase	YP_002128631
	-	-
CLSK2333706		
Organism	**Protein name**	**Accession**
J.Firmicutes		
Bacteria; Firmicutes; Clostridia; Clostridiales; Peptococcaceae; Candidatus Desulforudis; Candidatus Desulforudis audaxviator		
Candidatus Desulforudis audaxviator MP104CCandidatus Desulforudis (1 proteins)	ATP dependent DNA ligase	YP_001716762
	-	-
CLSK962101		
Organism	**Protein name**	**Accession**
CActinobacteria		
Bacteria; Actinobacteria; Actinobacteria (class); Actinobacteridae; Actinomycetales; Micromonosporineae; Micromonosporaceae; Salinispora; Salinispora arenicola	-	-
Salinispora arenicola CNS-205 Salinispora (2 proteins)	DNA polymerase LigD ligase region	YP_001539124
Salinispora tropica CNB-440Salinispora (2 proteins)	ATP dependent DNA ligase	YP_001160776
	-	-
CLSK915249		
Organism	**Protein name**	**Accession**
CActinobacteria See CLSK2303611 above		
Bacteria; Actinobacteria; Actinobacteria (class); Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces; Streptomyces coelicolor		
Streptomyces avermitilis MA-4680 (plasmid)Streptomyces (2 proteins)	putative ATP-dependint DNA ligase	NP_828839
Streptomyces sp. HK1 (plasmid)Streptomyces (2 proteins)	putative ATP-dependent DNA ligase	YP_001661618
CLSK862724		
Organism	**Protein name**	**Accession**
A.Archaea		
Archaea; Euryarchaeota; Archaeoglobi; Archaeoglobales; Archaeoglobaceae; Archaeoglobus; Archaeoglobus fulgidus		

Archaeoglobus fulgidus DSM 4304Archaeoglobus (1 proteins)	DNA ligase, putative	NP_070553
J.Firmicutes		
Pelotomaculum thermopropionicum SIPelotomaculum (1 proteins)	ATP-dependent DNA ligase	YP_0012 11793
Thermoanaerobacter pseudethanolicus ATCC 33223Thermoanaerobacter (2 proteins)	ATP dependent DNA ligase	YP_00 1664477
CLSK820690		
Organism	**Protein name**	**Accession**
A.Archaea		
Archaea; Euryarchaeota; environmental samples		
uncultured methanogenic archaeon RC-Ienvironmental samples (1 proteins)	ATP-dependent DNA ligase	YP_686457
N.Alphaproteobacteria		
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bradyrhizobium; Bradyrhizobium japonicum		
Bradyrhizobium japonicum USDA 1lOBradyrhizobium (2 proteins)	DNA ligase	NP_774671
Bradyrhizobium sp. BTAil Bradyrhizobium (2 proteins)	putative ATP-dependent DNA ligase	YP_001243518
CLSK808255		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Sinorhizobium/Ensifer group; Sinorhizobium; Sinorhizobium medicae		
Sinorhizobium medicae WSM419Sinorhizobium (2 proteins)	DNA polymerase LigD ligase region	YP_001326990
Sinorhizobium meliloti 1021 (plasmid)Sinorhizobium (2 proteins)	putative ATP-dependent DNA ligase protein	NP_437750
CLSK806855		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Rhizobium/Agrobacterium group; Agrobacterium; Agrobacterium tumefaciens		
Agrobacterium tumefaciens str. C58 (plasmid)Agrobacterium (3 proteins)	ATP-dependent DNA ligase	NP_396032
Rhizobium leguminosarum bv. trifolii WSM1325	DNA polymerase	YP_002973496

(plasmid)Rhizobium (10 proteins)	LigD, ligase domain protein	
Rhizobium leguminosarum bv. trifolii WSM2304 (plasmid)Rhizobium (10 proteins)	DNA polymerase LigD, ligase domain protein	YP_002278005
CLSK390680		
Organism	**Protein name**	**Accession**
N.Alphaproteobacteria		
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; Mesorhizobium; Mesorhizobium loti		
Mesorhizobium loti MAFF303099Mesorhizobium (3 proteins)	hypothetical protein	NP_108282

Some exemplary ligases are identified and their GI or accession numbers are provided in Table 1C below:

TABLE 1C

CV DNA Ligase, GenBank ID AAC96909. 1, from Paramecium bursaria Chlorella virus 1: MAITKPLLAATLENIEDVQFPCLATPKIDGIRSVKQTQMLSRTFKPIRNSVMNRLL TELLPEGSDGEISIEGATFQDTTSAVMTGHKMYNAKFSYYWFDYVTDDPLKKYI DRVEDMKNYITVHPHILEHAQVKIIPLIPVEINNITELLQYERDVLSKGFEGVMIRK PDGKYKFGRSTLKEGILLKMKQFKDAEATIISMTALFKNTNTKTKDNFGYSKRST HKSGKVEEDVMGSIEVDYDGWFSIGTGFDADQRRDFWQNKESYIGKMVKFKY FEMGSKDCPRFPVFIGIRHEEDR
MnM DNA Ligase, GenBank ID YP_333052.1, from Burkholderia pseudomallei 1710b (equivalent sequence to ABA50091) MSGVPYGFKPNLAATLTKPELIKFPVWASPKIDGIRCVFFGGVAYSRSLKPIPNPV VQEFAKAYANLLEGLDGELTVGSPTDANCMQNSMAVMSKAAAPDFTFHVFDW FHPAQAHIEFWQRSDVVEDRIVQFYDRYPEVDIRAAPQVLCTSLAHLDTNEARW LADGYEGMMIRDHCGRYKFGRSTEREGGLVKVKRFTDAEAIVIGFEEEMHNANE AKRDATGRTERSTSKAGLHGKGTLGALWKNERGIVFNIGTGFTAAQRADYWA NHPSLFGKMVKFKHFDHGTVDAPRHPVFIGFRHPEDM
Hin DNA Ligase, GenBank ID P44121, from Haemophilus influenza MKFYRTLLLFFASSFAFANSDLMLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQ LLTRQGQRLSPPAYFIKDFPPFAIDGELFSERNHFEEISTITKSFKGDGWEKLKLYV FDVPDAEGNLFERLAKLKAHLLEHPTTYIEIIEQIPVKDKTHLYQFLAQVENLQGE

GWVRNPNAPYERKRSSQILKLKTARGEECTVIAHHKGKGQFENVMGALTCKN HRGEFKIGSGFNLNERENPPPIGSVITYKYRGITNSGKPRFATYWREKK
DLX DNA Ligase, artificial ligase derived from Hin DNA ligase from Haemophilus influenza: MKFYRTLLLFFASSFAFANSDLMLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQ LLTRQGQRLSPPAYFIKDFPPFAIDGELFSERNHFEEISSITKSFKGDGWEKLKLYV FDVPDAEGNLFERLAKLKAHLLEHPTTYIEIIEQIPVKDKTHLYQFLAQVENLQGE GWVRNPNAPYERKRSSQILKLKTARGEECTVIAHHKGKGQFENVMGALTCKNH RGEFKIGSGFNLNERENPPPIGSVITYKYRGITNSGKPRFATYWREKK
DLXd DNA Ligase, artificial ligase derived from Hin D ligase from Haemophilus influenza: MKFYRTLLLFFASSFAFANSDLMLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQ LLTRQGQRLSPPAYFIKDFPPFAIDGELFSERNHFEEISSITKSFKGDGWEKLKLYV FDVPDAEGNLFERLAKLKAHLLEHPTTYIEIIEQIPVKDKTHLYQFLAQVENLQGE GWVRNPNAPYERKRSSQILKLKTARDEECTVIAHHKGKGQFENVMGALTCKNH RGEFKIGSGFNLNERENPPPIGSVITYKYRGITNSGKPRFATYWREKK
DLXd2 DNA Ligase (Gammaproteobacteria, Haemophilus influenza) (modified) MLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQLLTRQGQRLSPPAYFIKDFPPFA IDGELFSERNHFEEISSITKSFKGDGWEKLKLYVFDVPDAEGNLFERLAKLKAHLL EHPTTYIEIIEQIPVKDKTHLYQFLAQVENLQGEGVVVRNPNAPYERKRSSQILKL KTARDEECTVIAHHKGKGQFENVMGALTCKNHRGEFKIGSGFNLNERENPPPIGS VITYKYRGITNSGKPRFATYWREKK

[0086] The SFL is in one aspect an enzyme that can mediate the formation of a covalent bond between two polynucleotide termini, e.g., a 3'-OH terminus and a 5'-P04 terminus are joined together to form a phosphodiester bond. In some instances, DNA ligation entails any one or more of three sequential nucleotidyl transfer steps, discussed below. All three chemical steps depend on a divalent cation cofactor. In one aspect, the SFL is an ATP-dependent ligase or a NAD^+-dependent ligase.

[0087] Optionally, the SFL is Chlorella virus DNA ligase (ChVLig). Ho, et al, J Virol, 71(3): 1931-19374 (1997) or functional fragment or variant thereof. For example the SFL can comprise any one or more domains characteristic of a ligase, e.g., an N-terminal nucleotidyltransferase (NTase) domain and/or a C-terminal OB domain. The OB domain optionally comprises a five-stranded antiparallel beta-barrel plus an alpha-helix. Within the NTase domain is an adenylate-binding pocket composed of the six peptide motifs that define the covalent NTase enzyme family of polynucleotide ligases. Optionally, the NTase domain can comprise any one or more of the ligase amino acid

motifs I, la, III, Ilia, IV, V, and VI preferably all six motifs. Motif I (e.g., KxDGxR or a "KXDG" motif) optionally contains a lysine. Exemplary sequences for each motif in CV ligase are ATPKIDGIR (motif I), SRT (motif la), EGSDGEIS (motif III), YWFDY (motif Ilia), EGVMIR (motif IV), LLKMK (motif V). Motif 1 preferably contains a lysine residue. Other examples of motif I include CELKLDGLA, VEHKVDGLS, CEPKLDGLA, CELKLDGVA, AEIKYDGVR, CEYKYDGQR, VDYKYDGER, FEIKYDGAR, FEGKWDGYR, AREKIHGTN, ACEKVHGTN, ILTKEDGSL, and VEEKVDGYN. Examples of motif 1a include TRG, SRT, SRR, SRN, SRS, KRT, KRS, SKG and TRG. Examples of motif III include LEVRGEVF, VEVRGECY, LEVRGEVY, LEARGEAF, FMLDGELM, EGSDGEIS, FILDTEAV, FIIEGEIV, AIVEGELV, VVLDGEAV, YQVFGEFA, LVLNGELF, FTANFEFV and LILVGEMA. Examples of motif Ilia include FCYGV, FLYTV, TFYAL, ICHGL, NAYGI, FVYGL, KLYAI, YWFDY, YAFDI, FLFDL, NLFDV, WAFDL, YVFDI, FAFDI, ILLNA, AND FLFDV. Examples of motif IV include DGVVIK, DGIVIK, DGVVVK, DGTVLK, EGLIVK, EGVMIR, EGLMVK, EGVMVK, EGLMAK, EGVIAK, EGYVLK, EGVVIR, EGYVAV, and EGIIMK. Examples of motif V include AVAFK, AIAYK, ALAYK, AIAYK,WWKMK, LLKMK, WLKLK, WIKLK, WLKIK, WVKDK, AIKCK, IIKLR, HFKIK and IVKYV. The SFL optionally comprises all six motifs. Optionally all six motifs are found together in a naturally-occurring ligase, such as a SFL identified herein. Optionally, the SFL is not an RNA-capping enzyme.

[0088] The ligase optionally comprises any functional portion of a SFL. The ligase can be homologous to a SFL or any functional portion thereof, for example more than 75%, 85%, 90%, 95% or 99% homologous at the amino acid level.

[0089] Optionally, any of the ligation methods described herein is performed by an SFL that is not CV ligase or a functional fragment or derivative thereof. Optionally, the SFL is not T4 DNA ligase or functional fragment or variant thereof. Optionally, the SFL is less than 70%, 80% or 90% identical to T4 DNA ligase. In some examples, the SFL is not a ligase that was known by effective filing date of this application to efficiently ligate oligonucleotides shorter than 6 nucleotides in length. In some examples, the SFL is not a ligase that was known by January 11, 201 1, to efficiently ligate oligonucleotides shorter than 6 nucleotides in length.

[0090] In a typical assay, a probe that is 2, 3, 4, 5 or 6 nucleotides in length is ligated in template-dependent manner to a primer using an SFL. Optionally, the 3' end of the probe is ligated to the 5' end of the primer, or vice versa. Optionally, the SFL is CV

ligase. The efficiency of ligation is for example more than 5%, 10%, 20%, 30%, or 50%. In other instances a probe that is 6, 7, 8, 9, 10, 11 or 12 nucleotides in length is ligated in template-dependent manner to a primer using an SFL. Optionally, the 5' end of the probe is ligated to the 3' end of the primer, or vice versa. Optionally, the SFL is CV ligase. The efficiency of ligation is for example more than 80%, 90% or 95%.

[0091] In any ligation described herein, ligation product is optionally detected. Ligation can be detected by any known method or method described herein. For example the primer and/or template can be immobilized on a support and the probe labeled. The label on the ligated immobilized probe can be detected after non-ligated probe has been washed away. In other embodiments any one or more of the probe, primer or template is labeled. Any one or more of these reagents can optionally be immobilized.

[0092] In any ligation herein, the ligation can be repeated for a desired number of cycles, for example any reagent that has been subjected to a first ligation cycle is used as a reagent in a next ligation cycle. For example the primer, probe or template of a first ligation cycle can be used as primer, probe or template in a next ligation cycle. In some embodiments (e.g., ligation sequencing) the primer of a first cycle is used as primer in the next cycle. In other instances, the primer of a first cycle can be used a probe or template in the next, the probe of a first cycle can be used as primer or template, or the template of a fist cycle can be used as primer or probe the next.

[0093] The next cycle can be designed such that both ligated and unligated reagent of a first cycle can act as reagent in the next cycle. For example, the ligated and unligated primer of a first ligation cycle can be used as primer in the next cycle. Alternatively, the next cycle can be designed such that only a ligation product of the first cycle can act as a ligation reagent in the next cycle, and reagents that remain unligated in the first cycle will not act as reagents in the next cycle. In some examples, the unligated reagents of the previous cycle are "capped" or otherwise rendered unligatable before the next cycle of ligation is executed.

[0094] Any one or more ligases and/or ligation methods encompassed by the invention can optionally be used in one or more ligation assay formats, and/or are performed in the context of one or more specific ligation applications. Non-limiting examples of assay formats include: oligonucleotide ligation assay (OLA), a ligase chain reaction (LCR), a ligase detection reaction (LDR) and combination assays such as the OLA coupled with the polymerase chain reaction (PCR), e.g., OLA-PCR and PCR-OLA, the Combined Chain Reaction (CCR; a combination of PCR and LCR) and PCR-LDR

(see, e.g., Landegren et al, Science 241:1077-80, 1988; Barany, Proc. Natl. Acad. Sci. 88:189-93, 1991; Grossman etal, Nucl. Acids Res. 22(21):4527-34, 1994; Bi and Stambrook, Nucl. Acids Res. 25(14):2949-51, 1997; Zirvi et al, Nucl. Acids Res., 27(24):e40, 1999; U.S. Pat. No. 4,988,617; and PCT Publication Nos. WO 97/31256 and WO 01/92579. Non-limiting examples of specific applications include: amplification of template, detection and/or quantification of the presence of a particular nucleic acid, e.g., in a diagnostic sample, ligation sequencing, single nucleotide polymorphism (SNP) analysis, SNP genotyping, mutation detection, identification of single copy genes, detecting microsatellite repeat sequences, and DNA adduct mapping, among other things. See also Whitely et al, U.S. Pat. No. 4,883,750; Letsinger et al, U.S. Pat. No. 5,476,930; Fung et al, U.S. Pat. No. 5,593,826; Kool, U.S. Pat. No. 5,426,180; Landegren et al, U.S. Pat. No. 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15-3-29 (1982); and Namsaraev, U.S. patent publication 2004/01 10213.

[0095] In an embodiment, the method of ligation comprises or consists of a proximity ligation assay (PLA). PLAs typically involve at least three steps. The first step is typically the binding of first and second probes (e.g., antibody probes) to a ligand (e.g., a protein of interest) such that the probes are in close proximity to another. Each of the probes typically contain an oligonucleotide. The oligonucleotides are brought into proximity to one another with the binding of the probes and, in the second step, are then ligated to one another (e.g., the ligation event). The ligated oligonucleotides may then be amplified and detected to determine the presence of the ligand with a sample (e.g., a biological sample).

[0096] An exemplary PLA assay comprises the steps of determining the presence or absence of a target protein in a sample, comprising (a) contacting the target protein with at least a first and second binding agent, each binding agent having binding specificity for the protein and being adjoined to at least one polynucleotide, (b) ligating the oligonucleotides on the first and second binding agent to one another using a ligase to produce a target nucleic acid and amplifying the target nucleic acid; (c) detecting whether amplified target nucleic acid is present or not, and (d) concluding that the target protein is present in the sample if a significant amount of amplified target nucleic acid is detected, and/or concluding that the target protein is absent from the sample if a significant amount of amplified target nucleic acid is not detected. Optionally, step (d) alternatively or additionally comprises measuring or otherwise assessing the amount of

amplified target nucleic acid, and taking the amount of amplified nucleic acid as an indicator of the amount of target protein present in the sample.

[0097] In any methods described herein, polynucleotide ligation probes can be used for detection of a target nucleic acid. The ligation probe is optionally capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e., A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

[0098] The oligonucleotide ligation assay (OLA) is a convenient, highly-stringent method that permits distinction among known DNA sequence variants (Landegren, 1988). Multiplex analysis of highly polymorphic loci is useful for identification of individuals, e.g., for paternity testing and in forensic science, organ transplant donor-receiver matching, genetic disease diagnosis, prognosis, and pre-natal counseling, and other genetic-based testing which depend on the discrimination of single-base differences at a multiplicity of loci (Delahunty, 1996). For example, different assays where two PNA-DNA chimeras, a wild-type (WT) sequence chimera and a mutant sequence chimera, bear different fluorescent dyes. Only when the mutant sequence is present in the target sample, will the mutant sequence chimera ligate to the adjacently annealed second probe (oligo) if the mutant base pair is at the ligation site.

a) Sequence determination

[0099] In some embodiments, successive cycles of ligation can be performed, where the ligation product of a previous cycle is used as primer, probe and/or template in a succeeding cycle.

[00100] An exemplary use of such repetitive ligation is ligation sequencing. A template to be sequenced optionally contains a primer-binding region and polynucleotide region of unknown sequence. A primer with a ligatable terminus (e.g., a free 3' OH group or 5' phosphate group) is annealed to the primer binding region. An extension probe is hybridized to the template adjacently to the primer. The proximal nucleotide of the probe forms a complementary base pair with unknown nucleotide in the template. The extension probe is then ligated to the primer, optionally with an SFL, resulting in an extended duplex. Following ligation, the label attached to extension probe is optionally detected. The process is repeated for a desired number of cycles, using the ligation product of one cycle as the primer of the next cycle. Optionally, the extension probe has a non-ligatable distal terminus, and is then cleaved to provide a ligatable extended duplex.

[00101] In one example, the 3' terminus of the primer is ligated to the 5' terminus of the extension probe. The optional step of cleavage can be done for example at a phosphorothiolate linkage using $AgN0_3$ or another salt that provides Ag^+ ions, leaving a phosphate group at the 3' end of the extended duplex. Phosphatase treatment is used to generate an extendable probe terminus on the extended duplex.

[00102] In one-base encoding, the label corresponds to the identity of nucleotide X. Thus nucleotide Y is identified as the nucleotide complementary to nucleotide X. In other encodings, the label does not have a one-to-one correspondence with the identity of any particular nucleotide in the template.

[00103] Also provided are probe families for use in various ligation methods herein, e.g., sequencing. The probe families are optionally characterized in that each probe family comprises a plurality of labeled oligonucleotide probes of different sequence and, at each position in the sequence, a probe family comprises at least 2 probes having different bases at that position. Probes in each probe family comprise the same label. Preferably the probes comprise a scissile internucleoside linkage. The scissile linkage can be located anywhere in the probe. Preferably the probes have a moiety that is not extendable by ligase at one terminus. Preferably the probes are labeled at a position between the scissile linkage and the moiety that is not extendable by ligase, such that cleavage of the scissile linkage following ligation of a probe to an extendable probe

terminus results in an unlabeled portion that is ligated to the extendable probe terminus and a labeled portion that is no longer attached to the unlabeled portion.

[00104] In multiple-base encodings, the probes in each probe family preferably comprise at least j nucleosides **X**, wherein j is at least 2, and wherein each **X** is at least 2-fold degenerate among the probes in the probe family. Probes in each probe family further comprise at least k nucleosides N, wherein k is at least 2, and wherein N represents any nucleoside. In general, j + k is equal to or less than 100, typically less than or equal to 30. Nucleosides **X** can be located anywhere in the probe. Nucleosides **X** need not be located at contiguous positions. Similarly nucleosides N need not be located at contiguous positions. In other words, nucleosides **X** and N can be interspersed. Nevertheless, nucleosides **X** can be considered to have a 5'→3' sequence, with the understanding that the nucleosides need not be contiguous. For example, nucleosides **X** in a probe of structure $\mathbf{X_A}$**NXGNNXCN** would be considered to have the sequence AGC. Similarly, nucleosides N can be considered to have a sequence.

[00105] Nucleosides **X** can be identical or different but are not independently selected, i.e., the identity of each **X** is constrained by the identity of one or more other nucleosides **X** in the probe. Thus in general only certain combinations of nucleosides **X** are present in any particular probe and within the probes in any particular probe family. In other words, in each probe, the sequences of nucleosides **X** can only represent a subset of all possible sequences of length j. Thus the identity of one or more nucleotides in **X** limits the possible identities for one or more of the other nucleosides.

[00106] Nucleosides N are preferably independently selected and can be A, G, C, or T (or, optionally, a degeneracy-reducing nucleoside). Preferably the sequence of nucleosides N represents all possible sequences of length k, except that one or more N may be a degeneracy-reducing nucleoside. The probes thus contain two portions, of which the portion consisting of nucleosides N is referred to as the unconstrained portion and the portion consisting of nucleosides **X** is referred to as the constrained portion. As described above, the portions need not be contiguous nucleosides. Probes that contain a constrained portion and an unconstrained portion are referred to herein as partially constrained probes. Preferably one or more nucleosides in the constrained portion is at the proximal end of the probes, i.e., at the end that contains the nucleoside that will be ligated to the extendable probe terminus, which can be either the 5' or 3' end of the oligonucleotide probe in different embodiments of the invention.

[00107] Since the constrained portion of any oligonucleotide probe can only have certain sequences, knowing the identity of one or more of the nucleosides in the constrained portion of a probe, either by itself, or optionally in combination with the identity of the label on the probe, provides information about one or more of the other nucleotides in the constrained portion. The information may or may not be sufficient to precisely identify one or more of the other nucleosides, but it will be sufficient to eliminate one or more possible nucleotide combinations and/or permutations in the constrained portion. Optionally, the information is not sufficient to eliminate any possible identity of any one individual nucleotide in the constrained portion. In certain preferred embodiments, knowing the identity of one nucleoside in the constrained portion of a probe is sufficient to precisely identify each of the other nucleosides in the constrained portion, i.e., to determine the identity and order of the nucleosides that comprise the constrained portion.

[00108] As in the one-base-encoding sequencing methods described above, the most proximal nucleoside in an extension probe that is complementary to the template is ligated to an extendable terminus of an initializing oligonucleotide (in the first cycle of extension, ligation, and detection) and to an extendable terminus of an extended oligonucleotide probe in subsequent cycles of extension, ligation, and detection. Detection of the associated label determines the name of the probe family to which the newly ligated probe belongs. Since each position in the constrained portion of the probe is at least 2-fold degenerate, the name of the probe family does not in itself identify any nucleotide in the constrained portion. However, since the sequence of the constrained portion is one of a subset of all possible sequences of length j, identifying the probe family does eliminate certain possibilities for the sequence of the constrained portion. The constrained portion of the probe constitutes its sequence determining portion. Therefore, eliminating one or more possibilities for the identity of one or more nucleosides in the constrained portion of the probe by identifying the probe family to which it belongs eliminates one or more possibilities for the identity of a nucleotide in the template to which the extension probe hybridizes. In preferred embodiments of the invention the partially constrained probes comprise a scissile linkage between any two nucleosides.

[00109] In certain embodiments the partially constrained probes have the general structure $(X)_j(N)_k$, in which X represents a nucleoside, $(X)_j$ is at least 2-fold degenerate at each position such that X can be any of at least 2 nucleosides having different base-

pairing specificities, N represents any nucleoside, j is at least 2, k is between 1 and 100, and at least one N or X other than the X at the probe terminus comprises a detectable moiety. Preferably $(N)_k$ is independently 4-fold degenerate at each position so that, in each probe, $(N)_k$ represents all possible sequences of length k, except that one or more positions in $(N)_k$ may be occupied by a degeneracy-reducing nucleotide. Nucleosides in $(X)_j$ can be identical or different but are not independently selected. In other words, in each probe, $(X)_j$ can only represent a subset of all possible sequences of length j. Thus the identity of one or more nucleotides in $(X)_j$ limits the possible identities for one or more of the other nucleosides. The probes thus contain two portions, of which $(N)_k$ is the unconstrained portion and $(X)_j$ is the constrained portion.

[00110] In certain preferred embodiments of the invention the partially constrained probes have the structure 5'-(X)j(N)kNB*-3' or 3'-(X)j(N)kNB*-5', wherein N represents any nucleoside, NB represents a moiety that is not extendable by ligase, * represents a detectable moiety, $(X)_j$ is a constrained portion of the probe that is at least 2-fold degenerate at each position, nucleosides in $(X)_j$ can be identical or different but are not independently selected, at least one internucleoside linkage is a scissile linkage, j is at least 2, and k is between 1 and 100, with the proviso that a detectable moiety may be present on any nucleoside N or X other than the X at the probe terminus instead of, or in addition to, NB. The scissile linkage can be between two nucleosides in $(X)_j$, between the most distal nucleotide in $(X)_j$ and the most proximal nucleoside in $(N)_k$, between nucleosides within (N)k, or between the terminal nucleoside in (N)k and NB. Preferably the scissile linkage is a phosphorothiolate linkage.

[00111] A plurality of probe families is referred to as a "collection" of probe families. Probes in each probe family in a collection of probe families are labeled with a label that is distinguishable from labels used to label other probe families in the collection. Each probe family preferably has its own defined set of sequences, which optionally do not overlap with any other probe family. Preferably the combination of sets of defined sequences for probe families in a collection of probe families includes all possible sequences of the length of the sequence-determining portion. Preferably a collection of probe families comprises or consists of 4 distinguishably labeled probe families.

[00112] Preferably the sequence-determining portions of the probes in each probe family are the same length, and preferably the sequence-determining portions of probe families in a collection of probe families are of the same length. Preferably the

sequencing-determining portion is a constrained portion that is at least 2 nucleosides in length.

[00113] In some instances, a series of ligation cycles starting from a particular primer allows partial determination of a sequence, i.e., the identification of individual nucleotides spaced apart from one another in a template. Optionally, in order to gather more complete information, a plurality of reactions is performed in which each reaction utilizes a different initializing oligonucleotide i. The initializing oligonucleotides i bind to different portions of the binding region. Preferably the initializing oligonucleotides bind at positions such the extendable termini of the different initializing oligonucleotides are offset by 1 or more nucleotides from each other when hybridized to the template. For example, as shown in Fig. 3, sequencing reactions 1...N are performed. Initializing oligonucleotides ii. .. i_n have the same length and bind such that their terminal nucleotides hybridize to successive adjacent positions in the template. Extension probes ei...e_n thus bind at successive adjacent regions of the template and are ligated to the extendable termini of the initializing oligonucleotides. Terminal nucleotide of probe e_n ligated to i_n is complementary to nucleotide of polynucleotide region, i.e., the first unknown polynucleotide in the template. In the second cycle of extension, ligation, and detection, terminal nucleotide of probe $_{ei2}$ is complementary to nucleotide of polynucleotide region, i.e., the second nucleotide of unknown sequence. Likewise, terminal nucleotides of extension probes ligated to duplexes initialized with initializing oligonucleotides i_{12}, i_{13}, i_4, and so on, will be complementary to the third, fourth, and fifth nucleotides of unknown sequence. It will be appreciated that the initializing oligonucleotides may bind to regions progressively further away from the polynucleotide region rather than progressively closer to it.

[00114] The spacer function of the non-terminal nucleotides of the extension probes allows the acquisition of sequence information at positions in the template that are considerably removed from the position at which the initializing oligonucleotide binds without requiring a correspondingly large number of cycles to be performed on any given template.

a. *Capping*

[00115] In any one or more repetitive ligation methods herein, it is possible that fewer than all probes with extendable termini participate in a successful ligation reaction in each cycle of extension, ligation, and cleavage. It will be appreciated that if such probes participated in succeeding cycles, the accuracy of each nucleotide identification step

could progressively decline. In certain embodiments of the invention a capping step is included to prevent those extendable termini that do not undergo ligation from participating in future cycles. When sequencing in the 5'->3' direction using extension probes containing a 3'-0-P-S-5' phosphorothiolate linkage, capping may be performed by extending the unligated extendable termini with a DNA polymerase and a non-extendable moiety, e.g., a chain-terminating nucleotide such as a dideoxynucleotide or a nucleotide with a blocking moiety attached, e.g., following the ligation or detection step. When sequencing in the 3'->5' direction using extension probes containing a 3'-S-P-0-5' phosphorothiolate linkage, capping may be performed, e.g., by treating the template with a phosphatase, e.g., following ligation or detection. Other capping methods may also be used.

[00116] It is contemplated that any ligation assay or method herein can be highly multiplexed such that a large number of assays (e.g., greater than 1,000) can be performed in parallel, e.g., simultaneously.

[00117] Any multiplexed ligation assay can optionally be conducted on solid substrates where one or more ligation reagents (e.g., the ligase, template, probe and/or primer) can be immobilized on a solid support or surface. Optionally, the primers, probe and/or template can be attached to different portions of a solid substrate in the form of an array. Optionally, the template, primer or probe can be covalently attached to the solid substrate.

[00118] Optionally, one or more ligation reagents are labeled (e.g., template, probe and/or primer) so that ligation products may be discriminated from each other. Alternatively, ligation products can be distinguished based on: (i) size using electrophoresis or chromatography and/or (ii) detectable labels (Grossman, 1994). For example, multiplexed ligation assays can be conducted on a single sample in a single vessel.

[00119] Any of reagents herein (e.g., primer, probe and/or template) can be optionally immobilized on a solid phase. The solid phase optionally comprises a surface to which one or more reactants may be attached electrostatically, hydrophobically, or covalently. Representative solid phases include e.g.: nylon 6; nylon 66; polystyrene; latex beads; magnetic beads; glass beads; polyethylene; polypropylene; polybutylene; butadiene-styrene copolymers; silastic rubber; polyesters; polyamides; cellulose and derivatives; acrylates; methacrylates; polyvinyl; vinyl chloride; polyvinyl chloride; polyvinyl fluoride; copolymers of polystyrene; silica gel; silica wafers glass; agarose; dextrans; liposomes; insoluble protein metals; and, nitrocellulose. Representative solid phases can be formed

into appropriate articles such as beads (e.g., microparticles), tubes, strips, disks, filter papers, plates and the like.

[00120] In an embodiment, any one of the following can be attached to a solid support (e.g., a bead): the SFL, one or more polynucleotide reagents (e.g., an initializing probe, an extension probe, a target DNA or template, a 3' terminal or 5' terminal polynucleotide).

[00121] If so desired, one or more labels can be coupled to any reagent of interest, e.g., the SFL or an antibody that binds specifically to the SFL, one or more polynucleotides (e.g., the template, the 5'-terminal polynucleotide, the 3'-terminal polynucleotide, the template, the initializing probe, the extension probe, etc.

Compositions

[00122] Also provided is a polypeptide comprising or consisting essentially of the amino acid sequence of an SFL provided herein, generally provided as GenBank Accession Nos. in the Tables, or a fragment or variant thereof. The fragment or variant thereof preferably can ligate short oligonucleotides at an efficiency comparable to its parent enzyme. Optionally, the variants or fragments of the SFLs are at least 70%, 80, 90%, 95%, 99% identical to the parent SFL. In an example, the SFL is a ligase derived from Hin DNA ligase (e.g., DLX, DLXd or DLXd2) or any fragment or variant thereof that still retains one or more mutant residues shown in Figure 2, and/or has one or more C-terminal amino acids deleted, e.g., 22 C-terminal amino acids deleted. For example, the mutant Hin DNA ligase is at least 70% identical to Hin D ligase sequence provided in Figure 2 or in GenBank Accession No. P44121, which ligase comprises an amino acid mutation at position 193 of the Hin D ligase sequence provided in Figure 2 or in GenBank Accession No. P44121. Optionally the amino acid mutation consists of changing the glycine at position 193 to aspartic acid or glutamic acid.

[00123] Also provided is a nucleic acid encoding the novel SFLs or fragments or variants described herein. Also provided are genes of the novel SFL which comprise sequences that direct expression of the SFL. Also provided are nucleic acid vectors comprising a nucleic acid encoding the novel SFL and a host cell comprising such a vector. Also provided is an antibody that can bind specifically to the novel SFL but not to its naturally-occurring parent.

[00124] Optionally, the novel SFL or nucleic acid or vector or host cell or antibody is purified, isolated or recombinant.

[00125] Also provided is a method of making any one or more ligases described herein comprising: expressing the SFL in a host cell, e.g., by culturing said host cells under conditions such that the ligase is expressed; and optionally recovering or purifiying said ligase.

Kits

[00126] Also provided are kits comprising components for performing ligation reactions according to the disclosure. Kits can contain an SFL or functional variant or fragment thereof. Kits can alternatively or additionally include one or more oligonucleotide probes less than 12 nucleotides in length, (e.g., less than 8, 7, 6, 5, 4, 3 or 2 nucleotides in length), and/or primer oligonucleotides. In some embodiments, a kit can include CV ligase and one or more oligonucleotides probes less than 6 nucleotides in length. One or more probes for example comprises a 5'-phosphate and/or a label. The label is optionally attached to the 5' terminus, or alternatively to the 3' terminus. Optionally, one or more probes are cleavable.

[00127] The kits optionally provide one or more primers, probes and/or templates described herein. The primers and/or probes can have any one or more features or characteristics described herein. For example the probes can comprise a scissile (e.g., phosphorothiolate) linkage. The kits may contain a cleavage reagent suitable for cleaving phosphororothiolate linkages, e.g., AgN03 and appropriate buffers in which to perform the cleavage. The probes can comprise a trigger residue such as a nucleoside containing a damaged base or an abasic residue. The kits may contain a cleavage reagent suitable for cleaving a linkage between a nucleoside and an adjacent abasic residue and/or a reagent suitable for removing a damaged base from a polynucleotide, e.g., a DNA glycosylase. Certain kits contain oligonucleotide probes that comprise a disaccharide nucleotide and contain periodate as a cleavage reagent. In certain embodiments the kits contain a collection of distinguishably labeled oligonucleotide probe families or collections described herein. Optionally the kit does not comprise a polymerase. Optionally the kit does not comprise an enzyme other than a ligase, phosphatase or kinase. Optionally the kit does not comprise an enzyme other than a SFL that has one or more activities mentioned herein. The kits may include ligation reagents (e.g., ligase, buffers, etc.) and

instructions for practicing the particular embodiment of the invention. Appropriate buffers for the other enzymes that may be used, e.g., phosphatase, polymerases, may be included. In some cases, these buffers may be identical. Kits may also include a support, e.g. magnetic beads, which are either pre-attached to primers, primers or templates or are derivatised to be capable of attaching to such molecules. The beads may be functionalized with a primer for performing PCR amplification. Other optional components include washing solutions; vectors for inserting templates for PCR amplification; PCR reagents such as amplification primers, thermostable polymerase, nucleotides; reagents for preparing an emulsion; reagents for preparing a gel, etc. Kits can also comprise a plurality of distinguishably labeled probes, which can be labeled such that the identity of the label provides information about the sequence of the probe. Kits can also comprise other or additional compositions or reagents for use in ligation sequencing determination protocol. Optionally, the identity of the label provides the exact identity of a nucleotide occupying one degenerate position in the probe. For example, the identity of the label optionally eliminates one or more combinations or permutations of nucleotides at two or more degenerate positions. The probes are for example degenerate at two or more constrained positions, where the identity of a nucleotide at one constrained position eliminates at least one possible identity of a nucleotide at another constrained position. Optionally, knowing the identity of a constrained residue in that probe further eliminates at least one possible identity for another constrained residue in the probe.

[00128] Unless otherwise apparent from the context, any feature can be claimed in combination with any other, or be claimed as not present in combination with another feature. A feature can be for example any variation, step, feature, property, composition, method, step, degree, level, component, material, substance, element, mode, variable, aspect, measure, amount or embodiment.

[00129] Many features described herein are intended to be optional. If any feature is not explicitly indicated as being necessary, it is to be regarded as optional. Non-limiting examples of language indicating that a feature is optional include terms such as "variation," "where," "while," "when," "optionally," "include," "preferred," "especial," "recommended," "advisable," "particular," "should," "alternative," "typical," "representative," "various," "such as," "the like," "can," "may," "example," "embodiment," or " in an aspect" or "if or any combination and/or variation of such terms.

[00130] Any indication that a feature is optional is intended to provide adequate support for claims that include closed or exclusive or negative language (e.g., under 35 U.S.C. 112 or Art. 83 and 84 of EPC). Exclusive language specifically excludes the particular recited feature from including any additional subject matter. For example, if it is indicated that A can be drug X, such language is intended to provide support for a claim that explicitly specifies that A consists of X alone, or that A does not include any other drugs besides X. "Negative" language explicitly excludes the optional feature itself from the scope of the claims. For example, if it is indicated that element A can be X, such language is intended to provide support for a claim that explicitly specifies that A is not X.

[00131] Non-limiting examples exclusive or negative terms include "only," "solely," "consisting of," "consisting essentially of," "alone," "without", "in the absence of (e.g., other items of the same type, structure and/or function)" "excluding," "not", "doesn't", "cannot," or any combination and/or variation of such language.

[00132] Similarly, referents such as "a," "an," "said," or "the," are intended to support explicitly single or plural referents where so desired. Non-limiting examples of plural referents include "at least one," "one or more," "more than one," "two or more," "a multiplicity," "a plurality," "any combination of," "any permutation of," "any one or more of," etc.

[00133] All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference. Genbank records referenced by Genbank ID or accession number, particularly any polypeptide sequence, polynucleotide sequences or annotation thereof, are incorporated by reference herein. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

[00134] The following examples are provided for illustrative purposes and are not intended to limit the invention.

EXAMPLES

Example 1

[00135] This example describes ligation of short probes.

[00136] The oligonucleotide reagents were as follows. The short oligos in forward ligation (i.e., ligation of the 3' terminus of the probe to the 5' terminus of the primer) were: 8mer : 5'-CTCATTCG-3'; 6mer : 5'-TCATTCG-3' 5mer : 5'-ATTCG-3'; 4mer : 5'-TTCG-3'; 3mer : 5'-TCG-3'. Short oligos used for reverse ligation (i.e., ligation of the 5' terminus of the probe to the 3' terminus of the primer) were the same as forward ligation oligos except they have a 5' phosphate. All oligonucleotides were obtained from Integrated DNA Technologies. The forward primer was: P04-5'-CTGCTGTACCGTACATCCGC-3 '-6FAM. The reverse primer was: 5'-FAM-CTGCCCCGGGTTCCTCATTCTCT-3 '

[00137] Ligase Substrate Preparation: MyOne magnetic beads labeled with carboxylic acid, were obtained from Invitrogen. 5' amino labeled, 4 1-base oligonucleotide (CCA CTA CGC CTC CGC TTT CCT CTC TAT GGG CAG TCG GTG AT) was coupled to MyOne beads using standard amine coupling chemistry as taught in Nakajima et al, Bioconjugate Chem. 1995, 6(1), 123-130 (Figure lb). PCR was used to extend this oligonucleotide using (CTG CCC CGG GTT CCT CAT TCT CTA TTC GCT GCT GTA CCG TAC ATC CGC CTT GGC CGT ACA GCA GAT CAC CGA CTG CCC ATA GAG AGG). The following 105 base ligation substrate was now tethered to MyOne beads (CCA CTA CGC CTC CGC TTT CCT CTC TAT GGG CAG TCG GTG ATC TGC TGT ACG GCC AAG GCG GAT GTA CGG TAC AGC AGC GAA TAG AGA ATG AGG AAC CCG GGG CAG, Figure 1a). The amount of 105-base oligo tethered to the bead was measured by hybridization of a fluorescently labeled "primer" oligo, with 3' fluorescein label (6FAM) for detection by capillary electrophoresis. The amount of fluorescence denatured from the template was compared to a standard curve.

[00138] Ligation reactions were performed in buffer containing 50 mM Tris-HCl, 10 mM MgCL2, 1 mM ATP, 1 mM DTT and 5% w/v PEG 8000 at pH 7.5 (ligase buffer). Primer oligonucleotide was annealed to template in ligase buffer by heating to 85 °C for 10 minutes followed by slow cooling to room temperature. In the case of forward ligation, the primer was 5' phosphorylated and a 3' 6FAM labeled. In the case of reverse ligation, the primer was 5' 6FAM labeled. Ligation reactions were performed in a 96 well plate

using 2 nM template/primer, 2.0 μM ligase, 2-5 μM short oligonucleotide in a total volume of 50 μL. The ligation reactions proceeded for 20 minutes at 15 °C. The reactions were stopped using 1% v/v SDS followed by magnetic separation of the templated beads from the solution. Beads were washed three times in 100 μL of 1% SDS prior to washing with 100 uL of 100% formamide. 5 μL of the supernatant containing denatured, FAM labeled primer and ligation product were then analyzed by capillary electrophoresis. The ligation efficiency was calculated as the ratio of peak areas determined by CE, where FAM labeled, unligated and ligated primer were separated, i.e., Efficiency = ligated /(ligated + unligated).

[00139] Shown in Figures 3-5 are a series of ligations of probes of different lengths with a variety of SFLs. In each panel, the ligation reaction is plotted as a function of the probe length. These data clearly demonstrate that small footprint ligases can ligate probe substrates as short as two nucleotides. By utilizing SFLs, the size of a sequencing probe set (e.g., for ligation sequencing in SOLiD™) can be reduced from the current set of 1024 8-mers to as few as 16 (in the case of di-mers), 64 (in the case of trimers), or 256 in the case of tetramers.

[00140] In another experiment, the ligation efficiency of SF Ligase was compared using the same experimental system as above to T4 DNA ligase, using 8-mer ligation sequencing probes that were conjugated or not conjugated to dyes. SFL the highest overall ligation efficiency for both the dark (chase) probe and the labeled sequencing probe. This is true at the 200 nM concentration of probe, which is currently used in SOLiD. Specifically, a mixture of 1024 probes is used in SOLiD, where each individual probe of the 1024 probes is present at a final working concentration of 200 nM. The other concentrations were 2000 nM of enzyme and 1 nM bead template, where ligation was performed at 15°C for 30 minutes. Results are shown in Table 2.

Table 2

Maximum ligation efficiency of 8mer probes by various ligase enzymes

Ligase	Dark	Cy 5
SFL	0.91 ± 0.01	0.82 ± 0.01
T4	0.67 ± 0.02	0.62 ± 0.02

Example 2

[00141] The fidelity of a small-footprint ligase (CV ligase) was determined to be surprisingly better than T4 DNA ligase in DNA sequencing by ligation on the SOLiD sequencing system. A 35-nucleotide DNA template was covalently attached to beads, such each magnetic bead had about 100,000 covalently bound DNA molecules on average. Approximately 40 million beads were subjected to a ligation sequencing reaction at 20 °C. The sequencing reaction ligated 8-mer oligonucleotide "probes" labeled with fluorescent dye onto a primer, and a total of seven consecutive ligation or probing events were performed on each template molecule. The approximate number of ligation events were therefore 100,000 (i.e., number of template molecules per bead) x 40,000,000 (i.e., number of beads) x 7 (number of ligation events per template) = 2.8 x 10^{13}.

[00142] Results are given in Table 3 below. 0MM= Percentage of all ligation products (which are the result of seven consecutive ligation events) with no mismatches; 3MM = Percentage of all ligation products (which are the result of seven consecutive ligation events) with less than 3 mismatches.

Table 3

Ligase	0MM	3MM
T4	20%	57%
SF	27%	65%

The sequencing by ligation data demonstrates that 20% of the time the ligation product was perfectly complementary to the template and formed by seven consecutive ligation events that were all correct. An estimate of the ligation fidelity (expressed as the percentage probability that a ligation event is correct, or "p") can be calculated from the equation: 0MM rate = $(p/100)^n$ where p is the percentage ligation fidelity and n is the number of ligation events per final ligation product (here, seven). Thus for T4 DNA ligase, the ligation fidelity is 79.5% and for SFL the ligation fidelity is 83%.

We claim:

1. A method of ligation comprising enzymatically ligating an oligonucleotide ("probe") and a polynucleotide ("primer"), wherein: the probe is N nucleotide residues in length, where N is from 2 to 7.

2. The method of claim 1, comprising enzymatically ligating an oligonucleotide ("probe") and a polynucleotide ("primer"), wherein: the probe is N nucleotide residues in length, where N is 2 or 3.

3. The method of claim 1, comprising enzymatically ligating a polynucleotide ("primer") to an oligonucleotide ("probe"), wherein (i) the probe is 4 or 5 nucleotide residues in length, and (ii) the ligase is not Paramecium Bursaria Chlorella Virus ligase (CV ligase) or a derivative thereof, or the 3' end of the probe is ligated to the 5' end of the primer, and (iii) more than 30% of the probe or primer is ligated.

4. The method of claim 1, comprising enzymatically ligating a polynucleotide ("primer") to an oligonucleotide ("probe"), wherein (i) the probe is 6 or 7 nucleotide residues in length, and (ii) the ligase is not CV ligase or a derivative thereof, and (iii) more than 50% of the probe or primer is ligated.

5. The method of any one of the preceding claims, wherein ligation is performed by a ligase listed in Table 1A, IB or 1C.

6. The method of any one of the preceding claims, wherein the probe and the primer are not hybridized to the same polynucleotide ("template") if they are different polynucleotides,

7. The method of claim 5, wherein the probe and primer are single-stranded.

8. The method of claim 7, wherein the ligation is performed prior to amplification in a proximity ligation assay.

9. The method of any one of claims 1-4, comprising ligating a proximal terminus of the probe to a proximal terminus of the primer, wherein the probe and the primer are both hybridized to a polynucleotide ("template") such that their proximal termini are adjacently hybridized to each other.

10. A method of claim 1, wherein:

a) the probe and the primer are both hybridized to a polynucleotide ("template") such that their proximal termini are adjacently hybridized to each other, where the proximal terminus of the probe is ligated to the adjacently-hybridized proximal terminus of the primer;

b) the probe is N nucleotide residues in length, where N is 2 or 3.

11. A method of template-dependent ligation comprising enzymatically ligating an oligonucleotide ("probe") and a polynucleotide ("primer"), wherein:

a) the probe and primer are both hybridized to the template such that their proximal termini are adjacent to each other;

b) the probe is of length N, and comprises a proximal portion, wherein the proximal portionis (i) perfectly hybridized to the template and is (ii) L nucleotides long, where the probe's L+lth nucleotide is mismatched with the template; and

c) L is from 2 to 8, and furthermore is less than 6 if the proximal terminus of the probe is its 3' terminus.

12. The method of any one of the preceding claims, wherein ligation is achieved with a small footprint ligase ("SFL") at substantial efficiency, and optionally

(a) at least 10% of the probe or primer is ligated, or

(b) the SFL ligates the proximal termini of the primer and the probe at least 50% as efficiently as the SFL can ligate the proximal termini of the primer and a corresponding octanucleotide whose N proximal residues are identical to the probe, and whose distal 12-N residues are perfectly complementary to the template.

13. The method of any one of the preceding claims, wherein the SFL is CV ligase, or alternatively the SFL is not CV ligase or a derivative thereof.

14. The method of any one of the preceding claims, wherein the primer is not more than 6 nucleotides in length, for example not more 5, 4, 3 or 2 nucleotides.

15. The method of any one of the preceding claims, wherein the template, if present, is not more than 11 nucleotides in length, for example not more 10, 9, 8, 7, 6, 5, 4, 3 or 2 nucleotides.

16. The method of any one of the preceding claims, wherein the proximal terminus of the probe is its 3' terminus and the proximal terminus of the primer is its 5' terminus.

17. The method of any one of claims 1 to 14, wherein the proximal terminus of the probe is its 5' terminus and the proximal terminus of the primer is its 3' terminus.

18. The method of claim 16 or 17, wherein N is 4.

19. The method of claim 16 or 17, wherein N is 3.

20. The method of claim 16 or 17, wherein N is 2.

21. The method of any one of the preceding claims, wherein the probe is labeled.

22. The method of any one of the preceding claims, wherein the primer or template is labeled.

23. The method of any one of the preceding claims, wherein at least two of the probe, primer and template are labeled.The method of any one of the preceding claims, wherein at least one of the probe, the template and/or the primer is immobilized.

24. The method of any one of the preceding claims, wherein the ligation is repeated at least once.

25. The method of claim 22, wherein any ligation product of a previous ligation reaction is used as the primer of a next ligation, where optionally any unligated primer is rendered unligatable before initiating the next ligation.

26. The method of claim 22, further comprising detecting whether the probe has ligated to the primer before repeating the ligation.

27. The method of claim 24, wherein the method provides information about the template sequence.

28. The method of claim 24, wherein ligation is performed in the presence of multiple probes that are at least partially complementary to the same target region on the template adjacent to the proximal terminus of the primer.

29. The method of claim 26, wherein at least two of the multiple probes are labeled distinguishably from each other.

30. The method of claim 27, wherein identifying the label on a ligated probe provides information about the template sequence.

31. The method of claim 22, wherein any ligation product of the previous ligation reaction is used as the template of the next ligation reaction.

32. The method of claim 24, wherein the method comprises a ligase chain reaction.

33. The method of any one of claims 1-16 and 18-32, comprising enzymatically ligating the 5' end of the probe and the 3' end of the primer with CV ligase, wherein N is less than 6.

34. The method of claim 33, wherein N is 2, 3 or 4.

35. A method of template-dependent ligation of an oligonucleotide ("probe") with a polynucleotide ("primer"), where the length of the probe is less than 8 nucleotides.

36. The method of claim 35, wherein the primer is also less than 8 nucleotides, for example not more 6, 5, 4, 3 or 2 nucleotides.

37. The method of claim 35 or 36, wherein the primer is 4 nucleotides long.

38. The method of claim 35 or 36, wherein the primer is 3 nucleotides long.

39. The method of any one of claims 35 to 38, wherein the SFL is CV ligase.

40. A kit comprising a small footprint ligase ("SFL") and an oligonucleotide probe less than N nucleotides in length, where N is from 2 to 7.

41. The kit of claim 40, wherein the SFL is not CV ligase or a derivative thereof and N is less than 6.

42. The kit of any one of claims 40-41, wherein the probe comprises a 5'-phosphate.

43. The kit of any one of claims 40-42, wherein the probe comprises a label.

44. The kit of claim 43, wherein the label is attached to the 5' terminus.

45. The kit of claim 43, wherein the label is attached to the 3' terminus.

46. The kit of any one of claims 40-45, wherein the probe is cleavable.

47. The kit of any one of claims 40-46, wherein the kit comprises a plurality of distinguishably labeled probes.

48. The kit of claim 47, wherein the probes in said plurality are degenerate at one or more nucleotide positions.

49. The kit of claim 48, wherein the probes are labeled such that the identity of the label provides information about a nucleotide occupying a degenerate position in the probe.

50. The kit of claim 49, wherein the identity of the label provides the exact identity of a nucleotide occupying one degenerate position in the probe.

51. The kit of claim 48, wherein the identity of the label eliminates one or more combinations or permutations of nucleotides at two or more degenerate positions.

52. The kit of any one of claims 47 to 51, wherein the probes are degenerate at two or more constrained positions, where the identity of a nucleotide at one constrained position eliminates at least one possible identity of a nucleotide at another constrained position.

53. The kit of claim 52, wherein knowing the identity of a constrained residue in that probe further eliminates at least one possible identity for another constrained residue in the probe.

54. A mutant Hin (Haemophilus Influenzae) DNA ligase comprising a sequence of DLX, DLXd or DLXd2 shown in Table 1C.

55. A mutant Hin DNA ligase consisting essentially of a sequence of DLX, DLXd or DLXd2 shown in Table 1C.

56. A mutant Hin DNA ligase that has at least 70% identity to Hin D ligase sequence provided in Table 1C or in GenBank Accession No. P44121, which ligase comprises an amino acid mutation at position 193 of Hin D ligase sequence provided in Table 1C or in

GenBank Accession No. P44121, optionally wherein said amino acid mutation consists of changing the glycine at position 193 to aspartic acid or glutamic acid.

57. The ligase of claim 56, wherein the ligase has at least 80% identity to Hin **D** ligase.

58. The ligase of claim 56, wherein the ligase is at least 90% identical to Hin **D** ligase.

59. The ligase of claim 56, wherein the ligase is at least 95% identical to Hin **D** ligase.

60. The ligase of claim 56, wherein the ligase is at least 99% identical to Hin **D** ligase.

61. The ligase of any one of claims 54-60, wherein the ligase is isolated, purified or recombinant.

62. A nucleic acid molecule encoding the ligase of any one of claims 54-61.

63. The nucleic acid of claim 62, wherein the nucleic acid is isolated, purified or recombinant.

64. A vector comprising the nucleic acid molecule of claim 62.

65. The vector of claim 64, wherein the vector is isolated, purified or recombinant.

66. A host cell comprising the vector of claim 64.

67. The host cell of claim 66, wherein the host cell is isolated, purified or recombinant.

68. A method of making a ligase comprising:

a. culturing the isolated recombinant host cell of claim 66 or 67 under conditions such that the ligase is expressed; and
b. recovering said ligase.

FIGURE 1

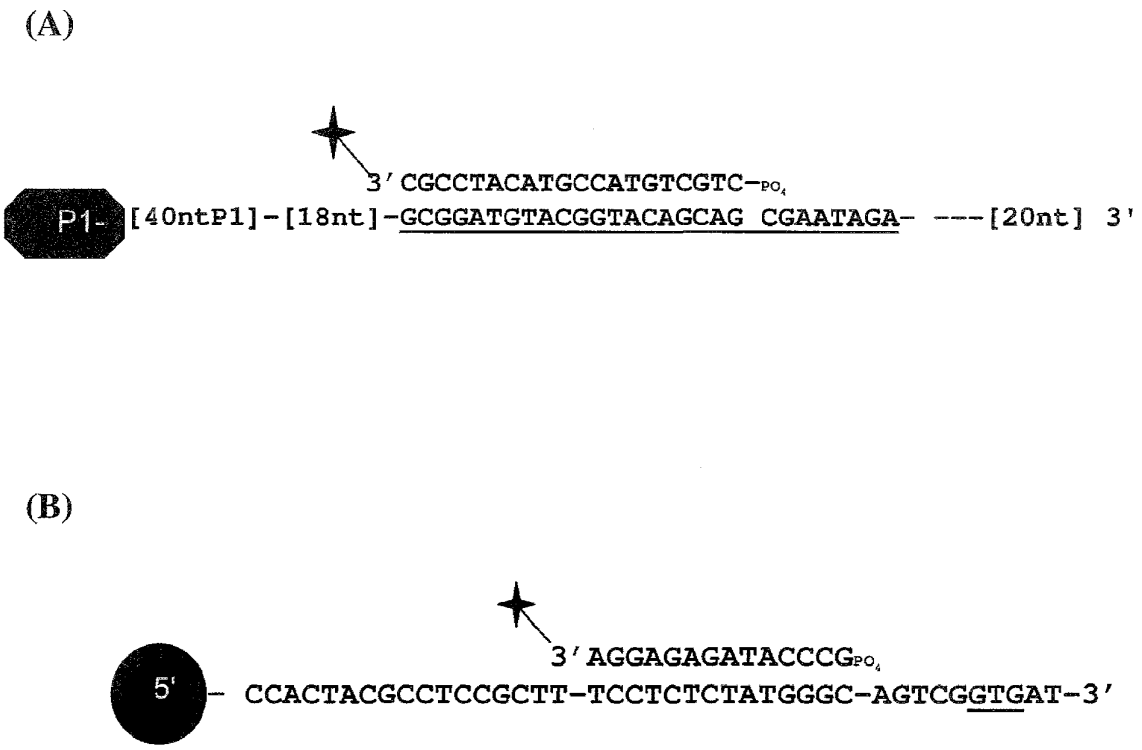

(A)
3′CGCCTACATGCCATGTCGTC–PO4
P1-
[40ntP1]-[18nt]-GCGGATGTACGGTACAGCAG CGAATAGA- ---[20nt] 3'
(B)
3′AGGAGAGATACCCGPO4
5'
CCACTACGCCTCCGCTT-TCCTCTCTATGGGC-AGTCGGTGAT-3′

FIGURE 2

CV DNA Ligase, GenBank ID AAC96909.1, from Paramecium bursaria Chlorella virus 1:

MAITKPLLAATLENIEDVQFPCLATPKIDGIRSVKQTQMLSRTFKPIRNSVMNRLLTELL
PEGSDGEISIEGATFQDTTSAVMTGHKMYNAKFSYYWFDYVTDDPLKKYIDRVEDMK
NYITVHPHILEHAQVKIIPLIPVEINNITELLQYERDVLSKGFEGVMIRKPDGKYKFGRST
LKEGILLKMKQFKDAEATIISMTALFKNTNTKTKDNFGYSKRSTHKSGKVEEDVMGSIE
VDYDGVVFSIGTGFDADQRRDFWQNKESYIGKMVKFKYFEMGSKDCPRFPVFIGIRHE
EDR

MnM DNA Ligase, GenBank ID YP_333052.1, from Burkholderia pseudomallei 1710b (equivalent sequence to ABA50091)

MSGVPYGFKPNLAATLTKPELIKFPVWASPKIDGIRCVFFGGVAYSRSLKPIPNPVVQEF
AKAYANLLEGLDGELTVGSPTDANCMQNSMAVMSKAAAPDFTFHVFDWFHPAQAHI
EFWQRSDVVEDRIVQFYDRYPEVDIRAAPQVLCTSLAHLDTNEARWLADGYEGMMIR
DHCGRYKFGRSTEREGGLVKVKRFTDAEAIVIGFEEEMHNANEAKRDATGRTERSTSK
AGLHGKGTLGALVVKNERGIVFNIGTGFTAAQRADYWANHPSLFGKMVKFKHFDHGT
VDAPRHPVFIGFRHPEDM

Hin DNA Ligase, GenBank ID P44121, from Haemophilus influenza

MKFYRTLLLFFASSFAFANSDLMLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQLLTR
QGQRLSPPAYFIKDFPPFAIDGELFSERNHFEEIS**T**ITKSFKGDGWEKLKLYVFDVPDAE
GNLFERLAKLKAHLLEHPTTYIEIIEQIPVKDKTHLYQFLAQVENLQGEGVVVRNPNAP
YERKRSSQILKLKTAR**G**EECTVIAHHKGKGQFENVMGALTCKNHRGEFKIGSGFNLNE
RENPPPIGSVITYKYRGITNSGKPRFATYWREKK

DLX DNA Ligase, artificial ligase derived from Hin DNA ligase from Haemophilus influenza:

MKFYRTLLLFFASSFAFANSDLMLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQLLTR
QGQRLSPPAYFIKDFPPFAIDGELFSERNHFEEIS**S**ITKSFKGDGWEKLKLYVFDVPDAEG
NLFERLAKLKAHLLEHPTTYIEIIEQIPVKDKTHLYQFLAQVENLQGEGVVVRNPNAPY
ERKRSSQILKLKTARGEECTVIAHHKGKGQFENVMGALTCKNHRGEFKIGSGFNLNER
ENPPPIGSVITYKYRGITNSGKPRFATYWREKK

DLXd DNA Ligase, artificial ligase derived from Hin D ligase from Haemophilus influenza:

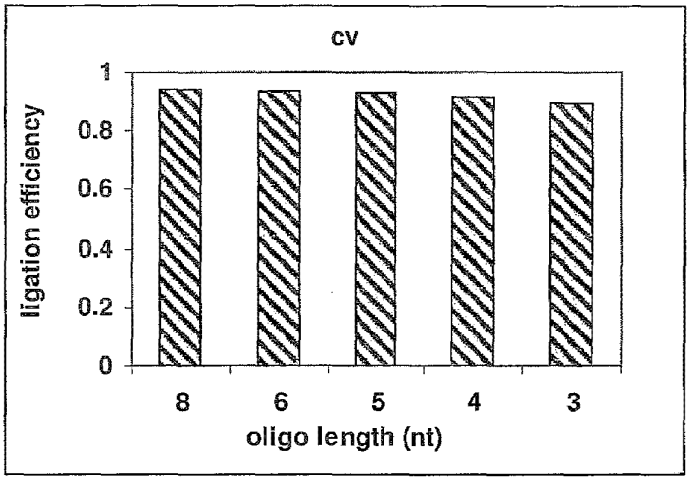
MKFYRTLLLFFASSFAFANSDLMLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQLLTR
QGQRLSPPAYFIKDFPPFAIDGELFSERNHFEEIS**S**ITKSFKGDGWEKLKLYVFDVPDAEG
NLFERLAKLKAHLLEHPTTYIEIIEQIPVKDKTHLYQFLAQVENLQGEGVVVRNPNAPY
ERKRSSQILKLKTAR**D**EECTVIAHHKGKGQFENVMGALTCKNHRGEFKIGSGFNLNER
ENPPPIGSVITYKYRGITNSGKPRFATYWREKK

DLXd2 DNA Ligase (Gammaproteobacteria, Haemophilus influenza) (modified)

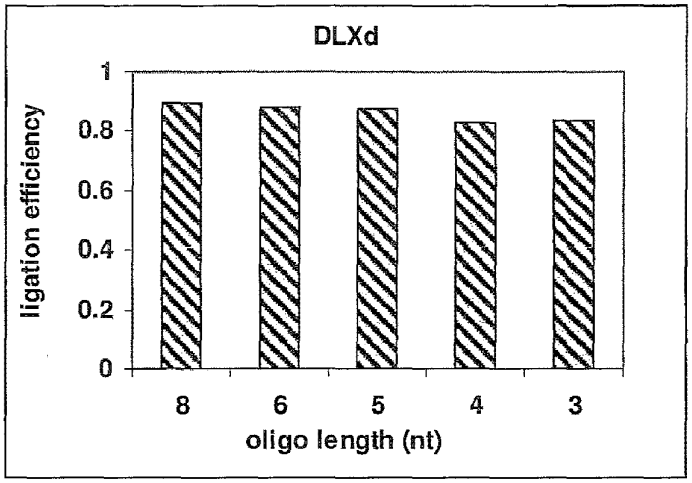
MLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQLLTRQGQRLSPPAYFIKDFPPFAIDGE
LFSERNHFEEIS**S**ITKSFKGDGWEKLKLYVFDVPDAEGNLFERLAKLKAHLLEHPTTYIE
IIEQIPVKDKTHLYQFLAQVENLQGEGVVVRNPNAPYERKRSSQILKLKTAR**D**EECTVIA
HHKGKGQFENVMGALTCKNHRGEFKIGSGFNLNERENPPPIGSVITYKYRGITNSGKPR
FATYWREKK

FIGURE 3

(A)


cv
ligation efficiency
1
0.8
0.6
0.4
0.2
0
8
6
5
4
3
oligo length (nt)

(B)


DLXd
ligation efficiency
1
0.8
0.6
0.4
0.2
0
8
6
5
4
3
oligo length (nt)

(C)

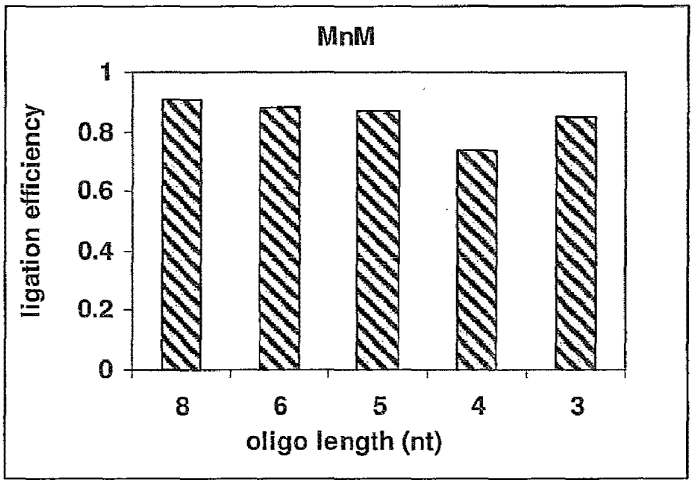
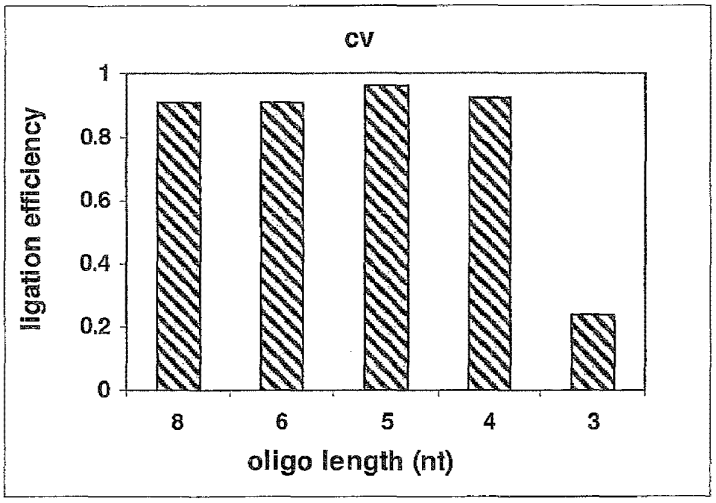
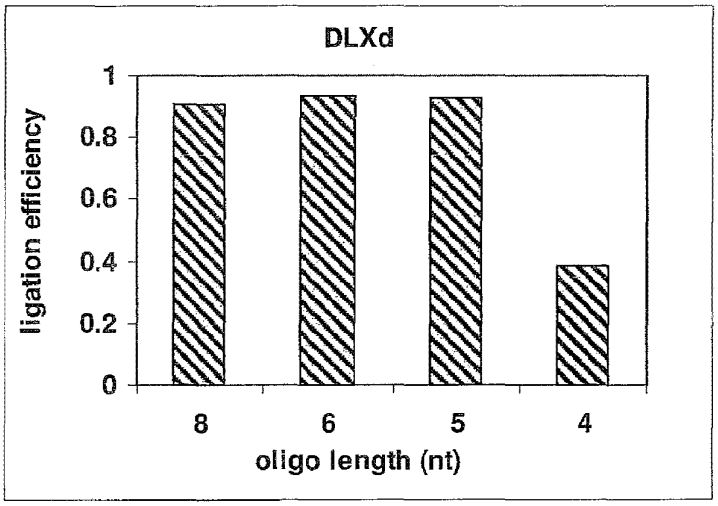

MnM
ligation efficiency
1
0.8
0.6
0.4
0.2
0
8
6
5
4
3
oligo length (nt)

FIGURE 4

(A)


cv
ligation efficiency
1
0.8
0.6
0.4
0.2
0
8
6
5
4
3
oligo length (nt)

(B)


DLXd
ligation efficiency
1
0.8
0.6
0.4
0.2
0
8
6
5
4
oligo length (nt)

(C)

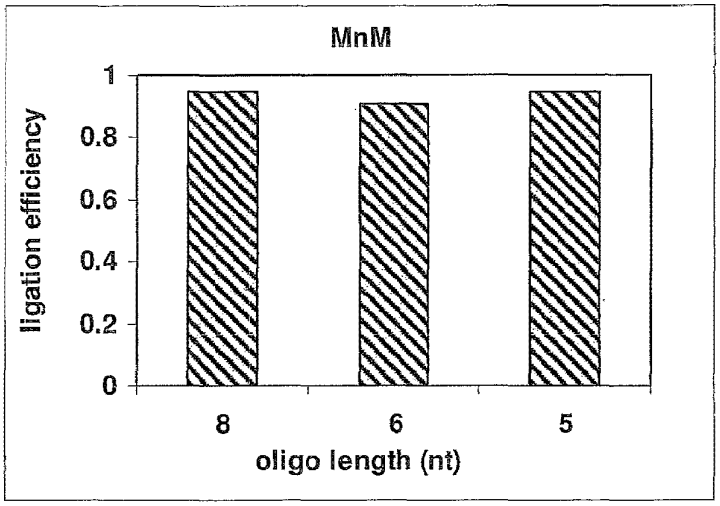
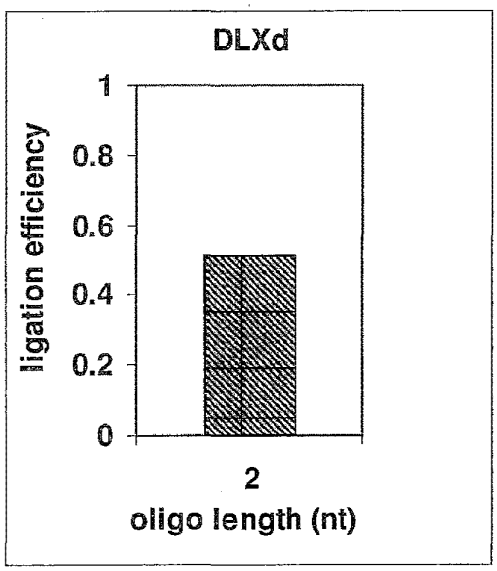

MnM
ligation efficiency
1
0.8
0.6
0.4
0.2
0
8
6
5
oligo length (nt)

FIGURE 5



Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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					

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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
	1	20140296081	A1	2014-10-02	Diehn et al.	Entire Document

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2012099832	WO	A2	2012-07-26	Hendricks	Entire Document	

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NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

	1	CROWLEY, E. et al. "Liquid biopsy: monitoring cancer-genetics in the blood" Nat Rev Clin Oncology (2013) 8:472-478	
	2	GREAVES, L.C. et al. "Quantification of mitochondrial DNA mutation load" Aging Cell (2009) 8(5): 566–572	
	3	Guardant Health, Inc. Response to Notice of Opposition in EP3378952 filed March 29, 2021.	
	4	Guardant Health, Inc. v. FMI Defendant's Answer and Counter Claims, filed January 14, 2021 (C.A. No. 20-1580 (LPS))	
	5	Guardant Health, Inc. v. FMI Defendant's Answering Brief in Opposition to Plaintiff Guardant Health, Inc's Motion for Preliminary Injunction, filed March 5, 2021 (C.A. No. 20-1580 (LPS))	
	6	Opposition to EP3524694, dated April 15, 2021 by Foundation Medicine, Inc.	
	7	Opposition to EP3524694, filed April 16, 2021 by Maiwald International	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2021-05-03
Name/Print	Timothy A. Hott	Registration Number	67740

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.**

Privacy Act Statement

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.

SUMMARY OF THE INTERVIEW

Applicant is appreciative of Examiner Horlick for extending the courtesy of a telephonic interview to Applicant's representative, Timothy Hott, on February 25, 2021. During the interview, arguments based on distinguishing features of the present claims over the cited prior art (Salk et al., US Patent No. 10,752,951) ("Salk") in view of the §102 and §103 rejections were presented. The Final Written Decision ("Decision") of the Patent Trial and Appeal Board ("Board") in IPR2019-000652 dated August 18, 2020 was also discussed.

Electronic Acknowledgement Receipt

EFS ID:	42623675
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	03-MAY-2021
Filing Date:	31-JUL-2020
Time Stamp:	21:23:43
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$4340
RAM confirmation Number	E202153L24472002
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	2021-05-03_GH0004US-CON5_RCETrans.pdf	1364284 f25d3de03dee0ccf055061470121a5e34312a41b	no	3
Warnings:					
Information:					
2		2021-05-03_GH0004US-CON5_RCEResponse.pdf	163539 ea82f363562efcec23f87a9988fcc737d3546d40	yes	12

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	6
Applicant summary of interview with examiner	7	7
Applicant Arguments/Remarks Made in an Amendment	8	12

Warnings:					
Information:					
3	Notice of concurrent proceedings / decisions	2021-05-03_GH0004US-CON5_NtcConcurrentProceedings_IC_Trial1580.pdf	98630 e910f6e71f46d58128eb63128f87ab882d5df334	no	2
Warnings:					
Information:					
4	Notice of concurrent proceedings / decisions	2021-05-03_GH0004US-CON5_NtcConcurrentProceedings_IV_Trial1580.pdf	275977 ef8919d43da092d637bfb0bb09646b0bb55a0385	no	5
Warnings:					
Information:					

5	Letter specifying the conditions for filing under 37CFR1.97	2021-05-03_GH0004US-CON5_IDSTrans.pdf	144270 371b38c49b1fc6a583058fc05dc5da1b58d2217e	no	4
Warnings:					
Information:					
6	Information Disclosure Statement (IDS) Form (SB08)	2021-05-03_GH0004US-CON5_SB08.pdf	1053810 cc376599f0f68becdf18810272bb75adbc4bcc86	no	4
Warnings:					
Information:					
7	Foreign Reference	WO_2012099832_A2.pdf	778659 b9088f1857dec7716802c1dbcf40e94e748526b2	no	79
Warnings:					
Information:					
8	Non Patent Literature	CROWLEY_NatRevClinOnc_2013_.pdf	1757154 42868307073f46f9d793304cc3ddd579b4db866e	no	13
Warnings:					
Information:					
9	Non Patent Literature	GREAVES_AgingCell_2009_566-572.pdf	196552 3b3a6326bd5821b8fc5bff9c6c95d920a5a749f5	no	7
Warnings:					
Information:					
10	Other Reference-Patent/App/Search documents	EP3378952_OppReply_2021-03-29.pdf	4241224 9c731daf95dc7abe71f59c79b834b16a1fc87f7e	no	76
Warnings:					
Information:					
11	Other Reference-Patent/App/Search documents	GH_FMI_Answer_2021-01-14.pdf	573488 7cb732f4a73f1f189aaed42c6943e347d019cb9a	no	73
Warnings:					
Information:					

12	Other Reference-Patent/App/Search documents	GH_FMI_AnsweringBrief_2021-03-05.pdf	426622 d36d25a8cbfe2bd799459e8018072192d8fb91f0	no	33
Warnings:					
Information:					
13	Other Reference-Patent/App/Search documents	EP3524694_FMI_Opposition_2021-04-15.pdf	4373772 06558436cf43ce1fca84bafb102e934d5fd5ca2e	no	58
Warnings:					
Information:					
14	Other Reference-Patent/App/Search documents	EP3524694_Maiwald_Opposition_2021-04-15.pdf	1915381 e0a000a029e7e6d6a8bc82fecb97bda581d40622	no	38
Warnings:					
Information:					
15	TrackOne Request	2021-05-03_GH0004US-CON5_T1Req.pdf	129248 8b6917b41471e70951b7dd404963a7e7a7245d2d	no	2
Warnings:					
Information:					
16	Fee Worksheet (SB06)	fee-info.pdf	35745 c2675e745fdecc02a55ecddfeb1a35ce3d5c399e	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	17528355	

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.

PATENT
Attorney Docket No. 42534-703.306/GH0004US-CON5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	AmirAli TALASAZ et al.	Confirmation No.:	2506
Application No.	16/945,124	Examiner:	Kenneth HORLICK
Filed:	July 31, 2020	Group Art Unit:	1637
Title: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		Customer No.	115823

NOTICE OF CONCURRENT PROCEEDINGS

Commissioner for Patents
U.S. Patent & Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

Pursuant to 37 CFR 1.56, MPEP 2001.06(c) and other duties of disclosure, Applicant hereby notifies the Office of pleadings by defendant Foundation Medicine, Inc. ("FMI") in the ongoing litigation proceeding *Guardant Health, Inc. v. Foundation Medicine, Inc.* Civil Action No. 1:20-CV-01580-LPS, alleging inequitable conduct for improper inventorship against Applicant. The proceeding was filed in the United States District Court for the District of Delaware. The patents at issue in the litigation are US 10,501,810; US 10,704,085; US 10,704,086; US 10,793,916; US 10,801,063; US 9,840,743; and US 9,834,822.

Publicly available copies of documents describing the abovementioned pleadings by FMI are submitted herewith. If at any point the Examiner would like to be provided with copies of any additional contents of any related proceeding, Applicant will readily provide such copies upon request subject to relevant protective orders.

If any additional fee is due or to credit any overpayment, please charge any fees incorrected in connection with this submission or credit any overpayment to Deposit Account 60-2231 (GH0004US-CON5).

Respectfully submitted,

Date: May 3, 2021

By: /Timothy A. Hott/

Timothy A. Hott
Reg. No. 67,740

GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063
Customer No. 115823

PATENT
Attorney Docket No. 42534-708.306/GH0004US-CON5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	AmirAli TALASAZ et al.	Confirmation No.:	2506
Application No.	16/945,124	Examiner:	Kenneth HORLICK
Filed:	July 31, 2020	Group Art Unit:	1637
Title:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	Customer No.	115823

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Commissioner for Patents
U.S. Patent & Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

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Copies of documents describing the abovementioned pleadings by FMI are submitted herewith. If at any point the Examiner would like to be provided with copies of any additional contents of any related proceeding, Applicant will readily provide such copies upon request subject to relevant protective orders.

If any additional fee is due or to credit any overpayment, please charge any fees incorrected in connection with this submission or credit any overpayment to Deposit Account 60-2231 (GH0004US-CON5).

Respectfully submitted,

Date: May 3, 2021

By: /Timothy A. Hott/

Timothy A. Hott
Reg. No. 67,740

GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063
Customer No. 115823

U.S. District Court
District of Delaware (Wilmington)
CIVIL DOCKET FOR CASE #: 1:20-cv-01580-LPS

Guardant Health, Inc. v. Foundation Medicine, Inc.
Assigned to: Judge Leonard P. Stark
Related Case: 1:17-cv-01616-LPS-CJB
Cause: 35:271 Patent Infringement

Date Filed: 11/23/2020
Jury Demand: Plaintiff
Nature of Suit: 830 Patent
Jurisdiction: Federal Question

Plaintiff

Guardant Health, Inc.

represented by **Brian E. Farnan**
Farnan LLP
919 North Market Street
12th Floor
Wilmington, DE 19801
(302) 777-0300
Fax: (302) 777-0301
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Justin L. Constant
Email: justin.constant@weil.com
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302-777-0338
Fax: 302-421-5870
Email: mfarnan@farnanlaw.com
ATTORNEY TO BE NOTICED

V.

Defendant

Foundation Medicine, Inc.

represented by **Karen Jacobs**
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1201 North Market Street
P.O. Box 1347
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(302)658-9200
Email: kjacobs@mnat.com
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P.O. Box 1347
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302-351-9106
Email: jtigan@mnat.com
ATTORNEY TO BE NOTICED

Counter Claimant

Foundation Medicine, Inc.

represented by **Karen Jacobs**
(See above for address)
LEAD ATTORNEY
ATTORNEY TO BE NOTICED

Jeremy A. Tigan
(See above for address)
ATTORNEY TO BE NOTICED

V.

Counter Defendant

Guardant Health, Inc.

represented by **Brian E. Farnan**
(See above for address)
LEAD ATTORNEY
ATTORNEY TO BE NOTICED

Derek C. Walter
(See above for address)
ATTORNEY TO BE NOTICED

Edward R. Reines
(See above for address)
ATTORNEY TO BE NOTICED

Garland T. Stephens

(See above for address)
ATTORNEY TO BE NOTICED

Justin L. Constant
(See above for address)
ATTORNEY TO BE NOTICED

Michael J. Farnan
(See above for address)
ATTORNEY TO BE NOTICED

Date Filed	#	Docket Text
11/23/2020	1	COMPLAINT filed with Jury Demand against Foundation Medicine, Inc. - Magistrate Consent Notice to Pltf. (Filing fee $400, receipt number ADEDC-3350084.) - filed by Guardant Health, Inc. (Attachments: # 1 Exhibit 1, # 2 Exhibit 2, # 3 Exhibit 3, # 4 Exhibit 4, # 5 Exhibit 5, # 6 Exhibit 6, # 7 Exhibit 7, # 8 Exhibit 8, # 9 Exhibit 9, # 10 Exhibit 10, # 11 Exhibit 11, # 12 Exhibit 12, # 13 Exhibit 13, # 14 Exhibit 14, # 15 Exhibit 15, # 16 Exhibit 16, # 17 Exhibit 17, # 18 Exhibit 18, # 19 Exhibit 19, # 20 Exhibit 20, # 21 Exhibit 21, # 22 Exhibit 22, # 23 Exhibit 23, # 24 Civil Cover Sheet)(kmd) (Entered: 11/24/2020)
11/23/2020	2	Notice, Consent and Referral forms re: U.S. Magistrate Judge jurisdiction. (kmd) (Entered: 11/24/2020)
11/23/2020	3	Report to the Commissioner of Patents and Trademarks for Patent/Trademark Number(s) US 10,501,810 B2; US 10,704,085 B2; US 10,704,086 B2; US 10,793,916 B2; US 10,801,063 B2; US 9,840,743 B2; US 9,834,822 B2. (kmd) (Entered: 11/24/2020)
11/23/2020	4	Summonses Issued (please complete the top portion of the form and print out for use/service). (kmd) (Entered: 11/24/2020)
11/25/2020		Case Assigned to Judge Leonard P. Stark. Please include the initials of the Judge (LPS) after the case number on all documents filed. (rjb) (Entered: 11/25/2020)
11/25/2020	5	SUMMONS Returned Executed by Guardant Health, Inc.. Foundation Medicine, Inc. served on 11/24/2020, answer due 12/15/2020. (Farnan, Michael) (Entered: 11/25/2020)
12/08/2020	6	MOTION for Pro Hac Vice Appearance of Attorney Edward R. Reines, Derek C. Walter, Garland T. Stephens, and Justin L. Constant - filed by Guardant Health, Inc.. (Farnan, Brian) (Entered: 12/08/2020)
12/10/2020	7	MOTION for Preliminary Injunction - filed by Guardant Health, Inc.. (Attachments: # 1 Text of Proposed Order, # 2 Rule 7.1.1 Certification, # 3 Certificate of Service)(Farnan, Brian) (Entered: 12/10/2020)
12/10/2020	8	[SEALED] OPENING BRIEF in Support re 7 MOTION for Preliminary Injunction filed by Guardant Health, Inc..Answering Brief/Response due date per Local Rules is 12/24/2020. (Farnan, Brian) (Entered: 12/10/2020)
12/10/2020		SO ORDERED, re 6 MOTION for Pro Hac Vice Appearance of Attorney Edward R. Reines, Derek C. Walter, Garland T. Stephens, and Justin L. Constant filed by Guardant Health, Inc. Signed by Judge Leonard P. Stark on 12/10/20. (ntl) (Entered: 12/10/2020)
12/10/2020	9	DECLARATION re 8 Opening Brief in Support *of Garland Stephens* by Guardant Health, Inc.. (Attachments: # 1 Exhibit 1, # 2 Exhibit 2, # 3 Exhibit 3, # 4 Exhibit 4, # 5 Exhibit 5, # 6 Exhibit 6, # 7 Exhibit 7, # 8 Exhibit 8, # 9 Exhibit 9, # 10 Exhibit 10)(Farnan, Brian) (Entered: 12/10/2020)
12/10/2020	10	[SEALED] DECLARATION re 8 Opening Brief in Support *of Gregory Cooper* by Guardant Health, Inc.. (Attachments: # 1 Exhibit 1, # 2 Exhibit 2, # 3 Exhibit 3, # 4 Exhibit 4, # 5 Exhibit 5, # 6 Exhibit 6, # 7 Exhibit 7, # 8 Exhibit 8, # 9 Exhibit 9, # 10 Exhibit 10, # 11 Exhibit 11, # 12 Exhibit 12, # 13 Exhibit 13, # 14 Exhibit 14, # 15 Exhibit 15, # 16 Exhibit 16, # 17 Exhibit 17, # 18 Exhibit 18, # 19 Exhibit 19, # 20 Exhibit 20, # 21 Exhibit 21, # 22 Exhibit 22, # 23 Exhibit 23)(Farnan, Brian) (Entered: 12/10/2020)
12/10/2020	11	DECLARATION re 8 Opening Brief in Support *of Daniel Simon* by Guardant Health, Inc.. (Farnan, Brian) (Entered: 12/10/2020)
12/11/2020	12	Joint STIPULATION TO EXTEND TIME to ANSWER complaint to January 14, 2021 - filed by Foundation Medicine, Inc.. (Jacobs, Karen) (Entered: 12/11/2020)
12/14/2020		SO ORDERED, re 12 Joint STIPULATION TO EXTEND TIME to ANSWER complaint to January 14, 2021 filed by Foundation Medicine, Inc. Signed by Judge Leonard P. Stark on 12/14/20. (ntl) (Entered: 12/14/2020)
12/15/2020		Pro Hac Vice Attorney Edward R. Reines,Derek C. Walter,Justin L. Constant for Guardant Health, Inc. added for electronic noticing. Pursuant to Local Rule 83.5 (d)., Delaware counsel shall be the registered users of CM/ECF and shall be required to file all papers. (mal) (Entered: 12/15/2020)
12/16/2020		Pro Hac Vice Attorney Garland T. Stephens for Guardant Health, Inc. added for electronic noticing. Pursuant to Local Rule 83.5 (d)., Delaware counsel shall be the registered users of CM/ECF and shall be required to file all papers. (mal) (Entered: 12/16/2020)
12/18/2020	13	REDACTED VERSION of 8 Opening Brief in Support by Guardant Health, Inc.. (Farnan, Michael) (Entered: 12/18/2020)
12/18/2020	14	REDACTED VERSION of 10 Declaration,, by Guardant Health, Inc.. (Attachments: # 1 Exhibits 1-23)(Farnan, Michael) (Main Document 14 replaced on 12/21/2020) (ntl). (Entered: 12/18/2020)
12/21/2020		CORRECTING ENTRY: Corrected main document added to D.I. 14 per request of counsel. (ntl) (Entered: 12/21/2020)
12/23/2020	15	NOTICE OF SERVICE of Guardant Health, Inc.'s First Set of Requests for Production of Documents and Things to FMI (Nos. 1-10) filed by Guardant Health, Inc..(Farnan, Brian) (Entered: 12/23/2020)
12/23/2020	16	NOTICE OF SERVICE of Foundation Medicine, Inc.'s First Set of Requests for Production of Documents and Things to Guardant Health, Inc. filed by Foundation Medicine, Inc..(Tigan, Jeremy) (Entered: 12/23/2020)
12/23/2020	17	MOTION for Pro Hac Vice Appearance of Attorney Eric J. Marandett, G. Mark Edgarton, Sophie F. Wang and Diane C. Seol - filed by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 12/23/2020)
12/23/2020		SO ORDERED, re 17 MOTION for Pro Hac Vice Appearance of Attorney Eric J. Marandett, G. Mark Edgarton, Sophie F. Wang and Diane C. Seol filed by Foundation Medicine, Inc. Signed by Judge Leonard P. Stark on 12/23/20. (ntl) (Entered: 12/23/2020)
12/23/2020	18	STIPULATION and [Proposed] Order Regarding Preliminary Injunction Discovery and Briefing re 7 MOTION for Preliminary Injunction by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 12/23/2020)
01/04/2021	19	SO ORDERED, re 18 STIPULATION and [Proposed] Order Regarding Preliminary Injunction Discovery and Briefing -- A Motion Hearing (remote) is set for 5/14/2021 at 9:00 AM. Signed by Judge Leonard P. Stark on 1/4/21. (ntl) (Entered: 01/04/2021)
01/06/2021	20	NOTICE OF SERVICE of Plaintiff's Response to Foundation Medicine, Inc.'s First Set of Requests for Production of Documents and Things filed by Guardant Health, Inc..(Farnan, Brian) (Entered: 01/06/2021)
01/06/2021	21	NOTICE OF SERVICE of Defendant Foundation Medicine, Inc.'s Responses and Objections to Plaintiff Guardant Health, Inc.'s First Set of Requests for Production of Documents and Things (Nos. 1-10) filed by Foundation Medicine, Inc..(Tigan, Jeremy) (Entered: 01/06/2021)
01/14/2021	22	ANSWER to 1 Complaint,, with Jury Demand , COUNTERCLAIM against Guardant Health, Inc. by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 1-37)(Tigan, Jeremy) (Entered: 01/14/2021)
01/25/2021	23	NOTICE OF SERVICE of (1) Deposition Notice of Gregory Cooper and (2) Deposition Notice of Daniel Simon filed by Foundation Medicine, Inc..(Simonetti, Sarah) (Entered: 01/25/2021)
02/04/2021	24	ANSWER to 22 Answer to Complaint, Counterclaim by Guardant Health, Inc..(Farnan, Michael) (Entered: 02/04/2021)
02/23/2021	25	PROPOSED ORDER Stipulated Protective Order by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 02/23/2021)
02/26/2021		SO ORDERED, re 25 Stipulated Protective Order filed by Foundation Medicine, Inc. Signed by Judge Leonard P. Stark on 2/25/21. (ntl) (Entered: 02/26/2021)
02/26/2021	26	[SEALED] ANSWERING BRIEF in Opposition re 7 MOTION for Preliminary Injunction filed by Foundation Medicine, Inc..Reply Brief due date per Local Rules is 3/5/2021. (Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	27	[SEALED] APPENDIX re 26 Answering Brief in Opposition -- *Volume 1 of 4 (Exhibits 1-50)* by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 1-16, # 2 Exhibit 17-32, # 3 Exhibit 33-42, # 4 Exhibit 43-50)(Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	28	[SEALED] APPENDIX re 26 Answering Brief in Opposition -- *Volume 2 of 4 (Exhibits 51-90)* by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 51-58, # 2 Exhibit 59-75, # 3 Exhibit 76-90)(Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	29	[SEALED] APPENDIX re 26 Answering Brief in Opposition -- *Volume 3 of 4 (Exhibits 91-124)* by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 91-100, # 2 Exhibit 101-112, # 3 Exhibit 113-124) (Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	30	[SEALED] APPENDIX re 26 Answering Brief in Opposition -- *Volume 4 of 4 (Exhibits 125-147)* by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 125-139, # 2 Exhibit 140-147)(Tigan, Jeremy) (Attachment 1 replaced on 3/4/2021) (ntl). (Entered: 02/26/2021)
02/26/2021	31	[SEALED] DECLARATION re 26 Answering Brief in Opposition -- *Declaration of Stacey Gabriel, Ph.D.* by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	32	[SEALED] DECLARATION re 26 Answering Brief in Opposition -- *Declaration of Gary Benson, Ph.D.* by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	33	[SEALED] DECLARATION re 26 Answering Brief in Opposition -- *Declaration of Elizabeth Mansfield, Ph.D.* by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	34	DECLARATION re 26 Answering Brief in Opposition -- *Declaration of Ameet Malik* by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 02/26/2021)
02/26/2021	35	DECLARATION re 26 Answering Brief in Opposition -- *Declaration of Neil D. Shore, M.D., F.A.C.S., C.P.I.* by Foundation Medicine, Inc.. (Tigan, Jeremy) (Main Document 35 replaced on 3/10/2021) (ntl). (Entered: 02/26/2021)

02/26/2021	56	DECLARATION re 26 Answering Brief in Opposition -- *Declaration of Martin Dietrich, M.D., Ph.D.* by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 02/26/2021)
03/04/2021		CORRECTING ENTRY: Corrected Attachment 1 added to D.I. 30 per request of counsel. (ntl) (Entered: 03/04/2021)
03/05/2021	57	STIPULATION TO EXTEND TIME for Plaintiff to file a Reply in Support of its Motion for Preliminary Injunction to 3/29/2021 - filed by Guardant Health, Inc.. (Farnan, Brian) (Entered: 03/05/2021)
03/05/2021	58	REDACTED VERSION of 26 Answering Brief in Opposition by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 03/05/2021)
03/05/2021	59	REDACTED VERSION of 27 Appendix by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 1-16, # 2 Exhibit 17-32, # 3 Exhibit 33-42, # 4 Exhibit 43-50)(Tigan, Jeremy) (Entered: 03/05/2021)
03/05/2021	60	REDACTED VERSION of 28 Appendix by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 51-58, # 2 Exhibit 59-75, # 3 Exhibit 76-90)(Tigan, Jeremy) (Entered: 03/05/2021)
03/05/2021	61	REDACTED VERSION of 29 Appendix by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 91-124)(Tigan, Jeremy) (Entered: 03/05/2021)
03/05/2021	62	REDACTED VERSION of 30 Appendix, by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit 125-147)(Tigan, Jeremy) (Entered: 03/05/2021)
03/05/2021	63	REDACTED VERSION of 31 Declaration by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 03/05/2021)
03/05/2021	64	REDACTED VERSION of 32 Declaration by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 03/05/2021)
03/05/2021	65	REDACTED VERSION of 33 Declaration by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 03/05/2021)
03/09/2021		SO ORDERED, re 37 STIPULATION TO EXTEND TIME for Plaintiff to file a Reply in Support of its Motion for Preliminary Injunction to 3/29/2021 filed by Guardant Health, Inc. Signed by Judge Leonard P. Stark on 3/9/21. (ntl) (Entered: 03/09/2021)
03/10/2021		CORRECTING ENTRY: Corrected document added to D.I. 35 per request of counsel. (ntl) (Entered: 03/10/2021)
03/29/2021	66	[SEALED] REPLY BRIEF re 7 MOTION for Preliminary Injunction filed by Guardant Health, Inc.. (Farnan, Brian) (Entered: 03/29/2021)
03/29/2021	67	[SEALED] DECLARATION re 46 Reply Brief *of Justin Odegaard* by Guardant Health, Inc.. (Attachments: # 1 Exhibit A, # 2 Exhibit B, # 3 Exhibit C, # 4 Exhibit D, # 5 Exhibit E, # 6 Exhibit F, # 7 Exhibit G, # 8 Exhibit H, # 9 Exhibit I, # 10 Exhibit J, # 11 Exhibit K, # 12 Exhibit L)(Farnan, Brian) (Entered: 03/29/2021)
03/29/2021	68	[SEALED] DECLARATION re 46 Reply Brief *of Justin Constant* by Guardant Health, Inc.. (Attachments: # 1 Exhibit 1, # 2 Exhibit 2, # 3 Exhibit 3, # 4 Exhibit 4, # 5 Exhibit 5, # 6 Exhibit 6, # 7 Exhibit 7, # 8 Exhibit 8)(Farnan, Brian) (Entered: 03/29/2021)
03/29/2021	69	[SEALED] DECLARATION re 46 Reply Brief *of Gregory Cooper, Ph.D.* by Guardant Health, Inc.. (Farnan, Brian) (Entered: 03/29/2021)
04/15/2021	50	STIPULATION and [Proposed] Order *Regarding Surreply Brief in Opposition to Guardant's Motion for Preliminary Injunction* by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 04/15/2021)
04/16/2021		CORRECTING ENTRY: Letter filed 4/15/2021 at D.I. 51 removed at request of filer. (dlb) (Entered: 04/16/2021)
04/16/2021	51	[SEALED] MOTION for Leave to File *Amended Answer and Counterclaims* - filed by Foundation Medicine, Inc.. (Attachments: # 1 Exhibit A, # 2 Exhibit B)(Tigan, Jeremy) (Entered: 04/16/2021)
04/16/2021	52	[SEALED] Letter to The Honorable Leonard P. Stark from Jeremy A. Tigan regarding Motion for Leave to File Amended Answer and Counterclaims - re 51 MOTION for Leave to File *Amended Answer and Counterclaims*. (Attachments: # 1 Exhibit 1)(Tigan, Jeremy) (Entered: 04/16/2021)
04/20/2021		SO ORDERED, re 50 STIPULATION and [Proposed] Order Regarding Surreply Brief in Opposition to Guardant's Motion for Preliminary Injunction filed by Foundation Medicine, Inc. Signed by Judge Leonard P. Stark on 4/20/21. (ntl) (Entered: 04/20/2021)
04/20/2021	53	Letter to The Honorable Leonard P. Stark from Jeremy A. Tigan regarding Request for Teleconference - re 19 SO ORDERED, Set Hearings. (Tigan, Jeremy) (Entered: 04/20/2021)
04/21/2021	54	REDACTED VERSION of 46 Reply Brief by Guardant Health, Inc.. (Farnan, Brian) (Entered: 04/21/2021)
04/21/2021	55	REDACTED VERSION of 47 Declaration, *of Justin Odegaard* by Guardant Health, Inc.. (Attachments: # 1 Exhibits A-L)(Farnan, Brian) (Entered: 04/21/2021)
04/21/2021	56	REDACTED VERSION of 48 Declaration, *of Justin Constant* by Guardant Health, Inc.. (Attachments: # 1 Exhibits 1-8)(Farnan, Brian) (Entered: 04/21/2021)
04/21/2021	57	REDACTED VERSION of 49 Declaration *of Gregory Cooper, Ph.D.* by Guardant Health, Inc.. (Farnan, Brian) (Entered: 04/21/2021)
04/21/2021	58	STIPULATION TO EXTEND TIME for Plaintiff to respond to Foundation Medicine, Inc.'s Motions for Leave to File Amended Answer and Counterclaims to 5/7/2021 - filed by Guardant Health, Inc.. (Farnan, Brian) (Entered: 04/21/2021)
04/22/2021	59	MOTION for Pro Hac Vice Appearance of Attorney Matthew Wolf - filed by Foundation Medicine, Inc.. (Tigan, Jeremy) (Entered: 04/22/2021)
04/22/2021	60	ORAL ORDER: Having reviewed FMI's recent letter, IT IS HEREBY ORDERED that: (1) the parties shall submit a joint status report (not to exceed four pages in total), on April 26, setting out their position(s) for how the forthcoming remote hearing should proceed and any disputes relating to it; and (2) the Court will discuss such issues with the parties by teleconference on April 27 at 4:45 p.m. The parties and any interested members of the public can access the teleconference by dialing 877-336-1829 and using the access code 1408971. ORDERED by Judge Leonard P. Stark on 4/22/21. (ntl) (Entered: 04/22/2021)
04/22/2021	61	ORAL ORDER: IT IS HEREBY ORDERED that this case is referred to Magistrate Judge Christopher J. Burke, pursuant to 28 U.S.C. § 636(b), to hear and resolve all discovery disputes and all issues regarding scheduling. ORDERED by Judge Leonard P. Stark on 4/22/21. (ntl) (Entered: 04/22/2021)
04/23/2021		SO ORDERED, re 59 MOTION for Pro Hac Vice Appearance of Attorney Matthew Wolf filed by Foundation Medicine, Inc. Signed by Judge Leonard P. Stark on 4/23/21. (ntl) (Entered: 04/23/2021)
04/23/2021		SO ORDERED, re (58 in 1:20-cv-01580-LPS, 533 in 1:17-cv-01616-LPS-CJB) STIPULATION TO EXTEND TIME for Plaintiff to respond to Foundation Medicine, Inc.'s Motions for Leave to File Amended Answer and Counterclaims to 5/7/2021 filed by Guardant Health, Inc. Signed by Judge Leonard P. Stark on 4/22/21. Associated Cases: 1:17-cv-01616-LPS-CJB, 1:20-cv-01580-LPS (ntl) (Entered: 04/23/2021)
04/26/2021	62	Joint Letter to The Honorable Leonard P. Stark from Jeremy A. Tigan regarding D.I. 53 - re 53 Letter. (Tigan, Jeremy) (Entered: 04/26/2021)
04/27/2021	63	ORAL ORDER: Having reviewed the joint status report filed in C.A. No. 20-1580 (D.I. 62), and recognizing that the parties are also litigating C.A. No. 17-1616 and address matters relating to that action in the status report, IT IS HEREBY ORDERED that: (1) the May 13 spoliation evidentiary hearing in 17-1616 and May 14 preliminary injunction hearing in 20-1580 will be held in-person in Delaware, as jointly requested by the parties; (2) the Court would prefer to hear from the forensic experts by live testimony rather than relying solely on expert reports and deposition testimony; (3) each side will be allocated up to 3.5 hours for its presentation at the spoliation hearing, to be used however it wishes; (4) any witness who will testify at the hearing will be sequestered during opening statements; (5) Guardant will be permitted to examine Dr. Eltoukhy first, on direct examination, before FMI will be permitted to call him as an adverse witnesses - this order of presentation will be more helpful to the Court than the alternative presented by FMI, even recognizing that FMI is the moving party and bears the burden of proof; and (6) each side will be allocated up to 3 hours for its presentation at the preliminary injunction hearing and, as requested jointly by the parties, the Court will hear from the parties on an issue-by-issue basis (similar to at a claim construction hearing). IT IS FURTHER ORDERED that the teleconference scheduled for later today is CANCELLED. ORDERED by Judge Leonard P. Stark on 4/27/21. Associated Cases: 1:17-cv-01616-LPS-CJB, 1:20-cv-01580-LPS (ntl) Modified on 4/27/2021 (ntl). (Entered: 04/27/2021)
04/27/2021	64	ORAL ORDER: Pursuant to 28 U.S.C. § 636, IT IS HEREBY ORDERED that the following motion is referred to Magistrate Judge Christopher J. Burke: Motion for Leave to Amend Answer and Counterclaims (D.I. 51). ORDERED by Judge Leonard P. Stark on 4/27/21. (ntl) (Entered: 04/27/2021)

PACER Service Center

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05/03/2021 12:55:59

PACER Login:	wg0135	Client Code:	51042.0005-f203
Description:	Docket Report	Search Criteria:	1:20-cv-01580-LPS Start date: 1/1/1972 End date: 5/3/2021
Billable Pages:	7	Cost:	0.70

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	16/945,124	Filing Date	2020-07-31	Docket Number (if applicable)	42534-708.306	Art Unit	1637
First Named Inventor	AmirAli TALASAZ			Examiner Name	Kenneth R. HORLICK		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

☒ The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 602231

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

X Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

Signature of Registered U.S. Patent Practitioner			
Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2021-05-03
Name	Timothy A. Hott	Registration Number	67740

This collection of information is required by 37 CFR 1.114. 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 12 minutes 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.

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

Privacy Act Statement

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 records.

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.

REMARKS

Claims 1-30 were pending prior to entry of the abovementioned amendments. Claims 1, 4-5, 14-18, 21, 25-28, and 30 have been amended to clarify the claimed subject matter. New claim 31 has been added. Claim 13 has been canceled without prejudice or disclaimer. These amendments are fully supported throughout the instant specification and original claims as filed. No new matter is added by any of these amendments.

I. Rejection under 35 U.S.C. §102(a)(2)

Claims 1-2, 9, 11-12, 14-15, 18-19, 21, 23, and 25-29 were rejected under 35 U.S.C. §102(a)(2) as allegedly being anticipated by Salk.

Applicant respectfully submits that claims 1 and 18 are novel over Salk, at least because Salk does not meet all of the elements of the instant claims. For example, Salk does not disclose, either in the claims or elsewhere in the specification, a method directed to the analysis of **cell-free deoxyribonucleic acid (cfDNA) molecules**, much less "providing a population of **cell-free deoxyribonucleic acid (cfDNA) molecules** having first and second complementary strands" and "tagging a plurality of the **cfDNA molecules** in the population with duplex tags comprising molecular barcodes from a set of molecular barcodes to produce tagged parent polynucleotides, wherein the duplex tags are attached to both ends of a molecule of the plurality of the **cfDNA molecules**", as recited in claim 1. As another example, Salk does not disclose, either in the claims or elsewhere in the specification, a method comprising "tagging a population of double-stranded **cell-free deoxyribonucleic acid (cfDNA)** molecules obtained or derived from a sample of a subject with a set of tags comprising molecular barcodes to produce tagged parent polynucleotides" as recited in claim 18. Nor does Salk disclose "wherein reducing or tracking the redundancy of the plurality of sequence reads comprises mapping at least a subset of the plurality of sequence reads to a reference sequence", as recited in claim 1.

As explained by the Board in the Decision, "**Schmitt [Salk] does not expressly teach that the target polynucleotide is cfDNA…"** (Decision, page 31, lines 6-7). Salk exclusively describes the use of **cellular DNA** samples throughout the specification (*see* Examples 1-4). To the extent that other types of nucleic acids are even mentioned in Salk, a person of ordinary skill in the art would **not** understand these portions of Salk to be contemplating application of Salk's Duplex Consensus Sequencing (DCS) method to the analysis of cfDNA.

Merely observing that others have applied fundamentally different methods to fetal aneuploidy (Salk, 1:47-50) does not constitute disclosure of application of the DCS method to cfDNA. Further, single molecule counting of circulating microRNAs (i.e., Salk's only disclosed circulating nucleic acid) does not constitute disclosure of applying the DCS method to cfDNA, at least because (1) circulating microRNA is not cfDNA, and (2) single molecule counting is not the DCS method.

Additionally, Salk does not disclose, among other aspects, at least "mapping at least a subset of the plurality of sequence reads to a reference sequence", as recited in claim 1.

Accordingly, Applicant respectfully requests that the §102 rejections of independent claims 1 and 18 be withdrawn.

Claims 2, 9, 11-12, and 14-15 and claims 19, 21, 23 and 25-29 depend from an include all elements of claims 1 and 18, respectively, and recite additional elements of particular advantage and utility. Salk does not meet all the elements of claims 1 and 18, much less the unique combination of claims 2, 9, 11-12, and 14-15 and claims 19, 21, 23, and 25-29. Thus, Applicant respectfully requests that the §102 rejections of these claims also be withdrawn.

II. Rejection Under 35 U.S.C. §103

Claims 3-6, 8, 10, 13, 16-17, 20, 22, 24, and 30 were rejected under 35 U.S.C. §103 as allegedly being unpatentable over Salk.

Initially, Applicant submits that claims 3-6, 8, 10, 13, 16-17, 20, 22, 24, and 30 are not obvious over Salk for at least the reasons that these claims depend from and include all the elements of claims 1 and 18, respectively, and recite additional elements of particular advantage and utility. Salk does not disclose or suggest all of the elements of claims 1 and 18, as discussed above, much less the combination of elements of claims 3-6, 8, 10, 13, 16-17, 20, 22, 24, and 30. Further, the teachings and disclosure of Salk provide no reasonable expectation of success for at least a method directed to the analysis of "**cell-free deoxyribonucleic acid (cfDNA) molecules**" as recited in claims 1 and 18, let alone dependents thereof. There are numerous aspects of Salk's method that would be recognized as incompatible with cfDNA analysis, including at least Salk's random fragmentation process which would otherwise result in destruction of cfDNA that is already present in only minute quantities, Salk's sample processing that instructs discarding fragments of the size expected in cfDNA, and Salk's requirement of excessive amounts of DNA

that are not available in cfDNA samples. The inapplicability of the Salk method to cfDNA samples was also recognized and documented in the literature. For example, in 2017 (nearly five years after the earliest claimed filing date of Salk), Perakis et al. noted that the method had yet to be applied to cfDNA samples. Perakis further stated that "[p]rospects of success are limited since the method is relatively inefficient when limited amounts of input DNA—as it is most likely the case for cfDNA—are used." Perakis et al., "Advances in Circulating Tumor DNA Analysis," *Adv. Clin. Chem.*, Vol. 80 (2017), p. 30 (cited in Applicant's IDS submitted July 31, 2020).

Moreover, assuming *arguendo* that the DCS method of Salk, either alone or in combination with another reference, is suitable to be applied toward cfDNA, Salk does not disclose, among other aspects, at least (1) "**reducing or tracking redundancy** of a plurality of sequence reads from the set of sequence reads … to determine distinct cfDNA molecules from among the tagged parent polynucleotides, wherein the distinct cfDNA molecules are determined based on (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, or (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, **wherein reducing or tracking the redundancy of the plurality of sequence reads comprises mapping at least a subset of the plurality of sequence reads to a reference sequence**", as recited in claim 1, or (2) "sorting a plurality of sequence reads from the set of sequence reads into **(i) families comprising paired reads** corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides, **and (ii) families comprising unpaired reads** corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides", as recited in claim 18. In particular, Salk does not disclose "reducing or tracking the redundancy of the plurality of sequence reads", much less doing so at least in part by "mapping … sequence reads to a reference sequence", as recited in claim 1. Further, in the DCS method, Salk merely discloses that a duplex consensus sequence is generated from single-stranded consensus sequences of **both strands** of a double-stranded DNA fragment, and that unpaired reads are discarded rather than being sorted into families. Hence, Salk does not disclose "sorting …

sequence reads into... families comprising unpaired reads", as recited in claim 18. Applicant respectfully submits that at least for the foregoing reasons, claims 3-6, 8, 10, 16-17, 20, 22, 24, and 30 are not obvious over the cited references. Accordingly, withdrawal of the rejections is respectfully requested.

Without conceding in the basis of the rejection, Applicant has canceled claim 13 without prejudice or disclaimer, thereby rendering moot the rejection of this claim under 35 U.S.C. §103.

III. Allowable Subject Matter

Applicant recognizes and appreciates the Examiner's acknowledgement of allowable claimed subject matter. The objection to claim 7 is believed to be moot based on the above discussion related to claim 1, from which claim 7 depends.

***

It shall be understood herein that any instance in which Applicant has addressed certain comments set forth by the Office shall not be construed as a concession to other comments or arguments advanced by the Office. Any circumstance in which Applicant has amended or canceled a claim also does not mean that Applicant concedes to the arguments or positions advanced by the Office with respect to that claim or other claims pending herein.

CONCLUSION

This communication fully addresses the rejections raised in the Office Action mailed on December 21, 2020. Applicant believes that the present application is now in condition for allowance and respectfully requests that the Examiner expedite the prosecution of this application to allowance. The Commissioner is authorized to charge any underpayment, or credit any overpayment, to Deposit Account No. 60-2231 (Attorney Docket No. GH0004US-CON5).

Respectfully submitted,
GUARDANT HEALTH, INC.

Date: May 3, 2021

By: /Timothy A. Hott/
Timothy A. Hott
Registration No.: 67740

GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063
Customer No. 115823

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

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

First Named Inventor:	AmirAli TALASAZ	Nonprovisional Application Number (if known):	16/945,124
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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 /Timothy A. Hott/	Date May 3, 2021
Name (Print/Typed) Timothy A. Hott	Practitioner Registration Number 67740

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.**

☐ *Total of _____ forms are submitted.

Privacy Act Statement

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 disclosure of these records is required by the Freedom of Information Act.
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 inspection 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.

Attorney Docket No. 42534-708.306/GH0004US-CON5
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	AmirAli TALASAZ et al.	Confirmation No.:	2506
Serial No.:	16/945,124	Art Unit:	1637
Filing Date:	July 31, 2020	Examiner:	Kenneth R. HORLICK
Title:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

A. ☒ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☒ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☐ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☐ a fee of $260.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $260.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure

statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 60-2231(Docket No. GH0004US-CON5).

Respectfully submitted,

Dated: May 3, 2021

By: /Timothy A. Hott/
Timothy A. Hott, Reg. No. 67740

Customer No. 115823
GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/945,124	Filing Date 07/31/2020	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ 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).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	05/03/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 30	Minus	** 30	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/TINA J BARDEN/
*** 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 number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. 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 12 minutes 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.

Electronic Patent Application Fee Transmittal

Application Number:	16945124
Filing Date:	31-Jul-2020
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Filer:	Timothy A Hott/Michelle Chan
Attorney Docket Number:	42534-708.306

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4200	4200
Pages:				
Claims:				
Miscellaneous-Filing:				
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**4340**

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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
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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
/K.R.H/	1	20140296081	A1	2014-10-02	Diehn et al.	Entire Document

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
/K.R.H/	1	2012099832	WO	A2	2012-07-26	Hendricks	Entire Document	

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NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

/K.R.H/	1	CROWLEY, E. et al. "Liquid biopsy: monitoring cancer-genetics in the blood" Nat Rev Clin Oncology (2013) 8:472-478	
/K.R.H/	2	GREAVES, L.C. et al. "Quantification of mitochondrial DNA mutation load" Aging Cell (2009) 8(5): 566–572	
/K.R.H/	3	Guardant Health, Inc. Response to Notice of Opposition in EP3378952 filed March 29, 2021.	
/K.R.H/	4	Guardant Health, Inc. v. FMI Defendant's Answer and Counter Claims, filed January 14, 2021 (C.A. No. 20-1580 (LPS))	
/K.R.H/	5	Guardant Health, Inc. v. FMI Defendant's Answering Brief in Opposition to Plaintiff Guardant Health, Inc's Motion for Preliminary Injunction, filed March 5, 2021 (C.A. No. 20-1580 (LPS))	
/K.R.H/	6	Opposition to EP3524694, dated April 15, 2021 by Foundation Medicine, Inc.	
/K.R.H/	7	Opposition to EP3524694, filed April 16, 2021 by Maiwald International	

If you wish to add additional non-patent literature document citation information please click the Add button [Add]

EXAMINER SIGNATURE

Examiner Signature	/KENNETH R HORLICK/	Date Considered	05/11/2021

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2021-05-03
Name/Print	Timothy A. Hott	Registration Number	67740

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.**

Privacy Act Statement

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.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

115823 7590 05/18/2021

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
HORLICK, KENNETH R	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
05/18/2021	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):

Patents@guardanthealth.com
patentdocket@wsgr.com

Office Action Summary	Application No. 16/945,124	Applicant(s) TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	AIA (FITF) 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 3 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 05/03/21.

☐ 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) 1-12 and 14-31 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) 1-6,8-12 and 14-31 is/are rejected.

8) ☑ Claim(s) 7 is/are objected to.

9) ☐ Claim(s) _____ 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 05/03/21.

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

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

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

2. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 05/03/21 has been entered.

MAINTAINED REJECTIONS

3. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

4. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(2) the claimed invention was described in a patent issued under section 151, or in an application for patent published or deemed published under section 122(b), in which the patent or application, as the case may be, names another inventor and was effectively filed before the effective filing date of the claimed invention.

Claims 1-2, 9, 11-12, 14-15, 18-19, 21, 23, 25-29, and 31 are rejected under 35 U.S.C. 102(a)(2) as being anticipated by Salk et al. (US 10,752,951).

Independent claims 1 and 18 are drawn to a method comprising: tagging a plurality of cfDNA molecules with tags comprising molecular barcodes; amplifying tagged molecules; sequencing at least a subset of amplified tagged molecules; - and reducing or tracking redundancy in sequence reads based on paired or unpaired reads and comprising mapping reads to a reference sequence (claim 1) - or - and sorting reads into families comprising paired and unpaired reads (claim 18).

Salk et al. discloses such methods; see Summary in column 3; column 20, line 50 to column 24, line 32; column 26, line 61 to column 28, line 64; and claims 1-28. Specifically, grouping reads into families is disclosed in the paragraph bridging columns 26 and 27, and mapping reads to a reference sequence is disclosed in column 28 at line 45 as well as in claims 2-4, 8-9, 13-14, and 26. Thus, the claimed methods cannot be distinguished from the methods disclosed and claimed in Salk et al.

5. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

Claims 3-6, 8, 10, 16-17, 20, 22, 24, and 30 are rejected under 35 U.S.C. 103 as being unpatentable over Salk et al.

While the further limitations of these dependent claims are not explicitly disclosed in the claims or specification of Salk et al., they clearly merely relate to routine optimization of known-important reaction parameters, which as well established in U.S. patent practice does not support unobviousness. Thus, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the application was filed to modify the method of Salk et al. in the manner of these claims.

REPLY TO ARGUMENTS

6. With respect to the above rejections, the arguments of the response filed 05/03/21 have been fully considered, but are not found persuasive. On page 8 of the response, it is argued that Salk et al. does not teach analysis of cfDNA molecules, but this is explicitly taught in claims 11-18; as one of ordinary skill in the art is aware, 'circulating' nucleic acid molecules are the same as 'cell-free' or 'cf' nucleic acid molecules. The response also argues on pages 8 and 9 that Salk et al. does not teach mapping sequence reads to a reference sequence, but as noted above this is disclosed in column 28 at line 45 as well as in claims 2-4, 8-9, 13-14, and 26. As far as sorting sequence reads into families which is mentioned on pages 10-11, it is noted above that this is disclosed in the paragraph bridging columns 26 and 27. Thus, it is submitted that the claimed methods are either anticipated or rendered obvious by Salk et al.

CONCLUSION

7. Claim 7 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims. No claims are allowable.

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to KENNETH R HORLICK whose telephone number is (571)272-0784. The examiner can normally be reached on Mon. - Thurs. 8:30 - 6:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

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 https://ppair-my.uspto.gov/pair/PrivatePair. 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.

05/13/21

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/945,124	TALASAZ et al.
	Examiner	Art Unit
	KENNETH R HORLICK	1637

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
inventor name search	09/02/2020	KH
reviewed parent applications and references therein	09/02/2020	KH
updated parent searches in USPAT and PGPUB	09/02/2020	KH
updated parent searches in USPAT and PGPUB	12/16/2020	KH
updated parent searches in USPAT and PGPUB	05/11/2021	KH

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637	

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

115823 7590 06/23/2021

Guardant Health / WSGR
650 Page Mill Road
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EXAMINER
HORLICK, KENNETH R

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
06/23/2021	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):

Patents@guardanthealth.com
patentdocket@wsgr.com

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 16/945,124	**Applicant(s)** TALASAZ et al.	
	Examiner CHERYL P GIBSON BAYLOR	**Art Unit** OPET	**AIA (FITF) Status** Yes

1. THE REQUEST FILED 03 May 2021 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 CHERYL GIBSON BAYLOR at (571)272-3213. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/CHERYL GIBSON BAYLOR/ Paralegal Specialist, OPET	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): AmirAli TALASAZ et al.	
Serial Number: 16/945,124	Confirmation No.: 2506
Filing Date: July 31, 2020	Customer No.: 115823
Title: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	Group Art Unit: 1637
	Examiner: Kenneth R. HORLICK

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

RESPONSE TO NON-FINAL OFFICE ACTION

Sir:

This communication is in response to the Non-Final Office Action mailed on May 18, 2021. The shortened statutory period for reply expires August 18, 2021, therefore this response is timely filed. Applicant respectfully requests reconsideration of the above-referenced application in view of the following remarks:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 8 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings in the above-referenced patent application. The foregoing amendments are without prejudice and do not constitute an admission regarding the patentability of the amended subject matter and should not so be construed. Applicant reserves the right to pursue the subject matter of the canceled claims in this or any other appropriate patent application.

Listing of Claims:

1. (Currently amended): A method, comprising:

(a) providing a population of cell-free deoxyribonucleic acid (cfDNA) molecules having first and second complementary strands;

(b) tagging a plurality of the cfDNA molecules in the population with duplex tags comprising molecular barcodes ~~from a set of molecular barcodes~~ to produce tagged parent polynucleotides, wherein the duplex tags are attached to both ends of a molecule of the plurality of the cfDNA molecules,

wherein the plurality of the cfDNA molecules are tagged with n different combinations of molecular barcodes, wherein n is at least 2 and no more than 100,000*z, wherein z is a mean of an expected number of duplicate molecules in the population of cfDNA molecules that map to identical start and stop positions on a reference sequence;

(c) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(d) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(e) reducing or tracking redundancy of a plurality of sequence reads from the set of sequence reads using at least sequencing information from the molecular barcodes of the duplex tags to determine distinct cfDNA molecules from among the tagged parent polynucleotides, wherein the distinct cfDNA molecules are determined based on (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, or (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from cfDNA molecules from among the tagged parent

polynucleotides, wherein reducing or tracking the redundancy of the plurality of sequence reads comprises mapping at least a subset of the plurality of sequence reads to [[a]] the reference sequence.

2. (Previously presented): The method of claim 1, wherein the population of cfDNA molecules is obtained or derived from a subject having cancer.

3. (Previously presented): The method of claim 1, wherein the population of cfDNA molecules comprises 1 nanogram (ng) to 100 ng of cfDNA molecules.

4. (Previously presented): The method of claim 1, wherein the molecular barcodes are ligated to the plurality of the cfDNA molecules using more than a 10X excess of duplex tags as compared to the population of cfDNA molecules, wherein at least 20% of the cfDNA molecules from the population are tagged with the duplex tags.

5. (Previously presented): The method of claim 1, wherein the molecular barcodes are ligated to the plurality of the cfDNA molecules using more than an 80X excess of duplex tags as compared to the population of cfDNA molecules.

6. (Original): The method of claim 5, wherein at least 40% of the cfDNA molecules from the population are tagged with the duplex tags.

7. (Currently amended): The method of claim 1, wherein z is between 2 and 8 ~~the tagging comprises non-uniquely tagging the plurality of the cfDNA molecules, wherein the cfDNA molecules that map to a mappable base position of a reference sequence are tagged with a number of different molecular barcodes ranging from at least 2 to fewer than a number of the cfDNA molecules that map to the mappable base position~~.

8. (Currently amended): The method of claim 1, ~~wherein the set of molecular barcodes comprises 5 to 10,000 different molecular barcode sequences, and~~ wherein the molecular barcodes ~~in the set of molecular barcodes~~ have a length of 5 to 20 base pairs.

9. (Previously presented): The method of claim 1, further comprising, prior to the sequencing, enriching at least a subset of the amplified progeny polynucleotides for target regions of interest to produce enriched progeny polynucleotides.

10. (Original): The method of claim 9, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2,

FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1, CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

11. (Previously presented): The method of claim 10, further comprising, prior to the sequencing, amplifying a plurality of the enriched progeny polynucleotides.

12. (Original): The method of claim 1, wherein the duplex tags are part of sequencing adapters.

13. (Canceled)

14. (Currently amended): The method of claim 1, wherein reducing or tracking the redundancy of the plurality of sequence reads comprises grouping the paired reads or the unpaired reads into families based at least in part on (i) the molecular barcodes <u>associated with</u> [[of]] the paired reads or the unpaired reads and (ii) sequence information at the start and stop positions of the paired reads or the unpaired reads.

15. (Previously presented): The method of claim 1, further comprising:

(f) determining quantitative measures of (i) paired reads or (ii) unpaired reads that map to a genomic locus of the reference sequence.

16. (Previously presented): The method of claim 15, further comprising:

(g) estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides that map to the genomic locus based on the quantitative measures of the paired reads and the unpaired reads.

17. (Previously presented): The method of claim 16, further comprising:

(h) detecting copy number variation in the population of cfDNA molecules by determining a normalized quantitative measure determined in (g) at each of one or more genomic loci.

18. (Previously presented): A method, comprising:

(a) tagging a population of double-stranded cell-free deoxyribonucleic acid (cfDNA) molecules obtained or derived from a sample of a subject with a set of tags comprising molecular barcodes to produce tagged parent polynucleotides;

(b) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(c) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(d) sorting a plurality of sequence reads from the set of sequence reads into (i) families comprising paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides, and (ii) families comprising unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides.

19. (Original): The method of claim 18, wherein the sample is blood, plasma, or serum.

20. (Previously presented): The method of claim 18, wherein the population of double-stranded cfDNA molecules comprises 1 nanogram (ng) to 100 ng of double-stranded cfDNA molecules.

21. (Previously presented): The method of claim 18, wherein the tagging comprises ligating the molecular barcodes to double-stranded cfDNA molecules.

22. (Previously presented): The method of claim 18, wherein the set of tags comprises 2 to 10,000 different molecular barcode sequences.

23. (Previously presented): The method of claim 18, further comprising, prior to the sequencing, enriching at least a subset of the amplified progeny polynucleotides for target regions of interest to produce enriched progeny polynucleotides.

24. (Original): The method of claim 23, wherein the target regions of interest comprise genetic sequences of a plurality of genes selected from the group consisting of ALK, APC, BRAF, CDKN2A, EGFR, ERBB2, FBXW7, KRAS, MYC, NOTCH1, NRAS, PIK3CA, PTEN, RB1, TP53, MET, AR, ABL1, AKT1, ATM, CDH1, CSF1R, CTNNB1, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, MLH1, MPL, NPM1, PDGFRA, PROC, PTPN11, RET, SMAD4, SMARCB1, SMO, SRC, STK11, VHL, TERT, CCND1, CDK4, CDKN2B, RAF1, BRCA1,

CCND2, CDK6, NF1, TP53, ARID1A, BRCA2, CCNE1, ESR1, RIT1, GATA3, MAP2K1, RHEB, ROS1, ARAF, MAP2K2, NFE2L2, RHOA, and NTRK1.

25. (Currently amended): The method of claim 18, wherein (d) further comprises ~~comprising, prior to (d),~~ reducing or tracking redundancy of a plurality of sequence reads from the set of sequencing reads, wherein the reducing or tracking comprises mapping at least a subset of the plurality of sequence reads to a reference sequence, and the reducing or tracking is based on (i) the molecular barcodes associated the paired reads or the unpaired reads and (ii) sequence information at the start and stop positions of the paired reads or the unpaired reads.

26. (Cancelled).

27. (Currently amended): The method of claim 25 [[26]], wherein reducing or tracking the redundancy of the plurality of sequence reads comprises determining a base call at one or more genomic loci for a plurality of sequence reads that map to the one or more genomic loci on the reference sequence.

28. (Currently amended): The method of claim 25 [[26]], wherein reducing or tracking the redundancy of the plurality of sequence reads comprises collapsing the plurality of sequence reads to produce consensus sequences representative of a sequence of the original double-stranded cfDNA molecules from among the tagged parent polynucleotides.

29. (Currently amended): The method of claim 25 [[26]], further comprising determining quantitative measures of at least two of (i) paired reads that map to one or more genomic loci, (ii) unpaired reads that map to one or more genomic loci, (iii) a read depth of the paired reads, and (iv) a read depth of the unpaired reads.

30. (Previously presented): The method of claim 29, further comprising estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides that map to a genomic locus of the reference sequence based on the quantitative measures of at least two of (i) paired reads, (ii) unpaired reads, (iii) a read depth of the paired reads, and (iv) a read depth of the unpaired reads at the genomic locus.

31. (Previously presented) The method of claim 1, wherein the distinct cfDNA molecules in (e) are determined based on (i) the paired reads and (ii) the unpaired reads.

Electronic Acknowledgement Receipt

EFS ID:	43546362
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	18-AUG-2021
Filing Date:	31-JUL-2020
Time Stamp:	20:27:26
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		2021-08-18_GH0004US-CON5_OA3response.pdf	162841 8f514bb4d07cd2295901c07e1b98d090cc217d36	yes	12

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Specification	2	2
	Claims	3	7
	Applicant Arguments/Remarks Made in an Amendment	8	12
Warnings:			
Information:			
	Total Files Size (in bytes):	162841	

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.

REMARKS

Claims 1-12 and 14-31 were pending prior to entry of the abovementioned amendments. Claims 1, 7-8, 14, 25, 27-29 have been amended to clarify the claimed subject matter and/or for claim dependency purposes. These amendments are fully supported throughout the instant specification, including at, for example, amended paragraph [00112], [00120], [00162]. No new matter is added by any of these amendments.

Incorporation by Reference

Paragraph [00112] of the specification has been amended to recite subject matter disclosed in U.S. Provisional Application No. 61/921,456, filed December 28, 2013 (the '456 application) at, for example, paragraph [0033]. The instant specification as filed provides a clear intent to incorporate by reference the '456 provisional application in its entirety (see paragraph [0001]). The material being added in the instant specification is incorporated by reference, and the amendment contains no new matter in compliance with 37 C.F.R. §1.57(g).

I. Rejection under 35 U.S.C. §102(a)(2)

Claims 1-2, 9, 11-12, 14-15, 18-19, 21, 23, 25-29, and 31 were rejected under 35 U.S.C. §102(a)(2) as allegedly being anticipated by Salk et al. U.S. Patent No. 10,752,951 (hereinafter "Salk").

1. Salk's priority documents do not support "cfDNA" as recited in claims 1 and 18.

Salk is a divisional application (U.S. Application No. 16/514,931) filed on July 17, 2019, claiming priority to USSN 16/120,091 (the '091 application). The '091 application claims priority to USSN 15/660,785 (the '758 application). The '785 application claims priority to USSN 14/386,800 (the '800 application), which is a U.S. national stage application of International Application No. PCT/US2013/032665, filed Mar. 15, 2013, which claims priority to U.S. Provisional Patent Application No. 61/613,413, filed Mar. 20, 2012; U.S. Provisional

Patent Application No. 61/625,623, filed Apr. 17, 2012; and U.S. Provisional Patent Application No. 61/625,319, filed Apr. 17, 2012.

In the present office action, the Office maintained the §102(a)(2) rejection raised in the Final Office Action of December 21, 2020, which referred to certain claims of Salk ("It is readily apparent that the noted instant claims are substantially identical to claims 1-28 of Salk et al. Thus, the claimed methods cannot be distinguished from the patented methods of Salk et al.," Office Action, p. 3). To the extent that the Salk claims are referenced with respect to the instant claims, Applicant submits that Salk does not disclose in its priority documents "cfDNA," or "circulating DNA" amongst other elements of Salk's claims. Accordingly, Applicant submits that Salk is not prior art under §102 (*See* MPEP 2136.06 "For prior art purposes, a U.S. patent or patent application publication that claims the benefit of an earlier filing date under 35 U.S.C. 120 of a prior nonprovisional application (i.e., a continuation, divisional, or continuation-in-part application) would be accorded the earlier filing date as its prior art date under pre-AIA 35 U.S.C. 102(e), **provided the earlier-filed application properly supports the subject matter relied upon in any rejection in compliance with 35 U.S.C. 112(a) or pre-AIA 35 U.S.C. 112, first paragraph**" (emphasis added); MPEP 211 "The disclosure of a continuation application must be the same as the disclosure of the prior-filed application; i.e., the continuation must not include anything which would constitute new matter if inserted in the original application.")

Indeed, the Patent Trial and Appeal Board ("Board") in its Final Written Decision ("Decision") dated August 18, 2020 in IPR2019-000652 stated, "**Schmitt does not expressly teach that the target polynucleotide is cfDNA . . .**" (Decision, page 31, lines 6-7). To be clear, the Schmitt patent referenced in the Decision was US 9,752,188, which issued from the '800 application. As discussed above, Salk claims priority to the '800 application. Therefore, the

priority documents of Salk do not support the disclosure of "cfDNA" as recited by the pending claims.

Moreover, the genus of circulating nucleic acids does not anticipate the species of cfDNA or circulating DNA.

2. Salk does not disclose the tagging method as recited in claim 1.

Without conceding in the basis of the rejection and solely to expedite prosecution, Applicant has amended claim 1 to clarify certain differences of the claimed subject matter over the cited reference. Applicant submits that claim 1 is not anticipated by Salk, at least because the asserted reference does not teach or disclose all the elements of claim 1, including at least "wherein the plurality of the cfDNA molecules are tagged with n different combinations of molecular barcodes, wherein n is at least 2 and no more than 100,000*z, wherein z is a mean of an expected number of duplicate molecules in the population of cfDNA molecules that map to identical start and stop positions on a reference sequence."

Accordingly, Applicant respectfully requests that the §102 rejections of independent claims 1 and 18 be withdrawn.

Claims 2, 9, 11-12, and 14-15 and claims 19, 21, 23, 25-29, and 31 depend from an include all elements of claims 1 and 18, respectively, and recite additional elements of particular advantage and utility. Salk does not meet all the elements of claims 1 and 18, much less the unique combination of claims 2, 9, 11-12, and 14-15 and claims 19, 21, 23, 25-39, and 31. Thus, Applicant respectfully requests that the §102 rejections of these claims also be withdrawn.

II. Rejection Under 35 U.S.C. §103

Claims 3-6, 8, 10, 16-17, 20, 22, 24, and 30 were rejected under 35 U.S.C. §103 as allegedly being unpatentable over Salk.

Applicant submits that claims 3-6, 8, 10, 16-17, 20, 22, 24, and 30 are not obvious over Salk for at least the reasons that these claims depend from and include all the elements of claims 1 and 18, respectively, and recite additional elements of particular advantage and utility. Salk

does not disclose or suggest all of the elements of claims 1 and 18, as discussed above, much less the combination of elements of claims 3-6, 8, 10, 13, 16-17, 20, 22, 24, and 30.

Therefore, Applicant respectfully requests that the §103 rejections of the aforementioned claims be withdrawn.

III. Claim Objection

Claim 7 was objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

Applicant submits that amended claim 1 is allowable upon withdrawal of the §102(a)(2) and §103 rejections, as discussed above. Claim 7 depends from and include all the elements of claim 1, and recites additional elements of particular advantage and utility.

Therefore, Applicant respectfully requests that the objection of claim 7 be withdrawn.

***

It shall be understood herein that any instance in which Applicant has addressed certain comments set forth by the Office shall not be construed as a concession to other comments or arguments advanced by the Office. Any circumstance in which Applicant has amended or canceled a claim also does not mean that Applicant concedes to the arguments or positions advanced by the Office with respect to that claim or other claims pending herein.

CONCLUSION

This communication fully addresses the rejections raised in the Office Action mailed on May 18, 2021. Applicant believes that the present application is now in condition for allowance and respectfully requests that the Examiner expedite the prosecution of this application to allowance. The Commissioner is authorized to charge any underpayment, or credit any overpayment, to Deposit Account No. 60-2231 (Attorney Docket No. GH0004US-CON5).

Respectfully submitted,
GUARDANT HEALTH, INC.

Date: August 18, 2021 By: /Timothy A. Hott/

Timothy A. Hott
Registration No.: 67740

GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063
Customer No. 115823

AMENDMENTS TO THE SPECIFICATION

Please amend paragraph **[00112]** of the specification as filed according to the following paragraph, which is marked to show the changes:

[00112] In certain embodiments, a population of polynucleotides in a sample of fragmented genomic DNA is tagged with n different unique identifiers, wherein n is at least 2 and no more than 100,000*z, wherein z is a measure of central tendency (e.g., mean, median, mode) of an expected number of duplicate molecules having the same start and stop positions. In certain embodiments, n is at least any of 2*z, 3*z, 4*z, 5*z, 6*z, 7*z, 8*z, 9*z, 10*z, 11*z, 12*z, 13*z, 14*z, 15*z, 16*z, 17*z, 18*z, 19*z, or 20*z (e.g., lower limit). In other embodiments, n is no greater than 100,000*z, 10,000*z, 1000*z or 100*z (e.g., upper limit). Thus, n can range between any combination of these lower and upper limits. In certain embodiments, n is between 5*z and 15*z, between 8*z and 12*z, or about 10*z. A haploid human genome equivalent has about 3 picograms of DNA. A sample of about 1 microgram of DNA contains about 300,000 haploid human genome equivalents. Improvements in sequencing can be achieved as long as at least some of the duplicate or cognate polynucleotides bear unique identifiers with respect to each other, that is bear different tags. However, in certain embodiments, the number of tags used is selected so that there is at least a 95% chance that all duplicate molecules starting at any one position bear unique identifiers. For example, in a sample comprising about 10,000 haploid human genome equivalents of fragmented genomic DNA, *e.g.*, cfDNA, z is expected to be between 2 and 8. Such a population can be tagged with between about 10 and 100 different identifiers, for example, about 2 identifiers, about 4 identifiers, about 9 identifiers, about 16 identifiers, about 25 identifiers, about 36 different identifiers, about 49 different identifiers, about 64 different identifiers, about 81 different identifiers, about 100 different identifiers.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. 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.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/945,124	Filing Date 07/31/2020	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ 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).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	08/18/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 30	Minus	** 30	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/VICTOR BARLOW/
*** 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 number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. 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 12 minutes 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.

Attorney Docket No. 42534-708.306/GH0004US-CON5
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	AmirAli TALASAZ et al.	Confirmation No.:	2506
Serial No.:	16/945,124	Art Unit:	1637
Filing Date:	July 31, 2020	Examiner:	Kenneth R. HORLICK
Title:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

A. ☐ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☒ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☒ a fee of $260.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $260.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure

statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $260.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 60-2231(Docket No. GH0004US-CON5).

Respectfully submitted,

Dated: August 19, 2021

By: /Timothy A. Hott/
Timothy A. Hott, Reg. No. 67740

Customer No. 115823
GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063

Attorney Docket No. 42534-708.306/GH0004US-CON5
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	AmirAli TALASAZ et al.	Confirmation No.:	2506
Serial No.:	16/945,124	Art Unit:	1637
Filing Date:	July 31, 2020	Examiner:	Kenneth R. HORLICK
Title:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS		

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

A. ☐ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

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-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☒ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☒ a fee of $260.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $260.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure

statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $260.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 60-2231(Docket No. GH0004US-CON5).

Respectfully submitted,

Dated: August 19, 2021

By: /Timothy A. Hott/
Timothy A. Hott, Reg. No. 67740

Customer No. 115823
GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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					

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

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
	1	20210222243	A1	2021-07-22	Bielas	Entire Document

If you wish to add additional U.S. Published Application citation information please click the Add button. Add

FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16945124
	Filing Date	2020-07-31
	First Named Inventor	AmirAli TALASAZ
	Art Unit	1637
	Examiner Name	Kenneth R. HORLICK
	Attorney Docket Number	42534-708.306

	1	Extended European search report and opinion dated 12/07/2020 for EP Application No. 20183626.9.
	2	Office action dated 06/23/2021 for US Application No. 16/913,965.

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2021-08-19
Name/Print	Timothy A. Hott	Registration Number	67740

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.**

Privacy Act Statement

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.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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					

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U.S.PATENT APPLICATION PUBLICATIONS Remove

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
	1	20210222243	A1	2021-07-22	Bielas	Entire Document

If you wish to add additional U.S. Published Application citation information please click the Add button. Add

FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16945124
	Filing Date	2020-07-31
	First Named Inventor	AmirAli TALASAZ
	Art Unit	1637
	Examiner Name	Kenneth R. HORLICK
	Attorney Docket Number	42534-708.306

	1	Extended European search report and opinion dated 12/07/2020 for EP Application No. 20183626.9.
	2	Office action dated 06/23/2021 for US Application No. 16/913,965.

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2021-08-19
Name/Print	Timothy A. Hott	Registration Number	67740

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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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.

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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)).

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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:	43551215
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	19-AUG-2021
Filing Date:	31-JUL-2020
Time Stamp:	13:18:23
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	Letter specifying the conditions for filing under 37CFR1.97	2021-08-19_GH0004US-CON5_IDSTrans.pdf	143481 a0cdc458b4555c80ade5f2bf21641874c39590de	no	4

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	2021-08-19_GH0004US-CON5_SB08.pdf	1053258 af4d3c72c1cbcd28c3a24474718700dd9dd229b4	no	4
Warnings:					
Information:					
3	Other Reference-Patent/App/Search documents	EESR_20183626_9_2020-12-07.pdf	1408104 7beb1b91e3e4f7c5af44f73745cb67b76a952c62	no	12
Warnings:					
Information:					
4	Other Reference-Patent/App/Search documents	OA_16913965_2021-06-23.pdf	640388 0983561611cc2a1beca353634f940e43ebdaa4c6	no	16
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	38177 943ecfa19ac2c9d681922307e7c512d42b1cfd18	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	3283408		

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.

Electronic Acknowledgement Receipt

EFS ID:	43551834
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	19-AUG-2021
Filing Date:	31-JUL-2020
Time Stamp:	13:53:51
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$260
RAM confirmation Number	E20218ID54071812
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Letter specifying the conditions for filing under 37CFR1.97	2021-08-19_GH0004US-CON5_IDSTrans.pdf	143481 a0cdc458b4555c80ade5f2bf21641874c39590de	no	4
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	2021-08-19_GH0004US-CON5_SB08.pdf	1053258 af4d3c72c1cbcd28c3a24474718700dd9dd229b4	no	4
Warnings:					
Information:					
3	Other Reference-Patent/App/Search documents	EESR_20183626_9_2020-12-07.pdf	1408104 7beb1b91e3e4f7c5af44f73745cb67b76a952c62	no	12
Warnings:					
Information:					
4	Other Reference-Patent/App/Search documents	OA_16913965_2021-06-23.pdf	640388 0983561611cc2a1beca353634f940e43ebdaa4c6	no	16
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	38179 cfb14380a7b9b6bb54a1df5ffad5a773c2ef1e2f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	3283410		

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.

Electronic Patent Application Fee Transmittal

Application Number:	16945124
Filing Date:	31-Jul-2020
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Filer:	Timothy A Hott/Michelle Chan
Attorney Docket Number:	42534-708.306

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**260**

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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**260**

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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 contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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
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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
/K.R.H/	1	20210222243	A1	2021-07-22	Bielas	Entire Document

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

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NON-PATENT LITERATURE DOCUMENTS Remove

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]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

/K.R.H/	1	Extended European search report and opinion dated 12/07/2020 for EP Application No. 20183626.9.
/K.R.H/	2	Office action dated 06/23/2021 for US Application No. 16/913,965.

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/KENNETH R HORLICK/	Date Considered	08/26/2021

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16945124
Filing Date	2020-07-31
First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

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	/Timothy A. Hott/	Date (YYYY-MM-DD)	2021-08-19
Name/Print	Timothy A. Hott	Registration Number	67740

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.**

Privacy Act Statement

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.

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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.

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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First Named Inventor	AmirAli TALASAZ
Art Unit	1637
Examiner Name	Kenneth R. HORLICK
Attorney Docket Number	42534-708.306

U.S.PATENTS Remove

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
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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
/K.R.H/	1	20210222243	A1	2021-07-22	Bielas	Entire Document

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

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NON-PATENT LITERATURE DOCUMENTS Remove

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]

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Examiner Signature	/KENNETH R HORLICK/	Date Considered	08/26/2021

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Signature	/Timothy A. Hott/	Date (YYYY-MM-DD)	2021-08-19
Name/Print	Timothy A. Hott	Registration Number	67740

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Alexandria, Virginia 22313-1450
www.uspto.gov

BIB DATA SHEET

CONFIRMATION NO. 2506

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
16/945,124	07/31/2020 RULE	435	1637	42534-708.306

APPLICANTS

GUARDANT HEALTH, INC., Redwood City, CA;

INVENTORS

AmirAli TALASAZ, Atherton, CA;
Helmy ELTOUKHY, Atherton, CA;
Stefanie Ann Ward MORTIMER, Morgan Hill, CA;

**** CONTINUING DATA ***************************

This application is a CON of 16/601,168 10/14/2019 PAT 10801063
which is a CON of 15/892,178 02/08/2018 PAT 10883139
which is a CON of 14/861,989 09/22/2015 PAT 9920366
which is a CON of PCT/US2014/072383 12/24/2014
which claims benefit of 61/948,509 03/05/2014
and claims benefit of 61/921,456 12/28/2013

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

08/07/2020

Foreign Priority claimed	❑ Yes ☑ No	❑ Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
35 USC 119(a-d) conditions met	❑ Yes ☑ No		CA	11	30	2
Verified and Acknowledged	/KENNETH R HORLICK/ Examiner's Signature	Initials				

ADDRESS

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304
UNITED STATES

TITLE

METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

FILING FEE RECEIVED		
2720	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	❑ All Fees ❑ 1.16 Fees (Filing) ❑ 1.17 Fees (Processing Ext. of time) ❑ 1.18 Fees (Issue) ❑ Other _____ ❑ Credit

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/945,124	TALASAZ et al.
	Examiner	Art Unit
	KENNETH R HORLICK	1637

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	09/09/2020	12/21/2020	05/18/2021	08/30/2021					
1	1	✓	✓	✓	=					
2	2	✓	✓	✓	=					
3	3	✓	✓	✓	=					
4	4	✓	✓	✓	=					
5	5	✓	✓	✓	=					
6	6	✓	✓	✓	=					
7	7	✓	O	O	=					
8	8	✓	✓	✓	=					
9	9	✓	✓	✓	=					
10	10	✓	✓	✓	=					
11	11	✓	✓	✓	=					
12	12	✓	✓	✓	=					
	13	✓	✓	-						
13	14	✓	✓	✓	=					
14	15	✓	✓	✓	=					
15	16	✓	✓	✓	=					
16	17	✓	✓	✓	=					
17	18	✓	✓	✓	=					
18	19	✓	✓	✓	=					
19	20	✓	✓	✓	=					
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21	22	✓	✓	✓	=					
22	23	✓	✓	✓	=					
23	24	✓	✓	✓	=					
24	25	✓	✓	✓	=					
	26	✓	✓	✓	-					
25	27	✓	✓	✓	=					
26	28	✓	✓	✓	=					
27	29	✓	✓	✓	=					
28	30	✓	✓	✓	=					
29	31			✓	=					

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/945,124	TALASAZ et al.
	Examiner	**Art Unit**
	KENNETH R HORLICK	1637

CPC								
Symbol							**Type**	**Version**
C12Q	/	1	/	6869			F	2013-01-01
C12Q	/	1	/	6886			I	2013-01-01
G16B	/	15	/	00			A	2019-02-01
C12Q	/	2600	/	158			A	2013-01-01
C12Q	/	2535	/	122			A	2013-01-01

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	29	
/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637	30 August 2021	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20210830

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/945,124	TALASAZ et al.
	Examiner	**Art Unit**
	KENNETH R HORLICK	1637

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C12P	/	19	/	34

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**
435	6.12

CROSS REFERENCES(S)							
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**						
435	91.2						

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	29	
/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637	30 August 2021	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

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CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	10	10	18	19	26	28								
2	2	11	11	19	20	27	29								
3	3	12	12	20	21	28	30								
4	4		13	21	22	29	31								
5	5	13	14	22	23										
6	6	14	15	23	24										
7	7	15	16	24	25										
8	8	16	17		26										
9	9	17	18	25	27										

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 29	
/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637 (Primary Examiner)	30 August 2021 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

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

NOTICE OF ALLOWANCE AND FEE(S) DUE

115823 7590 09/02/2021

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER
HORLICK, KENNETH R

ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 09/02/2021

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

TITLE OF INVENTION: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	12/02/2021

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

115823 7590 09/02/2021

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

TITLE OF INVENTION: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	12/02/2021

EXAMINER	ART UNIT	CLASS-SUBCLASS
HORLICK, KENNETH R	1637	435-006120

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

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EXAMINER	
HORLICK, KENNETH R	
ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 09/02/2021

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. 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 30 minutes 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, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. 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.

Privacy Act Statement

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 disclosure of these records is required by the Freedom of Information Act.
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 inspection 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.

Notice of Allowability	Application No. 16/945,124	Applicant(s) TALASAZ et al.	
	Examiner KENNETH R HORLICK	Art Unit 1637	AIA (FITF) Status Yes

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

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the response filed 08/18/21.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ 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.

3. ☑ The allowed claim(s) is/are 1-12,14-25 and 27-31 . As a result of the allowed claim(s), 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.**

4. ☐ 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)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 8/19/21; 8/19/21.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/945,124	TALASAZ et al.
	Examiner	Art Unit
	KENNETH R HORLICK	1637

CPC - Searched*		
Symbol	Date	Examiner
C12Q 1/6869	08/30/2021	KH

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
inventor name search	09/02/2020	KH
reviewed parent applications and references therein	09/02/2020	KH
updated parent searches in USPAT and PGPUB	09/02/2020	KH
updated parent searches in USPAT and PGPUB	12/16/2020	KH
updated parent searches in USPAT and PGPUB	05/11/2021	KH
updated parent searches in USPAT and PGPUB	08/30/2021	KH

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
NONE	NONE	08/30/2021	KH

/KENNETH R HORLICK/ Primary Examiner, Art Unit 1637	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): AmirAli TALASAZ et al.	Confirmation No.: 2506
Serial Number: 16/945,124	Customer No.: 115823
Filing Date: July 31, 2020	Group Art Unit: 1637
Title: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	Examiner: Kenneth R. HORLICK

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

AMENDMENT UNDER 37 C.F.R. §1.312

Sir:

This communication is submitted under 37 C.F.R. §1.312 after mailing of the Notice of Allowance on September 2, 2021.

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

115823 7590 09/02/2021
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650 Page Mill Road
Palo Alto, CA 94304

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

Michelle Chan	(Typed or printed name)
/mchan/	(Signature)
September 7, 2021	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

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APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	12/02/2021

EXAMINER	ART UNIT	CLASS-SUBCLASS
HORLICK, KENNETH R	1637	435-006120

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Timothy A. Hott
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: GUARDANT HEALTH, INC.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Redwood City, CA

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

☒ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 60-2231

5. **Change in Entity Status** (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Timothy A. Hott/ Date September 7, 2021

Typed or printed name Timothy A. Hott Registration No. 67740

Electronic Acknowledgement Receipt

EFS ID:	43702663
Application Number:	16945124
International Application Number:	
Confirmation Number:	2506
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Customer Number:	115823
Filer:	Timothy A Hott/Michelle Chan
Filer Authorized By:	Timothy A Hott
Attorney Docket Number:	42534-708.306
Receipt Date:	07-SEP-2021
Filing Date:	31-JUL-2020
Time Stamp:	20:05:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1200
RAM confirmation Number	E202197K06264080
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2021-09-07_GH0004US-CON5_312Amend.pdf	94747 6ae445ee321f4976c749a9d2be9ae0348cb9a7bf	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment after Notice of Allowance (Rule 312)	1	1
	Specification	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	2021-09-07_GH0004US-CON5_IssueFeeTrans.pdf	96767 d994213e0d86ae11138838faab0441c48ce102b1	no	1
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	38024 3ebea7edf6865c4db43e198cb11396baad54581d	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	229538		

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.

REMARKS

Paragraph [0001] of the specification is amended herein to update the status of an application. No new matter is being introduced.

CONCLUSION

Should the Examiner have any questions, the Examiner is respectfully encouraged to telephone the undersigned attorney. The Commissioner is authorized to charge any underpayment, or credit any overpayment, to Deposit Account No. 60-2231 (Attorney Docket No. GH0004US-CON5).

Respectfully submitted,
GUARDANT HEALTH, INC.

Date: September 7, 2021 By: /Timothy A. Hott/

Timothy A. Hott
Registration No.: 67740

GUARDANT HEALTH, INC.
505 Penobscot Drive
Redwood City, CA 94063
Customer No. 115823

AMENDMENTS TO THE SPECIFICATION

Please amend paragraph **[0001]** of the specification as follows:

[0001] This application is a continuation of U.S. Application No. 16/601,168, filed October 14, 2019 (now U.S. Patent No. 10,801,063, issued October 13, 2020), which is a continuation of U.S. Application No. 15/892,178, filed February 8, 2018 (now U.S. Patent No. 10,883,139, issued January 5, 2021), which is a continuation of U.S. Application No. 14/861,989, filed September 22, 2015 (now U.S. Patent No. 9,920,366, issued March 20, 2018), which is a continuation application of International Application No. PCT/US2014/072383, filed December 24, 2014, which application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/921,456, filed December 28, 2013, and U.S. Provisional Application No. 61/948,509, filed March 5, 2014, each of which is entirely incorporated herein by reference.

Electronic Patent Application Fee Transmittal

Application Number:	16945124
Filing Date:	31-Jul-2020
Title of Invention:	METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS
First Named Inventor/Applicant Name:	AmirAli TALASAZ
Filer:	Timothy A Hott/Michelle Chan
Attorney Docket Number:	42534-708.306

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1200	1200

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1200**

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Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

Courtesy Reminder for
Application Serial No: 16/945,124

Attorney Docket No: 42534-708.306
Customer Number: 115823
Date of Electronic Notification: 09/02/2021

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

An email notification regarding the correspondence was sent to the following email address(es) associated with your customer number:

Patents@guardanthealth.com
patentdocket@wsgr.com

To view your correspondence online or update your email addresses, please visit us anytime at **https://ppair-my.uspto.gov/pair/PrivatePair.**
If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov or call 1-866-217-9197.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): AmirAli TALASAZ et al.	
Serial Number: 16/945,124	Confirmation No.: 2506
Filing Date: July 31, 2020	Customer No.: 115823
Title: METHODS AND SYSTEMS FOR DETECTING GENETIC VARIANTS	Group Art Unit: 1637
	Examiner: Kenneth R. HORLICK

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

OK TO ENTER: /K.R.H/
/K.R.H/ (09/13/2021)

AMENDMENT UNDER 37 C.F.R. §1.312

Sir:

This communication is submitted under 37 C.F.R. §1.312 after mailing of the Notice of Allowance on September 2, 2021.

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	07/31/2020	AmirAli TALASAZ	42534-708.306	2506

115823 7590 09/20/2021

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
HORLICK, KENNETH R	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
09/20/2021	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):

Patents@guardanthealth.com
patentdocket@wsgr.com

Response to Rule 312 Communication	Application No.	Applicant(s)	
	16/945,124	TALASAZ et al.	
	Examiner	Art Unit	AIA (FITF) Status
	KENNETH R HORLICK	1637	Yes

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

1. ☑ The amendment filed on 07 September 2021 under 37 CFR 1.312 has been considered, and has been:

a) ☐ entered.

b) ☑ entered as directed to matters of form not affecting the scope of the invention.

c) ☐ disapproved because the amendment was filed after the payment of the issue fee.

Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

d) ☐ disapproved. See explanation below.

e) ☐ entered in part. See explanation below.

/KENNETH R HORLICK/
Primary Examiner, Art Unit 1637

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/945,124	10/19/2021	11149306	42534-708.306	2506

115823 7590 09/29/2021

Guardant Health / WSGR
650 Page Mill Road
Palo Alto, CA 94304

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

GUARDANT HEALTH, INC., Redwood City, CA;

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 USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

TWINSTRAND BIOSCIENCES, INC.,
Petitioner,

v.

GUARDANT HEALTH, INC.,
Patent Owner.

IPR2022-01400
Patent 11,149,306 B2

Before SUSAN L. C. MITCHELL, TINA E. HULSE, and MICHAEL A. VALEK, *Administrative Patent Judges.*

VALEK, *Administrative Patent Judge*.

DECISION
Granting Institution of *Inter Partes* Review
35 U.S.C. § 314

I. INTRODUCTION

TwinStrand Biosciences, Inc. ("Petitioner") filed a Petition requesting an *inter partes* review of claims 1–29 of U.S. Patent No. 11,149,306 B2 (Ex. 1001, "the '306 patent"). Paper 2 ("Pet."). Guardant Health, Inc. ("Patent Owner") filed a Preliminary Response. Paper 6 ("Prelim. Resp."). With our authorization, Petitioner filed a Reply to Patent Owner's Preliminary Response (Paper 7) and Patent Owner filed a Sur-reply to the Reply (Paper 8).

Considering the arguments and evidence of record, we determine that the Petition demonstrates "a reasonable likelihood that [P]etitioner would prevail with respect to at least 1 of the claims challenged in the petition." 35 U.S.C. § 314(a). Accordingly, we institute an *inter partes* review.

A. Related Proceedings

The parties state that the '306 patent has been asserted in *TwinsStrand Biosciences, Inc. et al. v. Guardant Health, Inc.*, 1-21-cv-01126 (D. Del.) and *Illumina, Inc. v. Guardant Health, Inc.*, 22-cv-00334 (D. Del.). Pet. 70; Paper 4, 1.

The parties identify the following IPRs involving patents related to the '306 patent: IPR2022-00746, IPR2022-01115, IPR2022-00747, IPR22-01116, and IPR2022-01152. Pet. 70; Paper 4, 1. Patent Owner additionally identifies a number of IPRs that it has filed, i.e., IPR2022-00449, IPR2022-00450, IPR2022-00816, IPR2022-00817, IPR2022-00935, IPR2022-01158, IPR2022-01159, and IPR2022-01388, as related matters. Paper 4, 1.

B. The '306 Patent

Genetic testing is useful for a number of diagnostic methods. Ex. 1001, 1:25–26. Disorders that are caused by rare genetic mutations (e.g., sequence variations) or changes in epigenetic markers, such as cancer and

partial or complete aneuploidy, may be detected or more accurately characterized with DNA sequence information. *Id.* at 1:26–30.

Early detection and monitoring of genetic diseases is often useful and needed in the successful treatment or management of a disease. Ex. 1001, 1:31–33. According to the '306 patent, one approach may include monitoring a sample derived from cell-free nucleic acids, which are polynucleotides that can be found in different types of bodily fluids. *Id.* at 1:33–36. Cell-free DNA ("cfDNA") may contain genetic aberrations, such as copy number variation or sequence variation, associated with a particular disease. *Id.* at 1:36–43.

The '306 patent explains that many methods have been developed to estimate copy number variation. Ex. 1001, 1:46–47. According to the Specification, most of those methods involve preparing a sample by converting the original nucleic acids into a sequenceable library, followed by massively parallel sequencing, and then conducting a bioinformatic analysis to estimate the copy number variation at one or more loci. *Id.* at 1:51–55.

The '306 patent states that although known methods for detecting cfDNA are able to reduce the errors introduced by the sample preparation and sequencing processes for the molecules that are converted and sequenced, these methods are not able to infer the counts of molecules that were converted, but not sequenced. Ex. 1001, 1:59–63. The '306 patent states this inability to count converted but unsequenced molecules "can dramatically and adversely affect the sensitivity that can be achieved." *Id.* at 1:63–67. Accordingly, the '306 patent relates to a method of tagging and counting both halves of double-stranded DNA and estimating the number of unseen molecules based on the number of Pairs (i.e., molecules where both

strands were identified) and Singlets (i.e., molecules where only one strand was identified) detected in a particular region. *See id.* at 2:1–18.

C. Illustrative Claims

Petitioner challenges claims 1–29 of the '306 patent. Of these, claims 1 and 17 are independent. Claims 1 and 17 read as follows:

> 1. A method, comprising:
>
> (a) providing a population of cell-free deoxyribonucleic acid (cfDNA) molecules having first and second complementary strands;
>
> (b) tagging a plurality of the cfDNA molecules in the population with duplex tags comprising molecular barcodes to produce tagged parent polynucleotides, wherein the duplex tags are attached at both ends of a molecule of the plurality of the cfDNA molecules, wherein the plurality of the cfDNA molecules are tagged with n different combinations of molecular barcodes, wherein n is at least 2 and no more than 100,000*z, wherein z is a mean of an expected number of duplicate molecules in the population of cfDNA molecules that map to identical start and stop positions on a reference sequence;
>
> (c) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;
>
> (d) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and
>
> (e) reducing or tracking redundancy of a plurality of sequence reads from the set of sequence reads using at least sequence information from the molecular barcodes of the duplex tags to determine distinct cfDNA molecules from among the tagged parent polynucleotides, wherein the distinct cfDNA molecules are determined based on (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, or (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand

having no second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, wherein reducing or tracking the redundancy of the plurality of sequence reads comprises mapping at least a subset of the plurality of sequence reads to the reference sequence.

17. A method, comprising:

(a) tagging a population of double-stranded cell-free deoxyribonucleic acid (cfDNA) molecules obtained or derived from a sample of a subject with a set of tags comprising molecular barcodes to produce tagged parent polynucleotides;

(b) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(c) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(d) sorting a plurality of sequence reads from the set of sequence reads into (i) families comprising paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides, and (ii) families comprising unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides.

Ex. 1001, 61:6–43 (claim 1), 62:45–65 (claim 17).

D. The Asserted Grounds of Unpatentability

Petitioner asserts that claims 1–29 would have been unpatentable on the following grounds:

Claims Challenged	35 U.S.C. §[1]	References/Basis
1–3, 5, 7, 9–14, 17–27, 29	103	Narayan[2] and Schmitt[3]
4, 6	103	Narayan, Schmitt, and Meyer[4]
8,	103	Narayan, Schmitt, and Craig[5]
15, 16, 28	103	Narayan, Schmitt, and Kivioja[6]

Petitioner also relies upon the Declaration of Paul T. Spellman, Ph.D. (Ex. 1002) ("Spellman Declaration").

Before turning to our analysis of these grounds, we address Patent Owner's argument that, notwithstanding the merits of the Petition, we should exercise discretion to deny institution under 35 U.S.C. § 325(d).

---

[1] The Leahy-Smith America Invents Act ("AIA"), Pub. L. No. 112–29, 125 Stat. 284 (2011), amended 35 U.S.C. §103, effective March 16, 2013. The '306 patent claims priority to a series of applications the earliest of which is a provisional application filed on December 28, 2013. Ex. 1001 code (60). Because the AIA became effective before the filing of the earliest of the application to which the '306 patent claims priority, we apply the AIA version of the statute.

[2] Narayan et al., *Ultrasensitive Measurement of Hotspot Mutations in Tumor DNA in Blood Using Error-Suppressed Multiplexed Deep Sequencing*, 72(14) CANCER RES. 3492–98 (Ex. 1082) ("Narayan").

[3] Schmitt et al., WO 2013/142389 A1, published Sept. 26, 2013 (Ex. 1009) ("Schmitt").

[4] Meyer et al., *Parallel Tagged Sequencing on the 454 Platform*, 3(2) NATURE PROTOCOLS 267–78 (2008) (Ex. 1005) ("Meyer").

[5] Craig et al., *Identification of Genetic Variants Using Bar-coded Multiplexed Sequencing*, 5(10) NATURE METHODS 887–93 (2008) (Ex. 1007) ("Craig").

[6] Kivioja et al., *Counting Absolute Numbers of Molecules Using Unique Molecular Identifiers*, 9 NATURE METHODS 72–76 (2012) (Ex. 1006) ("Kivioja").

II. DISCRETITION UNDER 35 U.S.C. § 325(d)

Patent Owner argues that we should exercise discretion to deny institution because "[t]he same art and arguments presented in this petition were considered and rejected by the Office multiple times" and Petitioner has "failed to . . . demonstrate material error." Prelim. Resp. 1–2. Petitioner disagrees. *See* Pet. 21–24.

A. Legal Standard

Section 325(d) provides that the Director may elect not to institute a proceeding if the challenge to the patent is based on matters previously presented to the Office. The statute states, in pertinent part, "[i]n determining whether to institute . . . the Director may take into account whether, and reject the petition . . . because, the same or substantially the same prior art or arguments previously were presented to the Office." 35 U.S.C. § 325(d).

The question of whether a petition presents art or arguments that are "the same or substantially the same" as art or arguments previously presented to the Office is a factual inquiry, which may be resolved by reference to the factors set forth in *Becton, Dickinson*.[7] The precedential section of that decision sets forth the following non-exclusive factors for consideration:

> (a) the similarities and material differences between the asserted art and the prior art involved during examination;
> (b) the cumulative nature of the asserted art and the prior art evaluated during examination;

[7] *Becton, Dickinson & Co. v. B. Braun Melsungen AG*, IPR2017-01586, Paper 8 (PTAB Dec. 15, 2017) (precedential as to § III.C.5, first paragraph) ("*Becton, Dickinson*").

> (c) the extent to which the asserted art was evaluated during examination, including whether the prior art was the basis for rejection;
>
> (d) the extent of the overlap between the arguments made during examination and the manner in which Petitioner relies on the prior art or Patent Owner distinguishes the prior art;
>
> (e) whether Petitioner has pointed out sufficiently how the Examiner erred in its evaluation of the asserted prior art; and
>
> (f) the extent to which additional evidence and facts presented in the Petition warrant reconsideration of the prior art or arguments.

Becton, Dickinson, 17–18.

Advanced Bionics[8] sets out a two-part framework for analyzing these factors. In the first part, we consider factors (a), (b), and (d) to determine whether the art and arguments presented in the petition are the same or substantially the same as those previously presented to the Office. *Advanced Bionics*, 8–10. "If, after review of factors (a), (b), and (d), it is determined that the same or substantially the same art or arguments previously were presented to the Office," then we move on to the second part of the analysis to determine "whether the petitioner has demonstrated a material error by the Office" in view of factors (c), (e), and (f). *Id.*

B. Advanced Bionics Part One

Patent Owner argues that the disclosure in "Schmitt was considered by the Examiner and applied as the primary reference in prosecution." Prelim. Resp. 10. We agree. Both parties acknowledge that Schmitt shares its specification with U.S. Patent No. 10,752,951 ("Salk"). Pet. 22; Prelim. Resp. 5. And it is undisputed that the Examiner asserted Salk in anticipation

[8] *Advanced Bionics, LLC v. MED-EL Electromedizinishe Geräte GmbH*, IPR2019-01469, Paper 6 at 10 (Feb. 13, 2020) (precedential) ("*Advanced Bionics*").

and obviousness rejections during prosecution. Pet. 17–18; Prelim. Resp. 6 (citing Ex. 1066, 539–41, 672–74). Thus, there is no dispute that the same disclosure the Petition relies upon in Schmitt was both before, and evaluated by,[9] the Examiner during prosecution.

Narayan, Craig, and Kivioja were also before the Examiner during prosecution. Each of these references was listed in an IDS. Ex. 1066, 122–221; *see also* Pet. 21 (acknowledging this fact). Thus, all of the same references in three of the Petition's four grounds and covering all but two of the challenged claims were previously presented to the Office. For these reasons, we determine that, on the whole, the Petition presents the same or substantially the same art as that previously presented to the Office and proceed to the second part of the *Advanced Bionics* framework.

C. Advanced Bionics Part Two

During prosecution, the Examiner rejected the then-pending claims as anticipated by and obvious over Salk, finding that Salk disclosed or rendered obvious each of the recited method steps. Ex. 1066, 539–40. Patent Owner's initial response (*id.* at 554–65) did not succeed in overcoming those rejections. *Id.* at 671–75 (maintaining rejections).

Patent Owner next responded by amending claim element 1(b) to recite tagging with n different combinations of barcodes, "wherein n is at least 2 and no more than 100,000*z, wherein z is a mean of an expected number of duplicate molecules in the population of cfDNA molecules that map to identical start and stop positions on a reference sequence." *Id.* at 687. Patent Owner argued that this amendment distinguished Salk '951. *Id.* at

[9] We will address the extent of the Examiner's evaluation of Schmitt's disclosure (i.e., *Becton, Dickinson* Factor (c)) when we turn to the second part of the *Advanced Bionics* analysis.

694. Patent Owner also argued that Salk was not prior art because its priority applications did not provide written description support for "cfDNA" and "circulating DNA" as recited in Salk's claims. Ex. 1066, 692–93. For support, Patent Owner pointed to the Board's statement in the final written decision in IPR2019-00652 (the "'652 IPR") that "'Schmitt ['188][10] does not expressly teach that the target polynucleotide is cfDNA.'" *Id.* at 693 (quoting Ex. 1074, 31). The Examiner subsequently withdrew the rejections without expressly noting the reasons for allowance. *See id.* at 708 (notice of allowance).

Petitioner urges that the Examiner erred in a number of respects. *See* Pet. 21–24; Reply 4–5. Most of these alleged errors relate to arguments Patent Owner made in its first response to the Examiner's rejections. As noted above, those initial arguments were not successful in overcoming the rejections, and the Examiner did not articulate any particular reasons for allowing the claims.

Nevertheless, based on its showing in Ground 1, we determine that Petitioner has sufficiently demonstrated a material error during the examination of claim 1. Claim 1 and its dependent claims were allowed shortly after Patent Owner's amendment to recite a range of barcode combinations n between 2 and 100,000*z in element 1(b). This suggests the Examiner determined that Salk and Schmitt '188 did not disclose or suggest the recited range. As explained below, however, the record at this stage of the proceeding supports Petitioner's argument that the number of barcode

[10] The "Schmitt" referenced in the '652 IPR decision is U.S. Patent No. 9,752,188 ("Schmitt '188"). Ex. 1074, 3 n. 2. Schmitt '188 claims priority to the international application publication (i.e., WO2013/142,389) referred to as "Schmitt" in this proceeding. Ex. 2008, code (87).

combinations for Schmitt's 3-mer hybrid tag embodiment falls within the recited range regardless of the value of z. *See infra* § III.E.1. To the extent the Examiner did not appreciate the relevance of the "hybrid method using a combination of sheared ends and shorter n-mer tags (such as 1 or 2 or 3 or 4 or more degenerate or semi-degenerate bases)" taught in Salk and Schmitt '188 to claim element 1(b), this constitutes material error. Ex. 2012 9:20–24; Ex. 1083 ¶ 30. Moreover, the Petition presents additional evidence regarding element 1(b), e.g., Ex. 1002 ¶ 166, that warrants reconsideration of Schmitt in this proceeding.

We also note that Patent Owner's argument during prosecution that Salk was not prior art because it lacks written description support for cfDNA is a different issue than whether the challenged claims are obvious over Schmitt and Narayan and the other references cited in the Petition. The Examiner's rejection relied at least in part on the disclosure in Salk's claims. *See* Ex. 1066, 674. The Petition, however, relies on the disclosure in Schmitt's specification (and even more specifically on the disclosure in a priority application, provisional application 61/625,623 ("Schmitt-623" (Ex. 1083)). *See infra* § III.D.2. At this stage of the proceeding, Patent Owner does not dispute that the disclosure in Schmitt is prior art to the challenged claims. Moreover, as explained below, the Petition relies on Narayan's disclosure of cfDNA in combination with Schmitt.[11] Thus, to the extent the

[11] In this regard, Petitioner's obviousness theory is consistent with the ground on which claims were determined to be unpatentable in the '652 IPR. There, the panel determined that "although Schmitt ['188] does not expressly teach that the target polynucleotide is cfDNA, [other references cited in combination with Schmitt '188] teach the extraction and analysis of cfDNA." Ex. 1074, 29; *see also id.* at 51 (finding, based on the record in that proceeding, "that an ordinarily skilled artisan screening cfDNA for cancer

Examiner did not consider the disclosure in Salk's specification to be prior art, or did not consider whether it would have been obvious to apply the methodology taught in Salk and Schmitt to cfDNA based on the teachings in other references such as Narayan, this was also a material error.

For these reasons, we find that Petitioner has demonstrated a material error, and therefore, will not exercise discretion to deny institution of *inter partes* review under 35 U.S.C. § 325(d).

III. ANALYSIS

A. Legal Standard

A patent claim is unpatentable under 35 U.S.C. § 103 if the differences between the claimed invention and the prior art are such that the claimed invention, as a whole, would have been obvious at the time the invention was made to a person having ordinary skill in the art to which the subject matter pertains. *See KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 406 (2007). The question of obviousness is resolved on the basis of underlying factual determinations, including: (1) the scope and content of the prior art; (2) any differences between the claimed subject matter and the prior art; (3) the level of skill in the art; and (4) objective evidence of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17–18 (1966).

"[A] patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *KSR*, 550 U.S. at 418. "[I]t can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention

---

mutations . . . would have looked to Schmitt's DCS method" to reduce errors and improve detection).

does." *Id.* Moreover, a person of ordinary skill in the art must have had a reasonable expectation of success of doing so. *PAR Pharm., Inc. v. TWI Pharms., Inc.*, 773 F.3d 1186, 1193 (Fed. Cir. 2014).

B. Person of Ordinary Skill in the Art

Petitioner asserts that a person of ordinary skill in the art ("POSA") at the time of the invention would have had

> (i) a Ph.D. in molecular biology, genetics, bioinformatics, or a related field, and have at least about two years of experience in the use and development of sequencing technologies; or (ii) a Master's degree in one of the same fields with at least about five years of the same experience.

Pet. 18 (citing Ex. 1002 ¶¶ 27–30). Patent Owner does not offer a proposed definition of the level of ordinary skill in the art at this stage of the proceeding.

On this record, we adopt Petitioner's uncontested definition of the level of ordinary skill in the art. We further note that the prior art itself is sufficient to demonstrate the level of skill in the art at the time of the invention. *See Okajima v. Bourdeau*, 261 F.3d 1350, 1355 (Fed. Cir. 2001) (explaining that specific findings regarding ordinary skill level are not required "where the prior art itself reflects an appropriate level and a need for testimony is not shown" (*quoting Litton Indus. Prods., Inc. v. Solid State Sys. Corp.*, 755 F.2d 158, 163 (Fed. Cir. 1985))).

C. Claim Construction

In an *inter partes* review, the Board applies the same claim construction standard that would be used to construe the claim in a civil action under 35 U.S.C. § 282(b). *See* 37 C.F.R. § 100(b). Under that standard, claim terms "are generally given their ordinary and customary meaning" as understood by a person of ordinary skill in the art at the time of

the invention. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312–13 (Fed. Cir. 2005) (en banc).

Element 1(b) recites a range of "different combinations of molecular barcodes" between 2 and "no more than 100,000*z, wherein z is a mean of an expected number of duplicate molecules in the population of cfDNA molecules that map to identical start and stop positions on a reference sequence." Ex. 1001, 61:15–21. Petitioner does not specify any formal claim construction, but contends a "POSA would understand that the population of cfDNA molecules that map to identical start and stop positions will vary, depending on the number of haploid gene equivalents (HGEs) in the sample." Pet. 19 (citing Ex. 1002 ¶¶ 131–133). Moreover, Petitioner contends that Schmitt discloses tagging with barcodes having a number of combinations that falls within the recited range regardless of the value of z. *See id.* at 29 (arguing that Schmitt discloses the use of "3-mer barcodes" having "4,096 different combinations" that "would fall between 2 and 100,000*z" regardless of "whether z is 1, 10, 100, 800, etc."). Patent Owner disputes Petitioner's showing for element 1(b), but does not seek any claim construction at this stage. Prelim. Resp. 19.

As explained below and based on the current record, Petitioner has shown sufficiently for institution that Schmitt discloses the use of barcodes having a number of different combinations that falls within the recited range regardless of the value of z. For this reason, it is unnecessary to expressly construe "z" or any other aspect of element 1(b) to decide the issues presented in the Petition. To the extent Patent Owner disagrees, it will have the opportunity to address this issue at trial.

Neither party refers to any other claim term in the claim construction sections of their papers. *See* Pet. 18–19; Prelim. Resp. 19. We agree that it is

unnecessary to expressly construe any claim term for purposes of rendering this Decision. *See Wellman, Inc. v. Eastman Chem. Co.*, 642 F.3d 1355, 1361 (Fed. Cir. 2011) ("[C]laim terms need only be construed 'to the extent necessary to resolve the controversy.'" (quoting *Vivid Techs., Inc. v. Am. Sci. & Eng'g, Inc.*, 200 F.3d 795, 803 (Fed. Cir. 1999))).

D. Cited References

1. Narayan

Narayan is a journal article titled "Ultrasensitive Measurement of Hotspot Mutations in Tumor DNA in Blood Using Error-Suppressed Multiplexed Deep Sequencing," and bearing a 2012 publication date. Ex. 1082, 3492.[12] Patent Owner does not dispute that Narayan is prior art in its Preliminary Response.

According to Narayan, "[d]etection of cell-free tumor DNA in the blood has offered promise as a cancer biomarker, but practical clinical implementations have been impeded by the lack of a sensitive and accurate method for quantification that is also simple, inexpensive, and readily scalable." *Id.* Narayan describes "an approach that uses next-generation sequencing ["NGS"] to quantify the small fraction of DNA molecules that contain tumor-specific mutations within a background of normal DNA in plasma." *Id.*

2. Schmitt

Schmitt is an international patent application entitled "Methods of Lowering the Error Rate of Massively Parallel DNA Sequencing Using Duplex Consensus Sequencing," and published on September 26, 2013.

[12] Unless stated otherwise, citations to page numbers refer to the reference page numbers.

Ex. 1009, code (43). Petitioner contends that Schmitt is prior art under 35 U.S.C. § 102(a)(1) and (a)(2), relying on the fact that Schmitt claims priority to Schmitt-623. Pet. 20–21. Petitioner contends that Schmitt is prior art as of the filing date of Schmitt-623, i.e., April 17, 2012, because the disclosures Petitioner relies upon in Schmitt "were carried forward from Schmitt-623" and "Schmitt-623 provides §112 support for at least on[e] claim in Schmitt." *Id.* (internal quotations omitted). Patent Owner does not dispute these contentions in its Preliminary Response.[13]

Schmitt describes a method called Duplex Consensus Sequencing ("DCS") that, according to Schmitt, "greatly reduces sequencing errors by independently tagging and sequencing each of the two strands of a DNA duplex." Ex. 1083, 47 (Abstract). Because the two strands of DNA are complementary, true mutations can be found at the same position on both strands. *Id.* As Schmitt explains:

> Comparing the sequence obtained from each of the two strands comprising a single molecule of duplex DNA facilitates differentiation of sequencing errors from true mutations. When an apparent mutation is, due to a PCR or sequencing error, the substitution will only be seen on a single strand. In contrast, with a true DNA mutation, complementary substitutions will be present on both strands.

Id. ¶ 62. According to Schmitt, its DCS "method uniquely capitalizes on the redundant information stored in double-stranded DNA, thus overcoming

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[13] The Petition cites to the disclosure in Schmitt-623, rather than Schmitt itself, as support for the asserted grounds. *See, e.g.*, Pet. 27–61 (citing Ex. 1083 to support statements regarding the teachings in Schmitt). The Preliminary Response also cites Schmitt-623 to respond to Petitioner's allegations. Prelim. Resp. 25 n.3. For ease of reference, we too cite to Schmitt-623. For purposes of our present analysis, we consider the disclosure in Schmitt-623 to be representative of the teachings in Schmitt.

technical limitations of prior methods of utilizing data from only one of the two strands." *Id.* at 47 (Abstract).

In Schmitt's DCS method, double-stranded DNA molecules are ligated to single molecule identifier ("SMI") adapter molecules. Ex. 1083 ¶¶ 9–10. In one embodiment, "[s]heared double-stranded DNA that has been end-repaired and T-tailed is combined with A-tailed SMI adaptors and ligated." *Id.* ¶ 11, Fig. 1. In this embodiment, "every adaptor contains a unique, double-stranded, complementary n-mer random tag on each end." *Id.*; *see also* ¶ 16 (describing the embodiment in Example 1 in which "the SMI sequence is a random degenerate nucleotide n-mer sequence which is 12 nucleotides in length"). In another embodiment, Schmitt discloses a "hybrid method using a combination of sheared ends and shorter n-mer tags (such as 1 or 2 or 3 or 4 or more degenerate or semi-degenerate bases)" to "serve as unique molecular identifiers." *Id.* ¶ 30. The labeled DNA fragments are then amplified (e.g., by PCR) and sequenced. *Id.* ¶ 42

Schmitt teaches that sequence reads are "grouped into families of paired target nucleic acid strands based on a common set of SMI sequences" to produce "an error corrected double-stranded consensus sequence." Ex. 1083 ¶¶ 43, 60. This data processing is described in Schmitt's Example 1. *See id.* ¶¶ 60–69. There, "[r]eads having common (i.e., identical) SMI sequences were grouped together, and were collapsed to generate a consensus read." *Id.* ¶ 60. "PCR consensus sequences arising from two complementary strands of duplex DNA" are then identified "by virtue of the complementary SMIs" that "identify the 'partner SMI.'" *Id.* ¶ 63; *see also id.* ¶ 13, Fig. 3 (showing how sequence reads "sharing a unique set of SMI tags are grouped into paired families with members having strand identifiers in either the αβ or βα orientation"). "Following partnering of two strands by

virtue of their complementary SMIs, the sequences of the strands are compared. Sequence reads at a given position are kept only if the read data from each of the two paired strands is in agreement." *Id.*; *see also id.* ¶¶ 60, 68 ("Sequence reads were considered only when the read data from each of the two strands is in perfect agreement").

3. Meyer

Meyer is a journal article titled "Parallel tagged sequencing on the 454 platform" and bearing a 2008 publication date. Ex. 1005, 267. Patent Owner does not dispute that Meyer is prior art in its Preliminary Response.

Meyer relates to a method called parallel tagged sequencing that allows for parallel sequencing of large numbers of double-stranded DNA samples on a next-generation sequencing system called the 454 Platform. *Id.*, Abstr. According to Meyer, the method involves blunt end repairing each DNA sample and then ligating sample-specific barcoding adapters to both ends of blunt-end repaired DNA molecules. *Id.* at 268, Fig. 1. The adapters "comprise single self-hybridized oligos containing a sequence tag and an *Srf*I restriction site." *Id.* at 267. The barcoded samples are pooled in equimolar ratios, and untagged molecules are excluded from sequencing through dephosphorylation and restriction digestion. *Id.*, Fig. 1. The sample pool is then sequenced and the sequence reads are sorted according to their tag sequences and the source of each DNA can then be traced using the tag sequences. *Id.*

4. Craig

Craig is a journal article titled "Identification of genetic variants using bar-coded multiplexed sequencing," and bearing an October 2008 publication date. Ex. 1007, 887. Patent Owner does not dispute that Craig is prior art in its Preliminary Response.

Craig relates to "a generalized framework for multiplexed resequencing of targeted human genome regions on the Illumina Genome Analyzer using degenerate indexed DNA bar codes ligated to fragmented DNA before sequencing" for simultaneously sequencing DNA from multiple individuals. Ex. 1007, 887. Craig refers to these bar codes as "indexes" and describes the use of "a six-base index with built-in redundancy for error correction." *Id.* According to Craig, "only 48 of the 4,096 possible nucleotide combinations" were synthesized for use in their experiment. Craig explains that this design "allowed us to control, tolerate and measure error base calling of the index" because "one, and in some cases two, sequencing errors could be tolerated without an index being incorrectly identified as being a different valid index. *Id.* at 888; *see also id.* at 13[14] (Supplementary Table 4 describing the design of the "DNA Indexes Appended to Each Adapter").

5. Kivioja

Kivioja is a journal article titled "Counting absolute numbers of molecules using unique molecular identifiers," and bearing a November 2011 publication date. Ex. 1006, 72. Patent Owner does not dispute that Kivioja is prior art in its Preliminary Response.

According to Kivioja, [d]etermining the relative abundance of two different molecular species or the absolute number of molecules [of DNA] in a single sample is challenging." Ex. 1006, 72. Kivioja describes "an absolute counting method that can use amplification but does not require detecting each original molecule or keeping track of the number of copies made." *Id.* In Kivioja's method, "each molecule in a population is first made unique" by

[14] This refers to the page number added to the exhibit.

labeling it with a unique molecular identifier ("UMI"). *Id.* "Upon deep sequencing, each UMI will be observed multiple times, and the number of original DNA molecules can be determined simply by counting each UMI only once. However, long before all UMIs are observed, increasingly precise estimates of the absolute molecule number can be made." *Id.* (citing Online Methods).

In its Online Methods section, Kivioja teaches that "the original number of molecules in a sample can be estimated as the sum of observed and unobserved UMIs" and "[t]he number of unobserved UMIs can be estimated based on the distribution of the copy numbers of the observed UMIs." *See* Ex. 1006, 4–5[15] (explaining that "the number of molecules from each gene was estimated by fitting a zero-truncated Poisson distribution to the UMI copy number distribution using the generalized additive models for location, scale and shape (GAMLSS) R package and adding the predicted number of unobserved UMIs to the observed UMI count").

E. Ground 1: Obviousness over Narayan and Schmitt

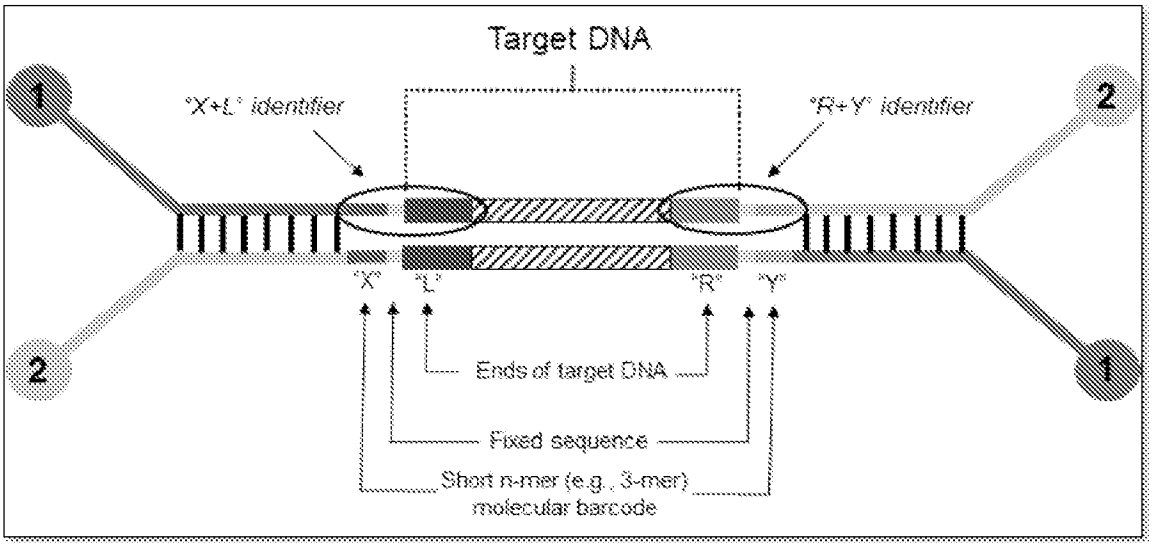
Petitioner asserts that claims 1–3, 5, 7, 9–14, 17–27 and 29 are unpatentable as obvious over Narayan and Schmitt. Pet. 25–58. Patent Owner opposes Petitioner's assertions. Prelim. Resp. 20–45. On this record, we determine that Petitioner has established a reasonable likelihood that it will prevail in showing that claims 1–3, 5, 7, and 9–14 are unpatentable as obvious over the cited references, but has not met its burden for the other claims in this ground.

[15] This refers to the page number added to the exhibit.

1. Analysis of Claims 1–3, 5, 7, and 9–14

We begin with claim 1. Petitioner contends claim 1 is rendered obvious by the application of Schmitt's DCS method to screen cfDNA for cancer as taught in Narayan. *See* Pet. 34. Petitioner argues that "Schmitt alone" discloses all of the steps of the method recited in claim 1. *Id.*

Regarding element 1(b), Petitioner relies on Schmitt's teaching of a "'hybrid method' of tagging, which uses a combination of the ends of the DNA fragments and 'a shorter n-mer tag'" such as a "3-mer" barcode that "together with the sequence information within the target DNA, serve as 'unique molecular identifiers.'" Pet. at 8–9 (quoting Ex. 1083 ¶¶ 30, 47, 75 (emphasis omitted)). Petitioner's declarant, Dr. Spellman, provides a diagram depicting Schmitt's hybrid tagging method that is reproduced below.



Id. at 9 (citing Ex. 1002 ¶ 89). In Dr. Spellman's diagram above, "the ends of the DNA fragment are designated 'L' and 'R,' and the short 3-mer molecular barcodes are labeled 'X' and 'Y.'" *Id.* According to Petitioner, the combination of X+L and R+Y "form unique molecular identifiers" even though the set of different 3-mer barcodes is "quite limited." *Id.*; *see also*

Pet. 28 (explaining that a "3-mer barcode would yield a set of 4^3 (i.e., 64) different molecular barcodes" providing "4,096 (i.e., 64^2) total" combinations of barcodes "considering the tagging occurs on both ends of the target molecule").

Petitioner offers evidence and argument for each of the other elements in claim 1 likewise premised on the use of 3-mer barcodes in Schmitt's DCS method. *See* Pet. 26–34 (citing evidence for elements 1(a)-(e)). Moreover, Petitioner articulates reasoning why "[a] POSA would have been motivated to use Schmitt's 3-mer hybrid tagging approach" as compared to other longer barcode embodiments taught in Schmitt. *Id.* at 35–36.

Petitioner further contends that a POSA would have had reason to combine Narayan's teachings regarding cfDNA with Schmitt's DCS method. *See* Pet 34–39. According to Petitioner,

> Narayan discloses using NGS for analyzing cfDNA from cancer patients, and Schmitt discloses methods of error correction for NGS by generating duplex consensus sequences (i.e., reducing or tracking redundancy in sequence reads). A POSA would have known that error correction is important when using NGS for diagnostic or screening applications as taught in Narayan, and would have been motivated to use cfDNA as disclosed in Narayan and in Schmitt's DCS methods. . . . A POSA would have understood that reducing or tracking sequence read redundancy using Schmitt's DCS would improve the sensitivity of sequence detection and thus been motivated to use it in in [sic] clinical settings as suggested by Narayan.

Id. at 34–35 (citing Ex. 1002 ¶187–189). Petitioner further contends that "[a] POSA would have expected to successfully use cfDNA . . . in Schmitt's DCS because the prior art expressly taught that DCS could 'enhance accuracy' when sequencing cfDNA from cancer patients" (*id.* at 37–38) and offers evidence supporting that a "POSA would have known that human

blood contains ample quantities of cfDNA for use in DCS." *Id.* at 5–6, 13–14, 39 (citing, e.g., Ex. 1002 ¶¶ 209–215).

Based on the current record, Petitioner's evidence and argument is sufficient to meet the burden for institution as to claim 1. Patent Owner raises several arguments to the contrary in its Preliminary Response, which are unavailing on the current record. We address those arguments below.

Patent Owner contends Petitioner has not shown that the cited art "discloses the tagging step of claim 1," i.e., element 1(b). Prelim. Resp. 42–45. Patent Owner refers to the final written decision in the '652 IPR, arguing that there "the Board considered Schmitt and found 'the relationship between 'z' and the number of tags 'n' was unknown'" in the prior art. *Id.* at 43 (quoting Ex. 1074, 47). According to Patent Owner, "Petitioner's challenge reflects the same deficiency the Office identified previously" because Petitioner does not "address the previously unknown relationship between n and z recited in the '306 patent claims." *Id.*

Based on the current record, we disagree. Contrary to Patent Owner's argument, claim 1 does not require a particular "relationship" between the number of tags and the expected number of duplicate molecules (z), nor does it require "tailoring" the recited method based on z. *See* Prelim. Resp. 19, 43. Rather, element 1(b) recites a range for "n" the number of "different combinations of molecular bar codes, wherein n is at least 2 and no more than 100,000*z." Ex. 1001, 61:16–17. Petitioner offers evidence, which is currently unrebutted, that n for Schmitt's 3-mer barcode is 4,096. Ex. 1002 ¶ 164. Thus, the record at this stage of the proceeding supports Petitioner's argument that Schmitt necessarily teaches a value for n that falls within the recited range "whether z is 1, 10, 100, 800, etc." Pet. 29.

The circumstances of this case are materially different from those in the '652 IPR. The upper bound of the range at issue in the '652 IPR was "100*z," which is three orders of magnitude narrower than the upper boundary recited in claim 1 of the '306 patent. Ex. 1074, 47. As Petitioner points out, the broader upper boundary at issue here means that the n for Schmitt's 3-mer barcode falls within the recited range even where z is 1. Pet. 29. This would not be the case for 100*z upper boundary at issue in the '652 IPR. Accordingly, Patent Owner's attempt to analogize the facts of this proceeding to the Board's determination in the '652 IPR is unpersuasive.

Patent Owner also argues that the Petition "fails to establish that the cited prior art discloses step **e** of claim 1" because "Schmitt does not mention unpaired reads as claimed and certainly does not disclose a method for identifying or sorting unpaired reads." *See* Prelim. Resp. 35–38. According to Patent Owner, "the Examiner allowed claims of [related U.S. Patent No. 11,118,221 B2 (the "'221 patent")] reciting substantially identical language finding that neither Schmitt nor any other prior art reference teaches or suggests the reducing or tracking step of claim 1" and "Petitioner gives no reason to second-guess the Examiner's findings." *Id.* at 36–37. Patent Owner also refers to our recent decision denying institution of *inter partes* review of the claims of the '221 patent. *See* Sur-reply 1–2.

As it relates to claim 1, Patent Owner's argument is unavailing. Element 1(e) of the '306 patent recites determining distinct cfDNA molecules "based on (i) paired reads . . . *or* (ii) unpaired reads . . . . " Ex. 1001, 61:29–36 (emphasis added).[16] On the current record, Petitioner

[16] By way of comparison, claim 29 depends from claim 1 and further limits element 1(e) by requiring the distinct cfDNA molecules be "determined

has sufficiently shown that the combination of Schmitt's DCS method with Narayan's cfDNA teaches or suggests the determination of cfDNA molecules based on paired reads. *See* Pet. 32–33 ("Schmitt discloses in a working example that 'consensus sequences were then *paired with their strand-mate*' and that the "complementary nature" of the double-stranded sequences "was used to *identify pairs of consensus groups* that arose from complementary DNA strands.") (quoting Ex. 1083 ¶¶ 60, 68). This is sufficient to meet element 1(e) here regardless of whether Schmitt teaches or suggests the determination of cfDNA molecules based on unpaired reads.

Patent Owner's attempt to analogize claim 1 of the '306 patent to the '221 patent claims ignores the fact that there is a significant difference in the pertinent claim language. *See* Sur-reply 2 (arguing that nothing "warrants a different conclusion regarding the parallel limitation[]" in element 1(e) of the '306 patent). Unlike the presently challenged claim 1, which recites determining distinct cfDNA molecules from paired reads *or* unpaired reads, element 1(e) of the '221 patent recites the generation of consensus sequences "from (i) paired reads . . . *and* (ii) unpaired reads . . . ." IPR2022-01152, paper 11, 13 (emphasis added).[17] For this reason, we do not agree with Patent Owner that the Office's previous consideration of the '221 patent

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based on (i) the paired reads *and* (ii) the unpaired reads." Ex. 1001, 64:29–31 (emphasis added).

[17] In IPR2022-01152, we determined "Petitioner ha[d] not sufficiently demonstrated that Schmitt's DCS method generates consensus sequences 'representative of the original cfDNA molecule . . . from . . . (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand' as recited in element 1(e)" of the '221 patent. IPR2022-01152, Paper 11, 16.

claims involved "substantially identical language" to that found in claim 1 here. *See* Prelim. Resp. 36.

Patent Owner also challenges Petitioner's rationale for combining Schmitt and Narayan, urging these references "present fundamentally different and seemingly incompatible[] methods and Narayan expressly disparages consensus sequencing approaches like Schmitt as poorly suited for cfDNA." *See* Prelim. Resp. 20–26. This argument too is unavailing on the current record. The Petition is premised on the application of Schmitt's DCS method to cfDNA. The mere fact that, in addition to teaching that cfDNA can be screened for cancer, Narayan describes a different method for analyzing it, does not provide a teaching away from the combination of references articulated in the Petition. Moreover, Petitioner articulates reasoning, which is supported by the testimony of Dr. Spellman and other evidence, explaining why a POSA would have been motivated to apply Schmitt's DCS method to cfDNA and would have had a reasonable expectation of successfully doing so. *See* Pet. 5–6, 13–14, 34–35, 37–39. Petitioner's evidence is largely unrebutted at this stage of the proceeding and sufficiently supported to meet the burden for institution.

Patent Owner's argument that "Narayan specifically disparages" consensus sequencing methods like DCS is not sufficiently developed and seems to overstate that reference's teaching based on the current record. *See* Prelim. Resp. 24–26. Patent Owner points to the following statement in Narayan:

> Kinde and colleagues recently described an elegant and powerful error reduction strategy that enables highly sensitive quantitation of DNA variants using massively parallel sequencing (21). However, this approach was not designed to

> analyze multiple amplicons from samples containing limited DNA and was not tested on clinical specimens.

Id. at 24 (quoting Ex. 1082, 3497). According to Patent Owner, this statement also applies to Schmitt's DCS method because it "bears the same fundamental design as Kinde." *Id.* Even so, on the current record, we do not agree that Narayan disparages such an approach. To the contrary, Narayan praises Kinde's methodology as "elegant," "powerful," and "highly sensitive," even if it had not been designed specifically for cfDNA or tested on clinical specimens. *See* Ex. 1082, 3497. Moreover, Petitioner offers evidence, including Kukita,[18] which it urges "expressly disclosed the potential benefit of using DCS for screening cfDNA from blood of cancer patients." Pet. 14, 38 (citing Ex. 1008, 2, 7). Patent Owner disagrees with Petitioner's evidence (*see* Prelim. Resp. 14–15), but we find on this record that Petitioner's evidence is sufficient for institution. Patent Owner's arguments to the contrary would benefit from further development at trial.[19]

Patent Owner's arguments regarding what it calls Petitioner's "routine blood draw theory" are also unavailing on the current record. *See* Prelim. Resp. 26–30. The Petition includes evidence and argument to show that a POSA would have understood that human blood contains sufficient amounts of cfDNA for use in DCS. *See* Pet. 5–6, 37, 39 (citing exhibits). As an

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[18] Kukita et al., *Quantitative Identification of Mutant Alleles Derived from Lung Cancer in Plasma Cell-Free DNA via Anomaly Detection Using Deep Sequencing Data*, 8 PLOS ONE METHODS 1–10 (2013) (Ex. 1008).

[19] We also remind the parties of the panel's observation in the '652 IPR that "the test for obviousness does not require 'actual success' in the prior art." Ex. 1074, 59 (quoting *Engo Pharm. Inc. v. Actavis LLC*, 992 F.3d 1365, 1379 (Fed. Cir. 2019)). The parties should consider this principle, and that panel's related findings (*see id.* at 58–63), as they develop their arguments and evidence at trial in this proceeding.

example, Petitioner explains that 1 µg of cfDNA can be obtained from 7–12 ml of plasma. *Id.* at 6 (citing Ex. 1002 ¶¶ 68–71). Patent Owner argues that this "amount of DNA comes out of thin air" because "neither [Schmitt nor Narayan] discusses 1 µg of DNA and no explanation is provided." Prelim. Resp. 28. We disagree. Dr. Spellman summarizes a number of references "teaching the use of [Next Generation Sequencing] on cfDNA extracted from [varying amounts of] plasma or blood" that are generally consistent with the exemplary sample amount of 1 µg of DNA in 7–12 ml of plasma referenced in the Petition. Ex. 1002 ¶ 72. This evidence, which is currently unrebutted, supports Petitioner's assertion a POSA "would have known that sufficient amounts of cfDNA could be extracted from blood" using routine techniques and "that more cfDNA, if needed, could be obtained by simply increasing the amount of plasma sample used for cfDNA extraction." Pet. 37 (citing Ex. 1002 ¶¶ 190–191; Ex. 1049: Ex. 1071, 718). To the extent Patent Owner disagrees, it will have the opportunity to respond more fully at trial.

Finally, Patent Owner criticizes Petitioner's reliance on "a 3-mer hybrid tag" as opposed to the longer 12 nucleotide tag employed in Schmitt Example 1. *See* Prelim. Resp. 30–35. According to Patent Owner, Petitioner "never explains why one would specifically pair a hybrid 3-mer with the proposed cfDNA samples or why a POSA would reasonably expect success in such a scenario." *Id.* at 32.

Patent Owner's argument is unavailing on the current record. Petitioner asserts that a "POSA would have appreciated that Schmitt's 3-mer hybrid tag embodiment offers advantages of simplified adapter synthesis, improved synthesis yield, and improved downstream PCR amplification compared to longer barcodes such as Schmitt's 12-mer or 20-mer embodiments." Pet. 35 (citing, e.g., Ex. 1002 ¶¶ 192–194). Petitioner

additionally asserts that a POSA "would have known that they could get more value per sequencing read when using Schmitt's 3-mer hybrid tags." *Id.* at 35–36 (citing, e.g., Ex. 1002 ¶¶ 194–196). These assertions are supported by Dr. Spellman's testimony and sufficient to meet Petitioner's burden at this stage of the proceeding. Patent Owner's arguments to the contrary, e.g., that Petitioner's publications "instruct avoiding tags less than 12 nucleotides" and "Schmitt teaches that tag diversity (e.g., tag size) should increase—rather than decrease—with increasing sample complexity," are not well developed at this stage and would benefit from further development at trial. *See* Prelim. Resp. 31–32.

Petitioner's showing for dependent claims 2, 3, 5, 7, and 9–14 is also sufficient to meet the burden for institution. The Petition presents evidence and argument to support Petitioner's assertion that the cited references teach or suggest the additional limitations recited in each of these claims. Pet. 44–55 (addressing additional limitations in claims 2, 3, 5, 7, and 9–14).[20] At this stage, Patent Owner does not make any arguments against Petitioner's showing for these dependent claims beyond its arguments for claim 1, which we have already explained are unavailing on the current record.

Accordingly, based on the record at this stage of the proceeding, Petitioner has established a reasonable likelihood it will prevail in

[20] For reasons explained in sections III.E.2 and III.H below, we are skeptical of the Petition's showing for claim 14 to the extent Petitioner contends that Schmitt's "pilot experiment" demonstrates "a quantitative measure of unpaired reads (more than 99%), as claimed." Pet. 54. However, claim 14 recites determining quantitative measures of paired reads "or" unpaired reads. Ex. 1001, 62:32. Thus, on the current record, Petitioner's showing relating to Schmitt's quantification of paired reads is itself sufficient to teach this limitation.

demonstrating that claims 1–3, 5, 7, and 9–14 would have been obvious over Schmitt and Narayan.

2. Analysis of Claims 17–27 and 29

While on the current record Petitioner has met its burden with respect to claims 1–3, 5, 7, and 9–14, the same is not true for claims 17–27 and 29. Independent claim 17 differs from claim 1 in several respects, including its recitation of "sorting a plurality of sequence reads from the set of sequence reads into (i) families comprising paired reads . . . *and* (ii) families comprising unpaired reads" in element 17(d). Ex. 1001, 62:55–65 (emphasis added). Claim 29 depends from claim 1 and further limits element 1(e) by reciting that "the distinct cfDNA molecules in (e) are determined based on (i) the paired reads *and* (ii) the unpaired reads." *Id.* at 65:29–31 (emphasis added). As discussed below, Petitioner has not sufficiently explained how the cited references teach or suggest the sorting of reads into families of unpaired reads as recited in element 17(d) or determining distinct cfDNA molecules based on unpaired reads as recited in claim 29.

For each of these elements, the Petition relies solely on the teachings in Schmitt. *See* Pet. 41–44 (claim 17); 58 (claim 29). Petitioner contends "a POSA would have known that Schmitt discloses 'sorting' the reads into paired and unpaired reads because Schmitt discloses generating duplex consensus sequence reads (paired reads), which requires 'sorting' the reads into paired reads and unpaired reads." *Id.* at 42. For support, Petitioner points to Figures 1 and 3 and the related disclosure in Schmitt, urging that it teaches sorting reads into families based on their SMI tag that are then sorted into paired families. *Id.* at 44–45 (citing Ex. 1083 ¶¶ 43, 63, Figs 1, 3).

Petitioner also relies on its showing for element 1(e). Pet. 41, 58. There, the Petition points to the disclosure in Schmitt's "working example

that 'consensus sequences were then *paired with their strand-mate*' and the 'complementary nature' of the double-stranded sequences 'was used to *identify pairs of consensus groups* that arose from complementary DNA strands." *Id.* at 32–33 (quoting Ex. 1083 ¶¶ 60, 68). Petitioner urges that "[b]y following this teaching in Schmitt, the POSA would have also determined distinct DNA molecules from sequence reads of a first tagged strand having no second complementary strand – i.e., unpaired reads." *Id.* at 33. Petitioner also references "a separate working example" found in the "Schmitt PCT," i.e., Ex. 1009.[21] *Id.* According to Petitioner, the data in Table 1 of Ex. 1009 shows that in this example "more than half of the total SSCS reads remained unpaired, while the other reads were paired." *Id.* at 33–34.

Patent Owner argues that Schmitt does not disclose the sorting of unpaired reads recited in element 17(d) or the use of unpaired reads recited in claim 29. *See* Prelim. Resp. 35–42. According to Patent Owner, "Schmitt does not identify unpaired reads, as that term is used in the challenged claims, at any step of its method." *Id.* at 38. Patent Owner asserts that "Schmitt describes grouping as forming families of reads that share a common SMI tag" and this process is "agnostic to whether reads are paired or unpaired."

Patent Owner further contends that the passages Petitioner cites from Schmitt's working example "discuss only selecting consensus sequences based on a stringent filtering step," i.e., where there is "'perfect agreement' between SMI sequences." *Id.* at 38–40 (quoting Ex. 1083 ¶¶ 60, 68). Patent

[21] Unlike its other arguments regarding Schmitt, for this argument Petitioner cites the text in Ex. 1009.

Owner argues that "sequences lack the requisite 'perfect agreement' where [a] sequence error occurs in the SMI or barcode." *Id.* at 50. Patent Owner cites evidence, as well as Petitioner's own argument, to show that "this problem was widely recognized in the scientific literature as specifically a problem in Schmitt's DCS method and identified as a major cause of the data loss (e.g., >99% of tags) Schmitt describes." *Id.* (citing Pet. 63 (relying on Ex. 1063, 2); Ex. 2013, 5–6).

Patent Owner also disputes Petitioner's reliance on the data in Table 1 Ex. 1009, urging that it does not suggest the sorting of unpaired reads recited in element 17(d) or the use of unpaired recited in claim 29 because it "provides information on the basis of gross nucleotide count" as opposed to reads, i.e., "a series of nucleotides produced by sequencing a tagged strand derived from an original cfDNA molecule." *Id.* at 41–42.

On the record at this stage of the proceeding, we determine that Patent Owner has the better argument. Beginning with element 17(d), the Petition points to two steps in Schmitt's DCS method for element 17(d): (1) the grouping of reads into families "by virtue of having a common (i.e., the same) SMI tag sequence;" and (2) the subsequent grouping of those families "into *paired families*" based on the orientation of their tag. *See* Pet. 43 (quotations omitted). The first of these steps groups reads based on their SMI tag without regard to whether they have a complementary sequence. Thus, it does not disclose sorting "sequence reads into paired reads and unpaired reads" as recited in element 17(d). In the second of these steps, paired families comprising complementary sequences are formed. While this may result in the sorting of reads or SSCSs into families of paired reads, Petitioner has not shown that it teaches or suggests the sorting of reads into "families comprising unpaired reads" as recited in element 17(d).

Petitioner's argument that the generation of duplex consensus sequences necessarily "requires" the sorting of unpaired reads is unavailing on the current record. *See* Pet. 42. Schmitt teaches that the complementary nature of its SMI tag sequences are "used to identify pairs of consensus groups that arose from complementary DNA strands." Ex. 1083 ¶ 68. However, the Petition cites no corollary disclosure suggesting the sorting of reads into families that lack a complementary strand, nor do Petitioner or Dr. Spellman explain why the generation of duplex consensus sequences would require the sorting of sequence reads into families of unpaired reads. *See* Pet. 42; Ex. 1002 ¶ 233.

Instead, the figures and disclosures cited in the Petition and Dr. Spellman's testimony show the grouping of families of reads with the same SMI into families of paired reads. *See* Pet. 42; Ex. 1002 ¶¶ 234–36. According to Schmitt, Figure 1 depicts that

> two types of PCR products are produced from each capture event. Those derived from one strand will have the α SMI sequence adjacent to flow-cell sequence 1 and the β SMI sequence adjacent to flow cell sequence 2. PCR products originating from the complementary strand are labeled reciprocally.

Ex. 1083 ¶ 11. Thus, Figure 1 shows PCR products (i.e., reads) grouped into families based on their SMI sequence with an "αβ SMI family" and the *complementary* "βα SMI family." *Id.* at 56 (Fig. 1). Schmitt Figure 3 "(a-c) shows sequence reads . . . sharing a unique set of SMI tags . . . grouped into *paired* families" and an "error-corrected consensus sequence . . . for each *duplex*" in (d). Ex. 1083 ¶ 13 (emphases added). Petitioner has not explained how either figure depicts "families of unpaired reads corresponding to

sequence reads . . . having no second tagged complementary strand" as recited in element 17(d).

On the current record, Petitioner's reliance on Schmitt Example 1 is also unavailing. Schmitt Example 1 describes the results of a "pilot experiment" in which "29,409 SMI partner pairs were found, indicative that fewer than 1% of tags had their corresponding partner pair tag present in the library." Ex. 1083 ¶ 68. Petitioner argues this means the "measure of unpaired reads" in that experiment was "more than 99%" and thus a POSA following these teachings would have necessarily generated consensus sequences from unpaired reads.[22] Pet. 54; *see also* Reply 3 (asserting that Schmitt paragraph 68 discloses ">99% unpaired sequence reads"). We are not convinced. As an initial matter, Schmitt Example 1 does not expressly describe the sorting of reads into families of unpaired reads as recited in element 17(d). Rather, Schmitt indicates that reads (i.e., "PCR duplicates") were grouped into families based on their SMI tag, SSCSs were created from those families, and then the "complementary nature of the double-stranded SMI sequences was used to identify" and form double-stranded consensus sequences, but "only when the read data from each of the two strands [wa]s in perfect agreement." *See* Ex. 1083 ¶¶ 67–68. Petitioner appears to assume that if a SMI partner pair was not found for a tag, the SSCS associated with that tag was necessarily generated from "unpaired reads . . . having no second tagged complementary strand" as recited in element 17(d). But neither the Petition, nor the testimony it cites from Dr. Spellman, provides

[22] The Petition presents this argument for the "quantitative measures" limitations in dependent claims 14 and 27. Pet. 54. We address it here because Petitioner cites the same underlying disclosure to support its arguments for element 17(d). *See* Pet. 42 (citing Ex. 1083 ¶ 68).

sufficient explanation to support that assumption. *See* Pet. 54 (citing Ex. 1002 ¶ 309).

This is particularly so because Schmitt explains that for at least some tagged reads the reason a partner pair was not found was that the complementary strands were not in "perfect agreement." *See* Ex. 1068 ¶ 68 (explaining that SMI partner pairs were found "after grouping of PCR duplicates" and "reads were considered only when the read data from each of the two strands is in perfect agreement"). In such instances, there would be a complementary strand, which seemingly undermines Petitioner's theory that the tags that did not form SMI partner pairs in Schmitt's working example were necessarily the result of unpaired reads.

In addition, Patent Owner cites evidence suggesting that the tags lacking a "corresponding partner tag" in Schmitt Example 1 may have resulted from sequencing errors in the SMI or barcode. *See* Prelim. Resp. 40–41 (Ex. 1063, 2; Ex. 2013, 5–6). Patent Owner relies on Stoler,[23] which explains that "sequencing errors within duplex tags" was known to be "one of the main causes of data loss" in DCS. Ex. 2013, 5–6. Indeed, both Petitioner and Dr. Spellman acknowledge that "sequencing errors in the barcode itself" were known to occur and "'can cause one tag to appear identical to another (crossover) or sufficiently alter a sequence tag such that it is unrecognizable (loss) and untraceable to the source material'" Pet. at 64; Ex. 1002 ¶ 355 (quoting Ex. 1063, 2). Based on the current record, it seems that in such instances the inability to form a SMI partner pair would result from the error in the SMI tag—not the lack of a complementary strand. The

[23] Stoler et al., *Streamlined Analysis of Duplex Sequencing Data with Du Novo*, 17:180 Genome Biology 1–10 (2016) (Ex. 2013).

fact that Petitioner does not account for the impact of such known sequencing errors in its analysis, further undermines its argument that Schmitt discloses ">99% unpaired sequence reads" as well as sorting between families of paired and unpaired reads. *See* Reply 2–3.[24]

We are also skeptical of Petitioner's arguments regarding the example reported in Table 1 of Exhibit 1009.[25] As Patent Owner points out, Table 1 appears to provide data based on "gross nucleotide count" for the overall experiment. *See* Prelim. Resp. 41–42. This supports Patent Owner's argument that the data reported for "reads" in the fourth and fifth rows of Table 1 is calculated "as a measure of the total number of nucleotides ('initial nucleotides') divided by the total 'SSCS [or DCS] nucleotides'" as opposed to "identifying paired and unpaired reads" (*id.* at 42), which in turn would seem to impact the "SSCS:DCS ratio" Petitioner relies upon for its arguments (*see* Pet. 33–34). Moreover, as explained above, Patent Owner

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[24] In its Reply, Petitioner argues that "sequence reads with errors in the SMI or barcode are still *unpaired sequence reads* because they do not have a partner pair." Reply. 4. But element 17(d) requires sorting into "families comprising unpaired reads corresponding to sequence reads generated from a first tagged strand *having no second tagged complementary strand* derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides." Ex. 1001, 62:60–65 (emphases added). We agree with Patent Owner that on the current record Petitioner has not sufficiently explained how these aspects of element 1(a) are met when a sequencing error in the SMI or barcode prevents the formation of a partner pair. *See* Sur-reply 2 (urging that "[e]rrors in the barcode may prevent SMI-based matching, but not describe an unpaired read *as claimed*").

[25] It is unclear whether the fact that Table 1 and the related example Petitioner cites appear in Schmitt (i.e., Exhibit 1009) but apparently not in the priority application Schmitt-623 (Ex. 1083), affects the availability of this disclosure as prior art to the challenged claims. The parties are encouraged to address this issue in their subsequent papers.

cites evidence suggesting errors in the barcode and the lack of perfect agreement between complementary strands result in SSCSs not being paired to form DCS even where there is a "second tagged complementary strand" as recited in element 17(d). The analysis in the Petition and the corresponding testimony of Dr. Spellman do not account for these issues, undermining the persuasiveness of Petitioner's argument. *See* Pet. 33–34, 44; Ex. 1002 ¶¶ 184–85, 237.

For these reasons, and based on the current record, Petitioner has not established a reasonable likelihood that it would ultimately prevail on its assertion that claim 17 would have been obvious over Narayan and Schmitt. Claims 18–27 depend from claim 17 and rely on the same showing for element 17(d). Thus, Petitioner has not met its burden for these claims for the same reasons noted for claim 17.

Petitioner's showing for claim 29 is deficient for similar reasons. As explained above, the DCS method taught in Schmitt generates an "error corrected double-stranded consensus sequence." Ex. 1083 ¶¶ 43, 60. Schmitt teaches that these consensus sequences are error corrected because "[s]equence reads at a given position are kept only if the read data from each of the two paired strands is in agreement." *Id.* ¶¶ 60, 63, 68. Since only those reads having a complementary sequence in complete agreement are ultimately used to generate Schmitt's duplex consensus sequences, the current record does not sufficiently show that Schmitt's DCS method determines distinct cfDNA molecules "based on (i) the paired reads *and (ii) the unpaired reads*" as recited in claim 29.

F. Ground 2: Obviousness over Narayan, Schmitt, and Meyer

Petitioner asserts that claims 4 and 6 are unpatentable as obvious over Narayan and Schmitt. Claims 4 and 6 depend from claim 1 and additionally

recite a minimum percentage of cfDNA molecules from the population, i.e., "at least 20%" (claim 4) and "at least 40%" (claim 6), that must be tagged with duplex tags.[26] Ex. 1001, 61:53–55, 60–62. Petitioner asserts that each limitation of claims 4 and 6 is taught by the combination of Schmitt, Meyer, and Narayan, and that a POSA would have been motivated to combine Narayan's cfDNA with Schmitt's DCS method achieving the specified level of tagging efficiency in view of Meyer's teaching of tagging 40–60% of DNA molecules. Pet. 58–61. Patent Owner disagrees, arguing that Petitioner fails to show that the cited references teach or suggest the tagging efficiency limitations of claims 4 and 6. Prelim. Resp. 45–47. Based on the current record, we agree with Patent Owner.

Petitioner relies solely on Meyers for the tagging efficiency limitations, asserting

> A POSA would have understood that Meyer discloses that 40-60% of the DNA fragments are tagged because (i) Meyer's Figure 1 depicts the DNA fragments tagged on both ends (Fig. 1), (ii) Meyer's adapter ligation quantification "step 18" would only detect adapter-ligated DNA fragments, and (iii) Meyer expressly discloses successfully achieving at least 40-60% tagged DNA fragments.

Pet. 60 (citing Ex. 1002 ¶ 340; Ex. 1005, 274, Fig. 1). Petitioner further asserts that Meyer's disclosure of an expected recovery "between 40 and 60" falls within the ranges recited in the tagging efficiency limitations. *Id.* at 59–60.

Petitioner asserts that a person of ordinary skill in the art would have been motivated to optimize adapter-DNA ligation for high efficiency using

[26] For ease of reference, we refer to these limitations collectively as the "tagging efficiency limitations."

routine methods for doing so. Pet. 60–61. Petitioner argues "a POSA would have expected to successfully tag 'at least 40%' of the DNA molecules" because "Meyer expressly discloses successfully tagging 40–60% of DNA molecules." *Id.* at 60 (citing Ex. 1002 ¶ 347; Ex. 1005, 274). And, according to Petitioner, Meyer's disclosure of 40–60% tagging efficiency is consistent with other references. *Id.* For example, Petitioner cites the KAPA data sheet,[27] which teaches "15–40% of input DNA is typically recovered as adapter-ligated molecules." *Id.* (quoting Ex. 1015, 4). Petitioner also cites Fisher[28] as disclosing "recovering 47% of the library as adapter-ligated DNA fragments." *Id.* (citing Ex. 1031, Fig. 3). Finally, Petitioner asserts that a POSA "would have expected Schmitt's sticky-end adapter-DNA ligation (using A- and T-overhangs) to be even more efficient than Meyer's blunt-end adapter-DNA ligation, which nevertheless achieved 40–60% adapter-ligated DNA fragments." *Id.* at 60–61 (citing Ex. 1002 ¶ 348; Ex. 1005, 273; Ex. 1083 ¶¶ 55–56). Even if the claimed amount is not expressly taught by the art, Petitioner asserts that tagging efficiency limitations would have been an obvious optimization of a result-effective variable. *See id.* at 62 (citing Ex. 1002 ¶ 348).

We are not persuaded by Petitioner's arguments. As an initial matter, we note that Ground 2 in the instant Petition relies on nearly identical or similar arguments set forth by Petitioner in related proceedings, which we have previously rejected. *Compare, e.g.,* IPR2022-01115, Paper 2, 30–31,

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[27] KAPA Technical Data Sheet for KAPA HTP Library Preparation Kit. Ex. 1015 ("KAPA data sheet").

[28] Fisher et al., *A Scalable, Fully Automated Process for Construction of Sequence-Ready Human Exome Targeted Capture Libraries*, 12:R1 GENOME BIOLOGY 1–15 (2011). Ex. 1031 ("Fisher").

35, 38, *with* Pet. 58–61. Thus, for the reasons stated in the decisions denying institution in those proceedings, we also find Petitioner's showing for the tagging efficiency limitations in this proceeding to be deficient. *See, e.g.*, IPR2022-01115, Paper 14, 12–19.

That is, although Meyer teaches that its particular protocol results in 40–60% tagging efficiency, Petitioner has not sufficiently explained why a person of ordinary skill in the art would have expected Meyer's tagging efficiency to apply to Schmitt's DCS method when used with Narayan's sequencing of cfDNA. For example, the Petition does not address the differences between Meyer's adapters and Schmitt's adapters and how those differences affect the tagging efficiency. Meyer's barcodes comprise "single self-hybridized palindromic oligonucleotides, carrying an *Srf* I restriction site in the middle (GCCCGGGC), a sequence tag at the 3' end and the reverse complementary tag sequence at the 5' end." Ex. 1005, Fig. 1. Schmitt, on the other hand, teaches a "hybrid method" of tagging that uses a combination of the sheared ends of the DNA fragments and a "shorter n-mer tag (such as 1 or 2 or 3 or 4 or more degenerate or semi degenerate bases) in the adaptor." Ex. 1083 ¶ 30.

In addition, the KAPA data sheet states that tagging efficiency "can be significantly lower for DNA of lower quality." Ex. 1015, 4. Fisher also explains that "the efficiency of adaptor ligation varies between samples, probably because of variation in input DNA quality." Ex. 1031, 5–6. Petitioner does not address how the quality of the cfDNA of Narayan compares to the quality of the genomic DNA samples of Meyer and Schmitt or how that would affect the expected tagging efficiency. The KAPA data sheet discloses sticky-end adapter DNA ligation, but KAPA teaches that only 0.5 to 15% tagging efficiency is expected when the starting sample is

100 pg–100 ng of "high quality DNA." Ex. 1015, 4. Thus, the KAPA data suggests that the use of sticky-end adapters, such as what Schmitt discloses, does not in and of itself teach or suggest the tagging efficiency limitations recited in claims 4 and 6 when using cfDNA.

We also determine that the current record does not sufficiently support Petitioner's assertion "that adapter-DNA tagging efficiency is a *result-effective variable* that only requires routine skill to optimize." Pet. 60 (citing Ex. 1002 ¶¶ 348). Dr. Spellman states "it was understood that increasing the concentration of adapters was a routine technique that could be used to maximize ligation efficiency such as by using a molar excess of adapters relative to DNA fragments in the ligation reaction." Ex. 1002 ¶ 263. But the other evidence Petitioner relies upon suggests that achieving a particular level of tagging efficiency is not as simple as varying the concentration of adapters and is also affected by other factors, e.g., the amount of starting sample and the quality of the DNA. *See* Ex. 1015, 4; Ex. 1031, 6. Thus, we are not persuaded that Petitioner has shown sufficiently that adapter-DNA tagging efficiency, particularly for the cfDNA recited in the challenged claims, is a simple result-effective variable that can be determined by routine optimization.

For these reasons, and based on the current record, Petitioner has not established a reasonable likelihood that it will ultimately prevail on its assertion that claims 4 and 6 would have been obvious over Narayan, Schmitt, and Meyer.

G. Ground 3: Obviousness over Narayan, Schmitt, and Craig

Petitioner asserts that claim 8 would have been obvious over Narayan, Schmitt, and Craig. Pet. 61–63. Claim 8 depends from claim 1 and additionally recites that "the molecular barcodes have a length of 5 to 20

base pairs." Ex. 1001, 61:64–65. Petitioner relies on Craig's disclosure of a set of "48 6-mer barcodes" and articulates reasoning supported by the testimony of Dr. Spellman to explain why a POSA would have been motivated to use Craig's "48 6-mer barcodes" in Schmitt's DCS method with a reasonable expectation of success. Pet. 61–63 (citing Ex. 1002 ¶¶ 355–62).

At this stage of the proceeding, and based on the current record, we determine that Petitioner's showing for claims 8 is sufficient to meet the burden for institution. Patent Owner does not presently dispute Petitioner's showing beyond its arguments for claim 1, which we have addressed above. *See* Prelim. Resp. 47.

H. Ground 4: Obviousness over Narayan, Schmitt, and Kivioja

Petitioner asserts that claims 15, 16, and 28 would have been obvious over Narayan, Schmitt, and Kivioja. Pet. 63–69. Claim 15 depends from claim 14 (which in turn depends from claim 1) and recites "(g) estimating with a programmed computer processor a quantitative measure of tagged parent polynucleotides that map to the genomic locus based on the quantitative measure of the paired reads and the unpaired reads." Ex. 1001, 62:34–39. Claim 16 depends from claim 15 and additionally recites "(h) detecting copy number variation in the population of cfDNA molecules by determining a normalized quantitative measure determined in (g) at each of one or more genomic loci." *Id.* at 62:40–44. Claim 28 depends from a series of claims that depend from claim 17 and recites a limitation similar to that in claim 15. *See id.* at 64:22–28.

Petitioner relies on Kivioja's methods for calculating the total number of molecules that map to a genetic locus and detecting copy number for the additional limitations recited in claims 15, 16, and 28. *See* Pet. 63–69.

Moreover, Petitioner articulates reasoning, supported by the testimony of Dr. Spellman, to explain why a POSA would have been motivated to use Kivioja's quantification methods in conjunction with the other cited references. *Id.* at 64, 66–67 (citing Ex. 1002 ¶¶ 367–76). At this stage, Patent Owner does not dispute Petitioner's showing for these claims beyond its arguments for independent claims 1 and 17. *See* Prelim. Resp. 47.

At this stage of the proceeding, and based on the current record, we determine that Petitioner's showing for claims 15 and 16 is sufficient to meet the burden for institution.[29] However, we express some skepticism to the extent the use of Kivioja's quantification methods may require quantitative measures of the number of unpaired reads that map to a genomic locus of the reference sequence. Petitioner's argument that Schmitt discloses a quantitative measure of unpaired reads is premised on its assumption that the tags that lacked partner pairs in Schmitt's "pilot experiment" corresponded to unpaired reads as recited in the challenged claims. *See* Pet. 54 (arguing that "Schmitt discloses . . . a quantitative measure of unpaired reads (more than 99%)"). As explained above, and based on the record at this stage of the proceeding, Petitioner has not sufficiently shown that this is the case. *See supra* § III.E.2. This in turn may impact Petitioner's arguments for combining Kivioja's methods with Schmitt and Narayan. In any event, these issues would benefit from further development, and we encourage the parties to address them in their subsequent papers at trial.

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[29] Petitioner's showing for the additional limitation recited in claim 28 is also sufficient at this stage of the proceeding. However, as explained above, Petitioner has not met its burden for claim 17 from which claim 28 indirectly depends.

IV. CONCLUSION

Based on the current record, we determine Petitioner has shown a reasonable likelihood that it will prevail in establishing that at least one claim of the '306 patent is unpatentable. Accordingly, we institute review of all claims challenged on all of the grounds in the Petition. *See* Patent Trial & Appeal Board Consolidated Trial Practice Guide November 2019 ("CTPG," *available at* https://www.uspto.gov/TrialPracticeGuideConsolidated), 64.

At this stage of the proceeding, the Board has not made a final determination as to the patentability of any challenged claim. Our view with regard to any conclusion reached in the foregoing analysis could change upon completion of the record.

V. ORDER

Accordingly, it is:

ORDERED that pursuant to 35 U.S.C. § 314, an *inter partes* review is hereby instituted as to claims 1–29 of the '306 patent based on the unpatentability challenges presented in the Petition;

FURTHER ORDERED that pursuant to 35 U.S.C. § 314(c) and 37 C.F.R. § 42.4, notice is hereby given of the institution of a trial commencing on the entry date of this decision.

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Paper 41
Entered: January 26, 2024

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

TWINSTRAND BIOSCIENCES, INC.,
Petitioner,

v.

GUARDANT HEALTH, INC.,
Patent Owner.

IPR2022-01400
Patent 11,149,306 B2

Before SUSAN L. C. MITCHELL, TINA E. HULSE, and MICHAEL A. VALEK, *Administrative Patent Judges.*

VALEK, *Administrative Patent Judge.*

JUDGMENT
Final Written Decision
Determining No Challenged Claims Unpatentable
35 U.S.C. § 318(a)
Denying Patent Owner's Motion to Strike
Denying Patent Owner's Motion to Exclude
37 C.F.R. § 42.64(c)

I. INTRODUCTION

Twin Strand Biosciences, Inc. ("Petitioner") filed a Petition requesting an *inter partes* review of claims 1–29 of U.S. Patent No. 11,149,306 B2 (Ex. 1001, "the '306 patent"). Paper 2 ("Pet."). We instituted trial on all of the grounds in the Petition. Paper 9, 44 ("Dec.").

Guardant Health, Inc. ("Patent Owner") subsequently filed its Response to the Petition (Paper 18, "Resp."), Petitioner filed its Reply (Paper 25, "Reply") and Patent Owner filed its Sur-reply (Paper 30, "Sur-reply."). In addition, with our authorization, Patent Owner filed a motion to strike certain exhibits submitted with the Reply (Paper 29, "MTS"), which Petitioner has opposed (Paper 31, "MTS Opp."). Patent Owner also filed a motion to exclude (Paper 32 ("MTE")), which Petitioner has opposed (Paper 34 ("MTE Opp."). We held a hearing on November 17, 2023, and a transcript is of record. Paper 40 ("Tr.").

After considering the parties' arguments and evidence, we find that Petitioner has not shown by a preponderance of the evidence that the challenged claims of the '306 patent are unpatentable. *See* 35 U.S.C. § 316(e). Moreover, we deny Patent Owner's motions to strike and exclude.

II. BACKGROUND

A. Real Parties in Interest

Petitioner identifies itself as a real party in interest and the University of Washington as a potential real party in interest. Pet. 69. Patent Owner identifies itself as a real party in interest. Paper 19, 1.

B. The '306 Patent

Genetic testing is useful for a number of diagnostic methods. Ex. 1001, 1:25–26. Disorders that are caused by rare genetic mutations (e.g., sequence variations) or changes in epigenetic markers, such as cancer and

partial or complete aneuploidy, may be detected or more accurately characterized with DNA sequence information. *Id.* at 1:26–30.

Early detection and monitoring of genetic diseases is often useful and needed in the successful treatment or management of a disease. Ex. 1001, 1:31–33. According to the '306 patent, one approach may include monitoring a sample derived from cell-free nucleic acids, which are polynucleotides that can be found in different types of bodily fluids. *Id.* at 1:33–36. Cell-free DNA ("cfDNA") may contain genetic aberrations, such as copy number variation or sequence variation, associated with a particular disease. *Id.* at 1:36–43.

The '306 patent explains that many methods have been developed to estimate copy number variation. Ex. 1001, 1:46–47. According to the Specification, most of those methods involve preparing a sample by converting the original nucleic acids into a sequenceable library, followed by massively parallel sequencing, and then conducting a bioinformatic analysis to estimate the copy number variation at one or more loci. *Id.* at 1:51–55.

The '306 patent states that although known methods for detecting cfDNA are able to reduce the errors introduced by the sample preparation and sequencing processes for the molecules that are converted and sequenced, these methods are not able to infer the counts of molecules that were converted, but not sequenced. Ex. 1001, 1:59–63. The '306 patent states this inability to count converted but unsequenced molecules "can dramatically and adversely affect the sensitivity that can be achieved." *Id.* at 1:63–67. Accordingly, the '306 patent relates to a method of tagging and counting both halves of double-stranded DNA and estimating the number of unseen molecules based on the number of Pairs (i.e., molecules where both

strands were identified) and Singlets (i.e., molecules where only one strand was identified) detected in a particular region. *See id.* at 2:1–18.

C. Illustrative Claims

Petitioner challenges claims 1–29 of the '306 patent. Of these, claims 1 and 17 are independent. Claims 1 and 17 read as follows:

> 1. A method, comprising:
>
> (a) providing a population of cell-free deoxyribonucleic acid (cfDNA) molecules having first and second complementary strands;
>
> (b) tagging a plurality of the cfDNA molecules in the population with duplex tags comprising molecular barcodes to produce tagged parent polynucleotides, wherein the duplex tags are attached at both ends of a molecule of the plurality of the cfDNA molecules, wherein the plurality of the cfDNA molecules are tagged with n different combinations of molecular barcodes, wherein n is at least 2 and no more than 100,000*z, wherein z is a mean of an expected number of duplicate molecules in the population of cfDNA molecules that map to identical start and stop positions on a reference sequence;
>
> (c) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;
>
> (d) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and
>
> (e) reducing or tracking redundancy of a plurality of sequence reads from the set of sequence reads using at least sequence information from the molecular barcodes of the duplex tags to determine distinct cfDNA molecules from among the tagged parent polynucleotides, wherein the distinct cfDNA molecules are determined based on (i) paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, or (ii) unpaired reads corresponding to sequence reads generated from a first tagged strand

having no second tagged complementary strand derived from cfDNA molecules from among the tagged parent polynucleotides, wherein reducing or tracking the redundancy of the plurality of sequence reads comprises mapping at least a subset of the plurality of sequence reads to the reference sequence.

17. A method, comprising:

(a) tagging a population of double-stranded cell-free deoxyribonucleic acid (cfDNA) molecules obtained or derived from a sample of a subject with a set of tags comprising molecular barcodes to produce tagged parent polynucleotides;

(b) amplifying a plurality of the tagged parent polynucleotides to produce amplified progeny polynucleotides;

(c) sequencing at least a subset of the amplified progeny polynucleotides to produce a set of sequence reads; and

(d) sorting a plurality of sequence reads from the set of sequence reads into (i) families comprising paired reads corresponding to sequence reads generated from a first tagged strand and a second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides, and (ii) families comprising unpaired reads corresponding to sequence reads generated from a first tagged strand having no second tagged complementary strand derived from double-stranded cfDNA molecules from among the tagged parent polynucleotides.

Ex. 1001, 61:6–43 (claim 1), 62:45–65 (claim 17).

D. The Asserted Grounds of Unpatentability

Petitioner asserts that claims 1–29 are unpatentable on the following grounds:

Claims Challenged	35 U.S.C. §[1]	References/Basis
1–3, 5, 7, 9–14, 17–27, 29	103	Narayan[2] and Schmitt[3]
4, 6	103	Narayan, Schmitt, and Meyer[4]
8,	103	Narayan, Schmitt, and Craig[5]
15, 16, 28	103	Narayan, Schmitt, and Kivioja[6]

In support of these grounds, Petitioner relies upon the Declaration of Paul T. Spellman, Ph.D. (Ex. 1002), submitted with the Petition, and the Declarations of Rahul Satija, D.Phil. (Ex. 1098) and Aleksandar Rajkovic, M.D., Ph.D. (Ex. 1099), submitted with the Reply. Patent Owner relies on the Declarations of Dr. John Quackenbush (Ex. 20115) and Dr. Ian Hageman (Ex. 2016).

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[1] The Leahy-Smith America Invents Act ("AIA"), Pub. L. No. 112–29, 125 Stat. 284 (2011), amended 35 U.S.C. §103, effective March 16, 2013. The '306 patent claims priority to a series of applications the earliest of which is a provisional application filed on December 28, 2013. Ex. 1001 code (60). Because the AIA became effective before the filing of the earliest of the application to which the '306 patent claims priority, we apply the AIA version of the statute.

[2] Narayan et al., *Ultrasensitive Measurement of Hotspot Mutations in Tumor DNA in Blood Using Error-Suppressed Multiplexed Deep Sequencing*, 72(14) CANCER RES. 3492–98 (Ex. 1082) ("Narayan").

[3] Schmitt et al., WO 2013/142389 A1, published Sept. 26, 2013 (Ex. 1009) ("Schmitt").

[4] Meyer et al., *Parallel Tagged Sequencing on the 454 Platform*, 3(2) NATURE PROTOCOLS 267–78 (2008) (Ex. 1005) ("Meyer").

[5] Craig et al., *Identification of Genetic Variants Using Bar-coded Multiplexed Sequencing*, 5(10) NATURE METHODS 887–93 (2008) (Ex. 1007) ("Craig").

[6] Kivioja et al., *Counting Absolute Numbers of Molecules Using Unique Molecular Identifiers*, 9 NATURE METHODS 72–76 (2012) (Ex. 1006) ("Kivioja").

III. ANALYSIS

A. Legal Standard

A patent claim is unpatentable under 35 U.S.C. § 103 if the differences between the claimed invention and the prior art are such that the claimed invention, as a whole, would have been obvious at the time the invention was made to a person having ordinary skill in the art to which the subject matter pertains. *See KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 406 (2007). The question of obviousness is resolved on the basis of underlying factual determinations, including: (1) the scope and content of the prior art; (2) any differences between the claimed subject matter and the prior art; (3) the level of skill in the art; and (4) objective evidence of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17–18 (1966).

"[A] patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *KSR*, 550 U.S. at 418. "[I]t can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *Id.* Moreover, a person of ordinary skill in the art must have had a reasonable expectation of success of doing so. *PAR Pharm., Inc. v. TWi Pharms., Inc.*, 773 F.3d 1186, 1193 (Fed. Cir. 2014).

B. Person of Ordinary Skill in the Art

Relying on Dr. Spellman's testimony, Petitioner asserts that a person of ordinary skill in the art ("POSA") at the time of the invention would have had

> (i) a Ph.D. in molecular biology, genetics, bioinformatics, or a related field, and have at least about two years of experience in the use and development of sequencing technologies; or (ii) a

> Master's degree in one of the same fields with at least about five years of the same experience.

Pet. 18 (citing Ex. 1002 ¶¶ 27–30). Patent Owner agrees that a POSA would have had the knowledge suggested by Dr. Spellman, and its declarants apply "Dr. Spellman's definition of a POSA in their analyses." Resp. 6–7.

We adopt Petitioner's uncontested definition of the level of ordinary skill in the art. This definition is supported by the record (Ex. 1002 ¶¶ 27–30) and consistent with the disclosure in the '306 patent and references cited in the Petition.

C. Claim Construction

In an *inter partes* review, the Board applies the same claim construction standard that would be used to construe the claim in a civil action under 35 U.S.C. § 282(b). *See* 37 C.F.R. § 100(b). Under that standard, claim terms "are generally given their ordinary and customary meaning" as understood by a person of ordinary skill in the art at the time of the invention. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312–13 (Fed. Cir. 2005) (en banc).

In our Institution Decision, we noted Petitioner's assertions regarding a POSA's understanding of "z" in element 1(b) and its contention that Schmitt discloses a number of different combinations of molecular barcodes that was within the range recited in element 1(b) regardless of the z value. Dec. 14 (citing Pet. 19). Based on the record at that time, we explained that "[f]or this reason, it is unnecessary to expressly construe 'z' or any other aspect of element 1(b) to decides the issues present in the Petition." *Id.* We also noted that, at least at the institution stage, neither side had proposed any express claim construction. *Id.*

In its Response, Patent Owner asserts that "[n]o specific construction is believed necessary here to determine that the recited terms are disclosed in the cited prior art." Resp. 7. Nevertheless, Patent Owner offers a "discussion" to "provide[] context" regarding its interpretation of certain terms relating to tagging of cfDNA molecules in element 1(b). *Id.* at 7–8. According to Patent Owner, "claim construction must begin with the words of the claims themselves," and here the "language of claim 1 distinguishes the duplex tags" comprising molecular barcodes in element 1(b) "from the cfDNA molecules" to which they are attached. *Id.* at 8 (quoting *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 457 F.3d 1293, 1301 (Fed. Cir. 2006). In particular, Patent Owner points to the fact that claim 1 "expressly recites 'wherein the duplex tags are attached to both ends of a molecule of the plurality of the cfDNA molecules.'" *Id.* (quoting Ex. 1001, 61:13–14).

Petitioner does not directly reply to Patent Owner's argument that the claim language distinguishes between the duplex tags and cfDNA molecules. *See generally* Reply. Instead, Petitioner contends that Patent Owner "ignores the ['306 patent's definition of *duplex tags*." Reply 8. Moreover, Petitioner contends that the '306 patent "admits" that its methods encompass the tagging methodology, i.e., "Schmitt's hybrid tagging embodiment," it relies upon in the Petition. *Id.* at 9.

Thus, while neither party proposes any formal claim construction, some of their arguments regarding the application of Schmitt's teachings to element 1(b) present issues relating to the proper interpretation of element 1(b). We address those issues below in context of the parties' other arguments relating to element 1(b). No other claim construction is necessary for this decision.

D. Cited References

1. Narayan

Narayan is a journal article titled "Ultrasensitive Measurement of Hotspot Mutations in Tumor DNA in Blood Using Error-Suppressed Multiplexed Deep Sequencing," and bearing a 2012 publication date. Ex. 1082, 3492.[7] Patent Owner does not dispute, and we agree, that Narayan is prior art to the challenged claims here.

According to Narayan, "[d]etection of cell-free tumor DNA in the blood has offered promise as a cancer biomarker, but practical clinical implementations have been impeded by the lack of a sensitive and accurate method for quantification that is also simple, inexpensive, and readily scalable." *Id.* Narayan describes "an approach that uses next-generation sequencing to quantify the small fraction of DNA molecules that contain tumor-specific mutations within a background of normal DNA in plasma." *Id.*

2. Schmitt

Schmitt is an international patent application entitled "Methods of Lowering the Error Rate of Massively Parallel DNA Sequencing Using Duplex Consensus Sequencing," and published on September 26, 2013. Ex. 1009, code (43). Petitioner contends that Schmitt is prior art under 35 U.S.C. § 102(a)(1) and (a)(2), relying on the fact that Schmitt claims priority to Schmitt-623. Pet. 20–21. Petitioner contends that Schmitt is prior art as of the filing date of Schmitt-623, i.e., April 17, 2012, because the disclosures Petitioner relies upon in Schmitt "were carried forward from

[7] Unless stated otherwise, citations to page numbers refer to the reference page numbers.

Schmitt-623" and "Schmitt-623 provides §112 support for at least on[e] claim in Schmitt." *Id.* (internal quotations omitted). Patent Owner does not dispute these contentions and we agree that Schmitt is prior art to the challenged claims here.[8]

Schmitt describes a method called Duplex Consensus Sequencing ("DCS") that, according to Schmitt, "greatly reduces sequencing errors by independently tagging and sequencing each of the two strands of a DNA duplex." Ex. 1083, 47 (Abstr.). Because the two strands of DNA are complementary, true mutations can be found at the same position on both strands. *Id.* As Schmitt explains:

> Comparing the sequence obtained from each of the two strands comprising a single molecule of duplex DNA facilitates differentiation of sequencing errors from true mutations. When an apparent mutation is, due to a PCR or sequencing error, the substitution will only be seen on a single strand. In contrast, with a true DNA mutation, complementary substitutions will be present on both strands.

Id. ¶ 62. According to Schmitt, its DCS "method uniquely capitalizes on the redundant information stored in double-stranded DNA, thus overcoming technical limitations of prior methods of utilizing data from only one of the two strands." *Id.* at 47 (Abstr.).

In Schmitt's DCS method, double-stranded DNA molecules are ligated to single molecule identifier ("SMI") adapter molecules. Ex. 1083

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[8] The Petition cites to the disclosure in Schmitt-623, rather than Schmitt itself, as support for the asserted grounds. *See, e.g.*, Pet. 27–61 (citing Ex. 1083 to support statements regarding the teachings in Schmitt). The Preliminary Response also cites Schmitt-623 to respond to Petitioner's allegations. Prelim. Resp. 25 n.3. For ease of reference, we too cite to Schmitt-623. For purposes of our present analysis, we consider the disclosure in Schmitt-623 to be representative of the teachings in Schmitt.

¶¶ 9–10. In one embodiment, "[s]heared double-stranded DNA that has been end-repaired and T-tailed is combined with A-tailed SMI adaptors and ligated." *Id.* ¶ 11, Fig. 1. In this embodiment, "every adaptor contains a unique, double-stranded, complementary n-mer[9] random tag on each end." *Id.*; *see also* ¶ 16 (describing the embodiment in Example 1 in which "the SMI sequence is a random degenerate nucleotide n-mer sequence which is 12 nucleotides in length"). In another embodiment, Schmitt discloses a "hybrid method using a combination of sheared ends and shorter n-mer tags (such as 1 or 2 or 3 or 4 or more degenerate or semi-degenerate bases)" to "serve as unique molecular identifiers." *Id.* ¶ 30. The labeled DNA fragments are then amplified (e.g., by PCR) and sequenced. *Id.* ¶ 42

Schmitt teaches that sequence reads are "grouped into families of paired target nucleic acid strands based on a common set of SMI sequences" to produce "an error corrected double-stranded consensus sequence." Ex. 1083 ¶¶ 43, 60. This data processing is described in Schmitt's Example 1. *See id.* ¶¶ 60–69. There, "[r]eads having common (i.e., identical) SMI sequences were grouped together, and were collapsed to generate a consensus read." *Id.* ¶ 60. "PCR consensus sequences arising from two complementary strands of duplex DNA" are then identified "by virtue of the complementary SMIs" that "identify the 'partner SMI.'" *Id.* ¶ 63; *see also id.* ¶ 13, Fig. 3 (showing how sequence reads "sharing a unique set of SMI tags are grouped into paired families with members having strand identifiers in either the αβ or βα orientation"). "Following partnering of two strands by virtue of their complementary SMIs, the sequences of the strands are

[9] An n-mer is a sequence where n is the number of nucleotides. A 3-mer, for example, is a sequence of three nucleotides.

compared. Sequence reads at a given position are kept only if the read data from each of the two paired strands is in agreement." *Id.*; *see also id.* ¶¶ 60, 68 ("Sequence reads were considered only when the read data from each of the two strands is in perfect agreement").

3. Meyer

Meyer is a journal article titled "Parallel tagged sequencing on the 454 platform" and bearing a 2008 publication date. Ex. 1005, 267. Patent Owner does not dispute, and we agree, that Meyer is prior art to the challenged claims here.

Meyer relates to a method called parallel tagged sequencing that allows for parallel sequencing of large numbers of double-stranded DNA samples on a next-generation sequencing system called the 454 Platform. *Id.*, Abstr. According to Meyer, the method involves blunt end repairing each DNA sample and then ligating sample-specific barcoding adapters to both ends of blunt-end repaired DNA molecules. *Id.* at 268, Fig. 1. The adapters "comprise single self-hybridized oligos containing a sequence tag and an *Srf*I restriction site." *Id.* at 267. The barcoded samples are pooled in equimolar ratios, and untagged molecules are excluded from sequencing through dephosphorylation and restriction digestion. *Id.*, Fig. 1. The sample pool is then sequenced and the sequence reads are sorted according to their tag sequences and the source of each DNA can then be traced using the tag sequences. *Id.*

4. Craig

Craig is a journal article titled "Identification of genetic variants using bar-coded multiplexed sequencing," and bearing an October 2008 publication date. Ex. 1007, 887. Patent Owner does not dispute, and we agree, that Craig is prior art to the challenged claims here.

Craig relates to "a generalized framework for multiplexed resequencing of targeted human genome regions on the Illumina Genome Analyzer using degenerate indexed DNA bar codes ligated to fragmented DNA before sequencing" for simultaneously sequencing DNA from multiple individuals. Ex. 1007, 887. Craig refers to these bar codes as "indexes" and describes the use of "a six-base index with built-in redundancy for error correction." *Id.* According to Craig, "only 48 of the 4,096 possible nucleotide combinations" were synthesized for use in their experiment. Craig explains that this design "allowed us to control, tolerate and measure error base calling of the index" because "one, and in some cases two, sequencing errors could be tolerated without an index being incorrectly identified as being a different valid index. *Id.* at 888; *see also id.* at 13[10] (Supplementary Table 4 describing the design of the "DNA Indexes Appended to Each Adapter").

5. Kivioja

Kivioja is a journal article titled "Counting absolute numbers of molecules using unique molecular identifiers," and bearing a November 2011 publication date. Ex. 1006, 72. Patent Owner does not dispute, and we agree, that Kivioja is prior art to the challenged claims here.

According to Kivioja, [d]etermining the relative abundance of two different molecular species or the absolute number of molecules [of DNA] in a single sample is challenging." Ex. 1006, 72. Kivioja describes "an absolute counting method that can use amplification but does not require detecting each original molecule or keeping track of the number of copies made." *Id.* In Kivioja's method, "each molecule in a population is first made unique" by

[10] This refers to the page number added to the exhibit.

labeling it with a unique molecular identifier ("UMI"). *Id.* "Upon deep sequencing, each UMI will be observed multiple times, and the number of original DNA molecules can be determined simply by counting each UMI only once. However, long before all UMIs are observed, increasingly precise estimates of the absolute molecule number can be made." *Id.* (citing Online Methods).

In its Online Methods section, Kivioja teaches that "the original number of molecules in a sample can be estimated as the sum of observed and unobserved UMIs" and "[t]he number of unobserved UMIs can be estimated based on the distribution of the copy numbers of the observed UMIs." *See* Ex. 1006, 4–5[11] (explaining that "the number of molecules from each gene was estimated by fitting a zero-truncated Poisson distribution to the UMI copy number distribution using the generalized additive models for location, scale and shape (GAMLSS) R package and adding the predicted number of unobserved UMIs to the observed UMI count").

E. Ground 1: Obviousness over Narayan and Schmitt

Petitioner asserts that claims 1–3, 5, 7, 9–14, 17–27 and 29 are unpatentable as obvious over Narayan and Schmitt. Pet. 25–58. Patent Owner opposes Petitioner's assertions. Resp. 10–60.

For the reasons explained below, we find that Petitioner has not shown by a preponderance of the evidence that any of these claims would have been obvious over the asserted combination of Narayan and Schmitt. More specifically, for independent claim 1, and those claims that depend from claim 1, Petitioner has not sufficiently shown that any of these references teach or suggest "tagging a plurality of the cfDNA molecules . . .

[11] This refers to the page number added to the exhibit.

with duplex tags comprising molecular barcodes . . . wherein the duplex tags are attached to both ends of a molecule of the plurality of the cfDNA molecules," as recited in element 1(b). Ex. 1001, 61:10–14. For independent claim 17 and those claims that depend from claim 17, Petitioner has not sufficiently shown that any of these references teach or suggest "sorting a plurality of sequence reads from the set of sequence reads into (i) families comprising paired reads . . . *and* (ii) families comprising unpaired reads" as recited in element 17(d). Ex. 1001, 62:55–65 (emphasis added). Because these issues are dispositive for all of the challenged claims, our analysis below focuses on the parties' arguments and evidence pertaining to these claim elements.

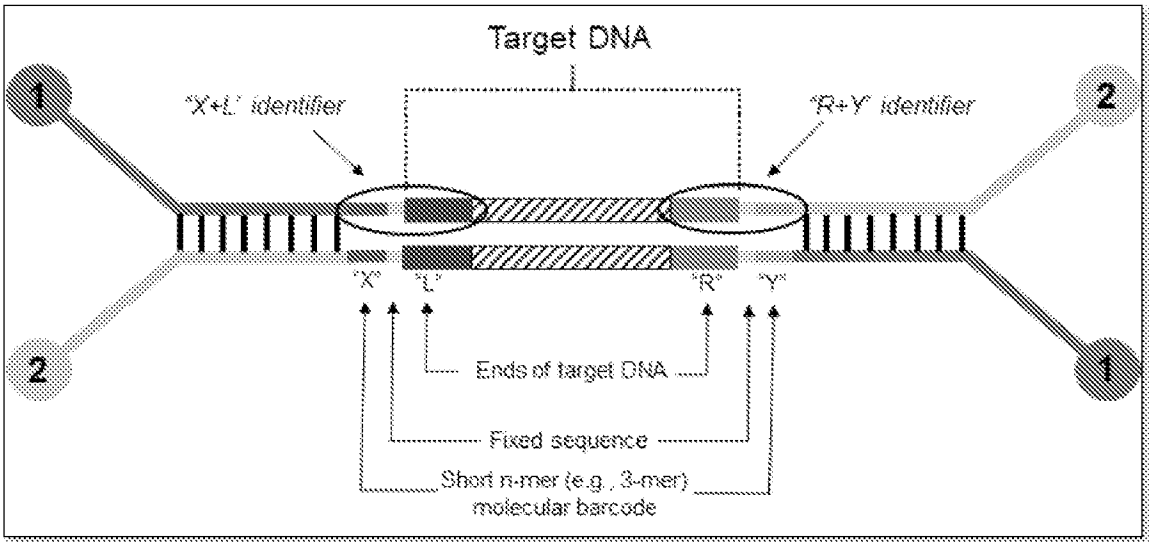
1. Analysis of Claims 1–3, 5, 7, 9–14, and 29

Petitioner contends claim 1 is rendered obvious by the application of Schmitt's DCS method to screen cfDNA for cancer as taught in Narayan. *See* Pet. 25, 34. That is, Petitioner asserts that "Narayan discloses isolating cfDNA . . . from the blood of cancer patient, sequencing it, and analyzing the sequence reads." *Id.* at 26. In the asserted combination, Petitioner contends a POSA would have substituted Schmitt's DCS method for the sequencing method used in Narayan. *Id.* at 34. According to Petitioner, "Schmitt alone" discloses all of the steps, i.e., "tagging, amplifying, sequencing, and reducing or tracking redundancy of sequencing reads," of the method in claim 1. *Id.*

Regarding element 1(b), Petitioner relies on Schmitt's teaching of a "'hybrid method' of tagging, which uses a combination of the ends of the DNA fragments and 'a shorter n-mer tag'" such as a "3-mer barcode" that "together with the sequence information within the target DNA, serve as 'unique molecular identifiers.'" Pet. at 8–9 (quoting Ex. 1083 ¶¶ 30, 47, 75

(emphasis omitted)). We refer to this as "Schmitt's hybrid method" or "Schmitt's hybrid embodiment" herein.

Petitioner's declarant, Dr. Spellman, provides the following diagram depicting Schmitt's hybrid embodiment.



Id. at 9 (citing Ex. 1002 ¶ 89). In Dr. Spellman's diagram above, "the ends of the DNA fragment are designated 'L' and 'R,' and the 3-mer molecular barcodes are labeled 'X' and 'Y.'" *Id.*

Petitioner asserts that Schmitt's hybrid embodiment discloses "'duplex tags comprising molecular barcodes' that are 'attached to both ends' of a cfDNA molecule" as those terms are recited in element 1(b). Pet. 27. Petitioner contends that "duplex tags" are "tags that differently label the complementary strands . . . of a double-stranded molecule." *Id.* (quoting Ex. 1001, 17:9–13). According to Petitioner,

> Schmitt's hybrid tag embodiment differently labels the complementary strands by tagging both ends of the molecule and relying on the asymmetrical nature of the duplex tags. And Schmitt's hybrid tag embodiment comprises molecular barcodes because the duplex tags comprise both the fragment DNA end sequences [i.e., L and R in the diagram above] and "a

> shorter n-mer tag" [i.e., X and Y in the diagram] to serve as "unique molecular identifiers."

Id. (citing Ex. 1002 ¶ 162; Ex. 1083 ¶¶ 11, 30).

For the "n different combinations of molecular barcodes" requirement of element 1(b), Petitioner points to Schmitt's disclosure of "3-mer barcodes." Pet. 28–29. Petitioner contends that this 3-mer barcode provides a total of 4,096 "different combinations of molecular barcodes, considering the tagging occurs on both ends of the target molecule." *Id.* at 28 (citing Ex. 1002 ¶ 164) (internal quotations omitted). Based on this calculation, Petitioner urges that Schmitt discloses an "n" with the recited range of "at least 2 and no more than 100,000*z" regardless of the z value for a given sample. *Id.* at 29 (citing Ex. 1002 ¶¶ 166–167).

In response, Patent Owner urges that "[t]he petition materials fail to establish the cited prior art discloses or teaches the tagging step of claim 1," i.e., element 1(b). Resp. 10. Patent Owner contends "[t]he petition materials acknowledge SMIs as the 'molecular barcodes' in Schmitt, and they acknowledge hybrid SMIs as the combination of shear sequence and exogenous n-mer sequence, but they improperly and inconsistently map only the exogenous 3-mer portion of the hybrid SMI in the discussion of the challenged claims." *Id.* (citing Pet. 28–29).

More specifically, Patent Owner argues that claim 1 does not encompass Schmitt's hybrid embodiment because "even if the hybrid SMI sequences are viewed as molecular barcodes in Schmitt's DCS process, those are not contained in adapter molecules or duplex tags that are attached to the DNA molecules." *Id.* at 18 (citing Ex. 2015 ¶ 59). According to Patent Owner, "the language of claim 1 specifies that the duplex tags are attached to the cfDNA molecules and distinguishes the 'cfDNA molecules' from the

'duplex tags" with the molecular barcodes." *Id.* (citing Ex. 2015 ¶ 60); *see also id.* at 8 (discussed *supra* § III(C)). But in Schmitt's hybrid embodiment, the SMI includes both the n-mer and a portion of the endogenous end sequence of the DNA molecule. *Id.*; *see also id.* at 16–17 (citing testimony from Dr. Spellman that Patent Owner contends "acknowledges the combination of the endogenous shear sequence and exogeneous 3-mer sequence together provide the SMI used for grouping and matching in Schmitt's DCS process"). Thus, urges Patent Owner, Petitioner's attempt to map this "amalgamation of sequences that together function as the SMI" to the duplex tag in element 1(b) "improperly conflates the DNA molecule with the attached duplex tags." *Id.* at 19 (citing Ex. 1002 ¶ 162.).

Patent Owner also asserts that Petitioner has not shown that the number of different combinations of the SMI in Schmitt's hybrid embodiment is within the recited range for "n" because Dr. Spellman's calculations only consider the nucleotides in the 3-mer tag and not those in the "shear sequences of the DNA molecule . . . included in the hybrid SMIs." *Id.* at 20–21.

In reply, Petitioner asserts that Patent Owner's arguments ignore the '306 "patent's definition of *duplex tags*" as "tags that differently label the complementary strands of a double-stranded molecule." Reply 8 (quoting Ex. 1001, 17:9–13). According to Petitioner, the "salient point" is that Schmitt teaches "tagging the double-stranded DNA molecules such that the tags *differently label the complementary strand* (i.e., 'duplex tags,' under the ']306 patent's definition."). *Id.* (citing Ex. 1002 ¶ 162; Ex. 1098 ¶¶ 38–40; Ex. 1097, 98:16–99:1). Moreover, Petitioner contends that the '306 "patent admits that tagging can be achieved using the *combination of a DNA barcode and one or more endogenous sequences* of the polynucleotide."

Reply 9 (citing Ex. 1001, 21:23–25, 21:39–42, 22:44–51, 34:55–58) (internal quotations omitted).

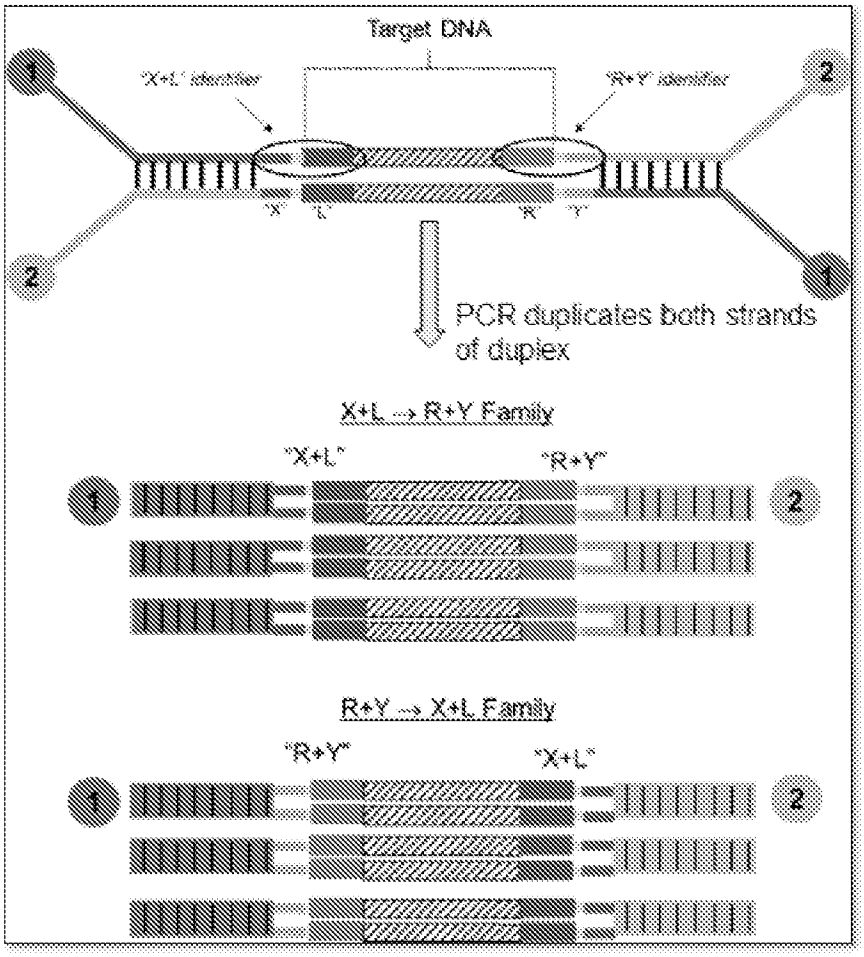
In sur-reply, Patent Owner urges that the Reply "never addresses the plain language" of claim 1 stating "that 1) the duplex tags include the molecular barcodes and 2) those duplex tags are attached to and distinguished from the 'cfDNA molecules.'" Sur-reply 3. Patent Owner argues that the functional description of duplex tags "in the specification as labeling complementary strands of cfDNA . . . in no way negates claim language placing structural limitations on duplex tags such as those recited in claim 1." *Id.* at 5.

Patent Owner further contends that Petitioner's argument that the '306 patent "admits that 'tagging' can be achieved by the combination of a DNA barcode and one or more endogenous sequences of the polynucleotide" (*see* Reply 9), "misquotes and mischaracterizes the '306 specification." Sur-reply 6. According to Patent Owner, none of the provisions Petitioner cites refer to tagging, but rather "describe molecular barcodes as exogenous sequences just like the claims." *Id.*

Having considered these arguments on the full trial record, we find Patent Owner has the better position because Petitioner's showing that element 1(b) is taught by Schmitt is unpersuasive. We begin with Patent Owner's argument concerning the distinction between the cfDNA molecules and the duplex tag comprising the molecular barcode found in the text of claim 1. Element 1(a) recites "a population of cell-free deoxyribonucleic acid (cfDNA) molecules." Ex. 1001, 61:7–9. Element 1(b) recites "tagging a plurality of the cfDNA molecules in the population" of element 1(a) "with duplex tags comprising molecular barcodes to produce tagged parent polynucleotides . . . wherein *the duplex tags are attached to both ends of a*

molecule of the plurality of the cfDNA molecules." *Id.* at 61:10–14 (emphasis added). We agree with Patent Owner and credit the testimony of its declarant that this claim language distinguishes the duplex tags from the ends of the cfDNA molecule to which they are attached. Resp. 8; Ex. 2015 ¶¶ 38–39, 60, 61. That is, the duplex tag is not part of the cfDNA molecule itself, but rather is "attached to both the ends" of that molecule by "tagging" it as recited in element 1(b).

We also agree with Patent Owner that given this distinction, claim 1 does not encompass Schmitt's hybrid embodiment. Resp. 18–20. As Petitioner notes, "[i]n Schmitt's hybrid-tag embodiment, the strands are grouped based on *the combination* of the short n-mer barcode and the end of the target DNA." Pet. 11; *see* Resp. 17 ("[T]he combination of the endogenous shear sequence and exogenous 3-mer sequence together provide the SMI used for grouping and matching in Schmitt's DCS process."). Petitioner and its declarant illustrate the grouping in Schmitt's hybrid embodiment with the following diagram. Pet. 12; Ex. 1002 ¶¶ 96–98.



Petitioner's diagram shows the PCR products produced from sequencing a "Target DNA" molecule that has been tagged with 3-mers X and Y according to Schmitt's hybrid embodiment. *See* Ex. 1002 ¶ 98 (describing diagram). A portion of the endogenous sequence of the DNA molecule labeled L and R is used in combination with the 3-mer sequence to group the resulting strands into two families labeled the "X+L→R+Y Family" and the "R+Y→X+L Family." Ex. 1002 ¶ 98; Ex. 2015 ¶ 62 (explaining that Petitioner's diagram shows that "the relied upon hybrid SMI sequences of Schmitt include sequence from the sample DNA molecule *not* contained in the adapter or the duplex tags").

Petitioner contends that Schmitt's X+L and R+Y identifiers, i.e., the SMIs, differently label the strands and therefore are the recited "duplex tags." *See* Pet. 27–28 ("duplex tags comprise *both* the fragment DNA end sequences and a shorter n-mer tag") (emphasis added) (internal quotations omitted). However, we credit Dr. Quackenbush's testimony that end sequences L and R are not "attached to both ends" of the cfDNA molecule by "tagging" it as recited in element 1(b). *See* Ex. 2015 ¶ 59–62 (explaining that Schmitt's hybrid SMI sequences include a portion of the sample DNA molecule, which is "not contained in adapter molecules or duplex tags that are attached to the DNA molecules"). Rather, end sequences L and R *are* the pre-existing, endogenous ends of that molecule. *Id.* Thus, Petitioner's theory of unpatentability does not account for the distinction the claim language draws between the cfDNA molecule and the duplex tags.

Petitioner's argument that the '306 patent defines "duplex tag" as a "tag that differently label the complimentary strands . . . of a double-stranded molecule" does not overcome this short-coming. Pet. 27; Reply 8 (citing Ex. 1001, 17:11–12). First, the same passage Petitioner cites for this definition further indicates those tags as molecules "attached to a polynucleotide" using various methods of "[t]agging" such as hybridization or ligation to attach the duplex tag to the polynucleotide. *See* Ex. 1001, 17:16–40. Thus, the Specification is consistent with the claim language in drawing a distinction between the tag and the polynucleotide to which it is attached. Second, even if we accept Petitioner's definition and agree that the combination of the end sequence and 3-mer in Schmitt's hybrid tag embodiment could be a "duplex tag," Petitioner's argument does not account for element 1(b)'s additional requirement that the duplex tag be "attached to both ends" of the cfDNA molecule.

Petitioner's argument that the '306 patent "admits that 'tagging' can be achieved using 'the *combination of a DNA barcode and one or more endogeneous sequences* of the polynucleotide" is unpersuasive because it conflates "identif[ying]" the cfDNA molecule with "tagging" it as recited in element 1(b). *See* Reply 9 (citing Ex. 1001, 21:23–25, 21:39–42, 22:44–51, 34:55–58). The Specification explains that a polynucleotide can be "uniquely *identified* in various ways," including by "a unique DNA barcode" or alternatively "by the combination of a DNA barcode and one or more endogenous sequences." Ex. 1001, 21:19–44 (emphasis added). However, none of the passages Petitioner cites describes the use of part of the polynucleotide's own sequence for purposes of identification as "tagging." To the contrary, these passages distinguish between a "tag" or "barcode," which is attached to the polynucleotide, and the endogenous sequence of polynucleotide itself. *See* Ex. 1001, 21:23–25 ("[A] polynucleotide can be uniquely identified by the combination of a DNA barcode and one or more endogenous sequences of the polynucleotide."); 21:39–42 ("Each polynucleotide in the sample can be identified by the combination of the DNA barcodes and about 10 base pair endogenous sequence on an end of the polynucleotide."); 22:44–51 ("[B]arcodes may be attached (e.g., by ligation) to individual molecules such that the combination of the barcode and the sequence it may be ligated to creates a specific sequence that may be individually tracked."); 34:55–58 (describing reads being grouped based on "various types of sequences, e.g., sequences of an oligonucleotide tag (e.g., a barcode), sequence of a polynucleotide fragments [sic], or any combinations").

Accordingly, while the Specification describes alternative embodiments that use a portion of the endogenous sequence to identify the

cfDNA molecule, Petitioner has not shown that claim 1 encompasses those embodiments given the distinction drawn in the claim language between the "duplex tags comprising molecular barcode" and the cfDNA "molecule" to which those tags are attached. Ex. 1001, 61:10–14. Indeed, it is well-settled that "claims need not be construed to encompass all disclosed embodiments when the claim language is clearly limited to one or more embodiments," as is the case here. *TIP Sys., LLC v. Phillips & Brooks/Gladwin, Inc.*, 529 F.3d 1364, 1375 (Fed. Cir. 2008); *see also Baran v. Med. Device Techs., Inc.*, 616 F.3d 1309, 1316 (Fed. Cir. 2010) ("It is not necessary that each claim read on every embodiment.").

Finally, we address a new theory Petitioner presented at the oral hearing. There, Petitioner changed its mapping of Schmitt's hybrid method to element 1(b), asserting that the alleged duplex tag did not include the ends of the cfDNA molecule in the SMI. Tr. 10:12–11:5. That is, Petitioner asserted that the duplex tag was *only* the "Y shaped adaptor," i.e., the "green and purple arms" in Petitioner's diagrams above, the "fixed sequence yellow portion," and the n-mer tag (X and Y), and did not include the ends of the target cfDNA molecule (L and R). *Id.*

We are not persuaded. First, this is an inappropriate new argument. The Petition presents a different mapping, alleging that the "duplex tag" comprises *both* the endogenous end sequence of the cfDNA molecule and the 3-mer barcode of Schmitt's hybrid tag embodiment. Pet. 27 ("the duplex tags comprise both the fragment DNA end sequences and 'a shorter n-mer tag' to serve as 'unique molecular identifiers'") (quoting Ex. 1083 ¶ 30, Ex. 1002 ¶¶ 162–163); *see also* Tr. 62:13–63:19 (Petitioner's counsel noting he was unaware of any explicit statement in the Petition that Schmitt's duplex tag comprised just the 3-mer without the end sequence). Nor did Petitioner

present this argument in its Reply in response to Patent Owner's arguments regarding element 1(b). *See* Reply 8–10. Rather, consistent with the mapping in the Petition, the Reply identifies "Schmitt's hybrid tags," i.e., the combination of the endogenous end sequence and the n-mer, as the duplex tags in element 1(b). *See* Reply 8 (urging that "duplex tags" are "tags that differently label the complementary strands," which "is precisely what Schmitt's hybrid tags do"). Because this argument was not previously presented, Patent Owner has not been afforded a sufficient opportunity to address it. Thus, Petitioner's new argument excluding the end sequences in Schmitt's hybrid tag embodiment from the alleged "duplex tags comprising molecular barcodes" has been waived.

Second, even if we were to consider it on the merits, Petitioner's new mapping for element 1(b) is inconsistent with Petitioner's allegations for element 1(e). Element 1(e) recites "reducing or tracking redundancy" in sequence reads "using at least sequencing information from the molecular barcodes of the duplex tags to determine distinct cfDNA molecules." Ex. 1001, 61:26–30. For these limitations, the Petition points to Schmitt's grouping of reads into families based on their SMI and asserts that Schmitt determines distinct cfDNA molecules based on "the complementary nature of the SMI on the two strands of the duplex." *See* Pet. 30–32 (internal quotations omitted). Thus, like Petition's mapping for element 1(b), Petitioner's allegations for element 1(e) rely on a duplex tag that includes both the 3-mer and the end sequence of the cfDNA molecule. Petitioner has not sufficiently explained, much less shown by a preponderance of the evidence, that Schmitt teaches or suggests the limitations in element 1(e) based on its new mapping of the 3-mer alone as duplex tags.

For these reasons, Petitioner has not shown by a preponderance of the evidence that the asserted combination of Narayan and Schmitt teaches or suggests element 1(b). Thus, Petitioner has not met its burden for claim 1. Petitioner has not met it burden for dependent claims 2, 3, 5, 7, 9–14, and 29 for the same reasons.

2. Analysis of Claims 17–27 and additional analysis of claim 29

Independent claim 17 differs from claim 1 in several respects, including its recitation of "sorting a plurality of sequence reads from the set of sequence reads into (i) families comprising paired reads . . . *and* (ii) families comprising unpaired reads" in element 17(d). Ex. 1001, 62:55–65 (emphasis added). Claim 29 depends from claim 1 and further limits element 1(e) by reciting that "the distinct cfDNA molecules in (e) are determined based on (i) the paired reads *and* (ii) the unpaired reads." *Id.* at 65:29–31 (emphasis added). As explained below, Petitioner has not met its burden to show that the asserted combination of references teaches or suggests sorting of reads into families of unpaired reads as recited in element 17(d) or determining distinct cfDNA molecules based on unpaired reads as recited in claim 29.

For each of these elements, the Petition relies solely on the teachings in Schmitt. *See* Pet. 41–44 (claim 17); 58 (claim 29). Petitioner contends "a POSA would have known that Schmitt discloses 'sorting' the reads into paired and unpaired reads because Schmitt discloses generating duplex consensus sequence reads (paired reads), which requires 'sorting' the reads into paired reads and unpaired reads." *Id.* at 42. As support, Petitioner points to Figures 1 and 3 and the related disclosure in Schmitt, urging that it teaches sorting reads into families based on their SMI tag that are then sorted into paired families. *Id.* at 44–45 (citing Ex. 1083 ¶¶ 43, 63, Figs 1, 3).

Petitioner also relies on its showing for element 1(e). Pet. 41, 58. There, the Petition points to the disclosure in Schmitt's "working example that 'consensus sequences were then *paired with their strand-mate*' and the 'complementary nature' of the double-stranded sequences 'was used to *identify pairs of consensus groups* that arose from complementary DNA strands." *Id.* at 32–33 (quoting Ex. 1083 ¶¶ 60, 68). Petitioner urges that "[b]y following this teaching in Schmitt, the POSA would have also determined distinct DNA molecules from sequence reads of a first tagged strand having no second complementary strand – i.e., unpaired reads." *Id.* at 33. Petitioner also references "a separate working example" found in the "Schmitt PCT," i.e., Ex. 1009. *Id.* (citing Ex. 1009 ¶¶ 120–137; Ex. 1002 ¶¶ 184–185). According to Petitioner, the data in Table 1 of Ex. 1009 shows that in this example "more than half of the total SSCS [i.e., single-strand consensus sequence] reads remained unpaired, while the other reads were paired." Pet. 33–34.

At the institution stage, we found Petitioner's showing unpersuasive in view of certain arguments in Patent Owner's Preliminary Response. *See* Dec. 30–37. Patent Owner reasserts those arguments in the Response. Resp. 55–60. In particular, Patent Owner argues that Schmitt does not disclose the sorting of unpaired reads recited in element 17(d) or the use of unpaired reads recited in claim 29. *See Id.* According to Patent Owner, "Schmitt does not identify unpaired reads, as that term is used in the challenged claims, at any step of its method." *Id.* at 57. Instead, Patent Owner contends "Schmitt describes grouping as forming families of reads that share a common SMI tag" and this process is "agnostic to whether reads are paired or unpaired." *Id.* at 57–58.

Moreover, Patent Owner points to reasons why Schmitt's identification of paired reads (i.e., reads with a common SMI tag) does not inherently results in the grouping of unpaired reads. According to Patent Owner, Schmitt describes "selecting consensus sequences based on a stringent filtering step," i.e., where there is "'perfect agreement' between SMI sequences." Resp. at 58–59 (quoting Ex. 1083 ¶¶ 60, 68). Patent Owner argues that "[s]equences lack the requisite 'perfect agreement' where a sequence error occurs in the SMI or barcode." *Id.* at 59. Patent Owner cites evidence, as well as Petitioner's own argument, to show that "this problem was widely recognized in the scientific literature as specifically a problem in Schmitt's DCS method and identified as a major cause of the data loss (e.g., >99% of tags) that Schmitt describes." *Id.* (citing Pet. 62 (relying on Ex. 1063, 2); Ex. 2013, 5–6).

Patent Owner also disputes Petitioner's reliance on the data in Table 1 of Ex. 1009, urging that it does not suggest the sorting of unpaired reads recited in element 17(d) or the use of unpaired reads recited in claim 29 because it "provides information on the basis of gross nucleotide count" as opposed to reads, i.e., "a series of nucleotides produced by sequencing a tagged strand derived from an original cfDNA molecule." Resp. 59–60.

In reply, Petitioner urges that a skilled artisan "reading Schmitt would understand from simple logic and knowledge in the art that there can only be three possible outcomes when amplifying a DNA template to generate sequence reads: (1) raw reads generated from both strands; (2) raw reads generated from the first or second strand only; or (3) no reads generated from either strand." Reply 11 (citing Ex. 1098 ¶¶ 48–50). According to Petitioner, "where Schmitt sorts sequence reads into families—either raw or consensus sequence reads—there will necessarily be groups comprising

either paired or unpaired sequence reads. Said another way, Schmitt necessarily sorts reads into paired *and unpaired reads*, because after sorting the paired reads all that remains is *unpaired reads*." *Id.* (citing Ex. 1083 ¶¶ 13, 60, 68, 71–72, Fig. 3; Ex. 1009 ¶ 128; Ex. 1002 ¶¶ 180–185, 231–237; Ex. 1098 ¶¶ 48–50).

In sur-reply, Patent Owner again points out that Petitioner's analysis does "not account for the impact of sequencing errors" and urges that the Reply does not address this deficiency. Sur-reply 25.

Having considered these arguments on the full trial record, we find Patent Owner has the better position and that Petitioner's arguments for element 17(d), and the similar element recited in claim 29, are unpersuasive. Beginning with element 17(d), the Petition points to two steps in Schmitt's DCS method for element 17(d): (1) the grouping of reads into families "by virtue of having a common (i.e., the same) SMI tag sequence;" and (2) the subsequent grouping of those families "into *paired families*" based on the orientation of their tag. *See* Pet. 43 (quotations omitted). The first of these steps groups reads based on their SMI tag without regard to whether they have a complementary sequence. Thus, it does not disclose sorting "sequence reads into paired reads and unpaired reads" as recited in element 17(d). In the second of these steps, paired families comprising complementary sequences are formed. While this may result in the sorting of reads or SSCSs into families of paired reads, Petitioner has not shown that it teaches or suggests the sorting of reads into "families comprising unpaired reads" as recited in element 17(d).

Petitioner's argument that the generation of duplex consensus sequences necessarily requires the sorting of unpaired reads is unpersuasive. *See* Pet. 42; Reply 11. Schmitt teaches that the complementary nature of its

SMI tag sequences is "used to identify pairs of consensus groups that arose from complementary DNA strands." Ex. 1083 ¶ 68. However, the Petition cites no corollary disclosure suggesting the sorting of reads into families that lack a complementary strand, nor do Petitioner or its declarants explain why the generation of duplex consensus sequences would require the sorting of sequence reads into families of unpaired reads. *See* Pet. 42; Ex. 1002 ¶ 233; Ex. 1098 ¶¶ 46-53.

Instead, the disclosures and testimony Petitioner cites show the grouping of families of reads with the same SMI into families of paired reads. *See* Pet. 42; Ex. 1002 ¶¶ 234–36. According to Schmitt, Figure 1 depicts that

> two types of PCR products are produced from each capture event. Those derived from one strand will have the α SMI sequence adjacent to flow-cell sequence 1 and the β SMI sequence adjacent to flow cell sequence 2. PCR products originating from the complementary strand are labeled reciprocally.

Ex. 1083 ¶ 11. Thus, Figure 1 shows PCR products (i.e., reads) grouped into families based on their SMI sequence with an "αβ SMI family" and the *complementary* "βα SMI family." *Id.* at 56 (Fig. 1). Schmitt Figure 3 "(a-c) shows sequence reads . . . sharing a unique set of SMI tags . . . grouped into *paired* families" and an "error-corrected consensus sequence . . . for each *duplex*" in (d). Ex. 1083 ¶ 13 (emphases added). Petitioner has not explained how either figure depicts "families of unpaired reads corresponding to sequence reads . . . having no second tagged complementary strand" as recited in element 17(d).

Petitioner's reliance on Schmitt Example 1 is also unavailing. Schmitt Example 1 describes the results of a "pilot experiment" in which "29,409

SMI partner pairs were found, indicative that fewer than 1% of tags had their corresponding partner pair tag present in the library." Ex. 1083 ¶ 68. Petitioner argues this means the "measure of unpaired reads" in that experiment was "more than 99%" and thus a POSA following these teachings would have necessarily generated consensus sequences from unpaired reads.[12] Pet. 54. We are not convinced. As an initial matter, Schmitt Example 1 does not expressly describe the sorting of reads into families of unpaired reads as recited in element 17(d). Rather, Schmitt indicates that reads (i.e., "PCR duplicates") were grouped into families based on their SMI tag, SSCSs were created from those families, and then the "complementary nature of the double-stranded SMI sequences was used to identify" and form double-stranded consensus sequences, but "only when the read data from each of the two strands [wa]s in perfect agreement." *See* Ex. 1083 ¶¶ 67–68. Petitioner appears to assume that if an SMI partner pair was not found for a tag, the SSCS associated with that tag was necessarily generated from "unpaired reads . . . having no second tagged complementary strand" as recited in element 17(d). *See* Reply 11 ("[W]here Schmitt sorts sequences reads into families—either raw or consensus sequence reads—there will necessarily be groups comprising either paired or unpaired sequence reads."). But neither Petitioner's briefing, nor the testimony Petitioner cites from its declarants, provides sufficient explanation to support that assumption. *See* Pet. 54 (citing Ex. 1002 ¶ 309); Reply 10–12 (citing Ex. 1098 ¶¶ 46–53).

[12] The Petition presents this argument for the "quantitative measures" limitations in dependent claims 14 and 27. Pet. 54. We address it here because Petitioner cites the same underlying disclosure to support its arguments for element 17(d). *See* Pet. 42 (citing Ex. 1083 ¶ 68).

This is because Schmitt explains that for at least some tagged reads the reason a partner pair was not found was that the complementary strands were not in "perfect agreement." *See* Ex. 1068 ¶ 68 (explaining that SMI partner pairs were found "after grouping of PCR duplicates" and "reads were considered only when the read data from each of the two strands is in perfect agreement"). In such instances, there would be a complementary strand albeit one with imperfect agreement to the corresponding strand, which undermines Petitioner's theory that the tags that did not form SMI partner pairs in Schmitt's working example were necessarily the result of unpaired reads.

Moreover, Patent Owner shows that a POSA would have understood that sequencing errors in the SMI result in reads that cannot be matched with their partner pair, not because of the lack of a complementary strand, but because of the error in the SMI. *See* Resp. 59–60 (Ex. 1063, 2; Ex. 2013, 5–6). Stoler[13] explains that "sequencing errors within duplex tags" were known to be "one of the main causes of data loss" in DCS. Ex. 2013, 5–6. Moreover, both Petitioner and Dr. Spellman acknowledge that "sequencing errors in the barcode itself" were known to occur and "can cause one tag to appear identical to another (crossover) or sufficiently alter a sequence tag such that it is unrecognizable (loss) and untraceable to the source material" Pet. at 64; Ex. 1002 ¶ 355 (quoting Ex. 1063, 2). In such instances, the inability to form a SMI partner pair results from the error in the SMI tag—not the lack of a complementary strand. That is, these reads are not

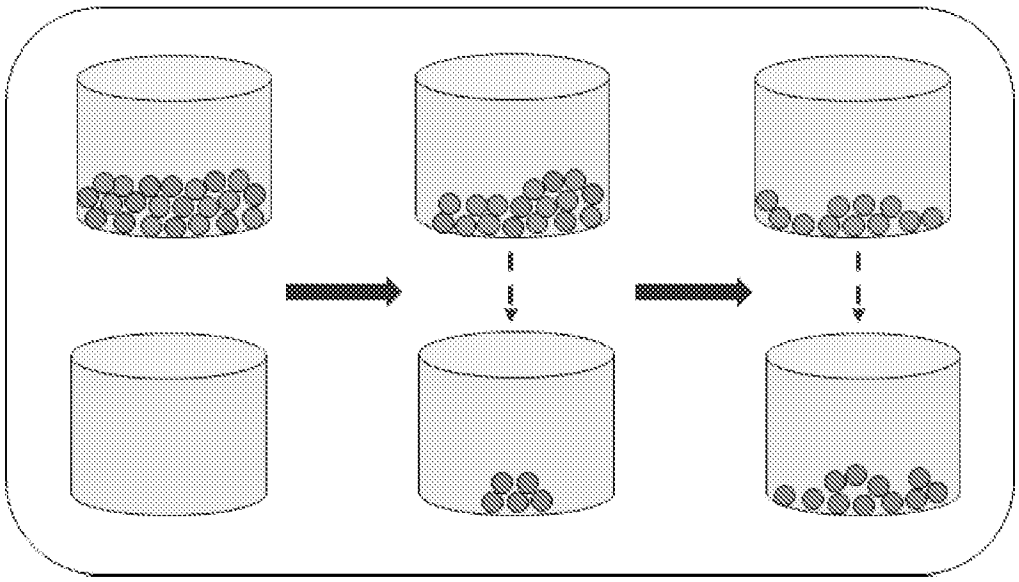
[13] Stoler et al., *Streamlined Analysis of Duplex Sequencing Data with Du Novo*, 17:180 GENOME BIOLOGY 1–10 (2016) (Ex. 2013).

"unpaired reads . . . having no second tagged complementary strand" as recited in element 17(d).

Petitioner's arguments regarding the additional example in Exhibit 1009 and the data in Table 1 are likewise unpersuasive. As Patent Owner points out, Table 1 appears to provide data based on "gross nucleotide count" for the overall experiment. Resp. 60; *see* Ex. 1009 ¶¶ 126–128. This supports Patent Owner's argument that the data reported for "reads" in the fourth and fifth rows of Table 1 is calculated "as a measure of the total number of nucleotides ('initial nucleotides') divided by the number of total 'SSCS [or DCS] nucleotides'" as opposed to "identifying paired and unpaired reads" (*id.*), which in turn would impact the "SSCS:DCS ratio" Petitioner relies upon for its arguments (*see* Pet. 33–34). Moreover, as explained above, Patent Owner cites evidence suggesting sequencing errors in the barcode and the lack of perfect agreement between complementary strands results in SSCSs not being paired to form DCS even where there is a "second tagged complementary strand" as recited in element 17(d). Ex. 1063, 2; Ex. 2013, 5–6; *see also* Ex. 1002 ¶ 355 (acknowledging that such errors "result in a sequencing read being erroneously assigned to the wrong barcode family"). Petitioner's argument and the corresponding testimony of Petitioner's declarants does not account for these issues. *See* Pet. 33–34, 44; Ex. 1002 ¶¶ 184–85, 237; Ex. 1098 ¶¶ 46–53.

The fact that Petitioner does not account for these issues undermines its argument that Schmitt necessarily teaches element 17(d) because "after sorting the paired reads, all that remains is *unpaired reads*." Reply. 11. The institution decision identified sequencing errors in the SMI as one shortcoming in Petitioner's showing for claim 17. Dec. 35–36. Petitioner's Reply does not dispute this point. *See* Reply 10–12. Instead, Petitioner and

its declarant, Dr. Satija, offer the following diagram analogizing Schmitt's sorting of reads to identify partner pairs to the sorting of red and green marbles representing paired and unpaired reads. *Id.* at 11–12 (citing Ex. 1098 ¶ 50).



According to Petitioner, the diagram above shows that "if one were to take a jar of red and green marbles [as shown on the far left] and only remove the red marbles [representing paired reads] to another jar, one would have sorted the marbles into a jar (family) of red marbles and a jar (family) of green marbles [representing unpaired reads]" as shown by the set of jars on the far right. *Id.* at 11.

The problem with Petitioner's marble analogy, and the related testimony of Dr. Satija, is that it ignores those instances where reads having a complementary strand (i.e., paired reads as recited in element 17(d) and claim 29) cannot be matched with their partner because of, e.g., a sequencing error in the SMI. Because of these and other instances where there is not perfect agreement between the strands in Schmitt's DCS method, it seems that at least some red marbles (paired reads) will remain mixed

together with the green marbles (unpaired reads).[14] For this reason, Dr. Satija's testimony on this point is unpersuasive. *See* Ex. 1098 ¶¶ 46–53. Thus, Petitioner has not shown that it is *necessarily* the case that Schmitt's DCS method will result in families of unpaired reads as recited in element 17(d) and claim 29. *See Par Pharma., Inc. v. TWI Pharms, Inc.*, 773 F.3d 1186, 1195 (Fed. Cir. 2014) (stating in an obviousness analysis, "the limitation at issue necessarily must be present, or the natural result of the combination of elements explicitly disclosed by the prior art").

Finally, Petitioner asserts that "Figure 5 of the Schmitt PCT [Exhibit 1009] demonstrates that raw sequence reads passing a quality threshold are available to be aligned to a reference genome" and therefore "a POSA would have known that the unpaired reads in Schmitt's methods *are not lost* in the process of pairing sequence reads." Reply 12 (citing Ex. 1009, Figs. 5A-B; Ex. 1098 ¶¶ 51–53). This is an inappropriate new argument. The Petition does not refer to Figure 5 of Exhibit 1009, nor is it clear how Petitioner's reliance on such is responsive to any argument in the Response. Thus, this argument has been waived. However, even if we were to consider it on its merits, Petitioner's new argument is unpersuasive because the mere fact that unpaired reads "are available to be aligned" and "are not lost" does not show sorting of such reads into families as recited in element 17(d).

For these reasons, Petitioner has not shown by a preponderance of the evidence that the asserted combination of Narayan and Schmitt teaches or suggests element 17(d). Thus, Petitioner has not met its burden for claim 17.

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[14] In this sense, the more apt comparison is the set of jars in the middle of Petitioner's diagram—where the jar on the top continues to be a mixture of red and green marbles even though some red marbles have been moved to the bottom jar.

For the same reasons, Petitioner has not met it burden for claims 18–27, which depend directly or indirectly from claim 17.

Petitioner's showing for claim 29 is similarly deficient. As explained above, the DCS method taught in Schmitt generates an "error corrected double-stranded consensus sequence." Ex. 1083 ¶¶ 43, 60. Schmitt teaches that these consensus sequences are error corrected because "[s]equence reads at a given position are kept only if the read data from each of the two paired strands is in agreement." *Id.* ¶¶ 60, 63, 68. Since only those reads having a complementary sequence that can be identified (e.g., because there is no sequencing error in the SMI) and are in complete agreement are ultimately used to generate Schmitt's duplex consensus sequences, Petitioner has not shown by a preponderance of the evidence that Schmitt's DCS method determines distinct cfDNA molecules "based on (i) the paired reads *and (ii) the unpaired reads*" as recited in claim 29. Thus, Petitioner has not met its burden for claim 29 both for this reason and the deficiency explained above for element 1(b).

F. Ground 2: Obviousness over Narayan, Schmitt, and Meyer

Petitioner asserts that claims 4 and 6 are unpatentable as obvious over Narayan and Schmitt. Claims 4 and 6 depend from claim 1 and additionally recite a minimum percentage of cfDNA molecules from the population, i.e., "at least 20%" (claim 4) and "at least 40%" (claim 6), that must be tagged with duplex tags.[15] Ex. 1001, 61:53–55, 60–62. Petitioner asserts that each limitation of claims 4 and 6 is taught by the combination of Schmitt, Meyer, and Narayan, and that a POSA would have been motivated to combine

[15] For ease of reference, we refer to these limitations collectively as the "tagging efficiency limitations."

Narayan's cfDNA with Schmitt's DCS method achieving the specified level of tagging efficiency in view of Meyer's teaching of tagging 40–60% of DNA molecules. Pet. 58–61.

Petitioner relies solely on Meyers for the tagging efficiency limitations, asserting

> A POSA would have understood that Meyer discloses that 40-60% of the DNA fragments are tagged because (i) Meyer's Figure 1 depicts the DNA fragments tagged on both ends (Fig. 1), (ii) Meyer's adapter ligation quantification "step 18" would only detect adapter-ligated DNA fragments, and (iii) Meyer expressly discloses successfully achieving at least 40-60% tagged DNA fragments.

Pet. 60 (citing Ex. 1002 ¶ 340; Ex. 1005, 274, Fig. 1). Petitioner further asserts that Meyer's disclosure of an expected recovery "between 40 and 60" falls within the ranges recited in the tagging efficiency limitations. *Id.* at 59–60.

Petitioner asserts that a POSA would have been motivated to optimize adapter-DNA ligation for high efficiency using routine methods for doing so. Pet. 60–61. Petitioner argues "a POSA would have expected to successfully tag 'at least 40%' of the DNA molecules" because "Meyer expressly discloses successfully tagging 40–60% of DNA molecules." *Id.* at 60 (citing Ex. 1002 ¶ 347; Ex. 1005, 274). And, according to Petitioner, Meyer's disclosure of 40–60% tagging efficiency is consistent with other references. *Id.* For example, Petitioner cites the KAPA data sheet,[16] which teaches "15–40% of input DNA is typically recovered as adapter-ligated

[16] KAPA Technical Data Sheet for KAPA HTP Library Preparation Kit. Ex. 1015 ("KAPA data sheet").

molecules." *Id.* (quoting Ex. 1015, 4). Petitioner also cites Fisher[17] as disclosing "recovering 47% of the library as adapter-ligated DNA fragments." *Id.* (citing Ex. 1031, Fig. 3). Finally, Petitioner asserts that a POSA "would have expected Schmitt's sticky-end adapter-DNA ligation (using A- and T-overhangs) to be even more efficient than Meyer's blunt-end adapter-DNA ligation, which nevertheless achieved 40–60% adapter-ligated DNA fragments." *Id.* at 60–61 (citing Ex. 1002 ¶ 348; Ex. 1005, 273; Ex. 1083 ¶¶ 55–56). Even if the claimed amount is not expressly taught by the art, Petitioner asserts that tagging efficiency limitations would have been an obvious optimization of a result-effective variable. *See id.* at 62 (citing Ex. 1002 ¶ 348).

At the institution stage, we found Petitioner's showing unpersuasive. *See* Dec. 39–41. We noted that Petitioner had not sufficiently explained why a POSA would have expected Meyer's tagging efficiency to apply to Schmitt's DCS method, particularly in light of evidence suggesting that tagging efficiency depends on the quality of the input DNA. *Id.* at 40–41. We also noted that Petitioner had not sufficiently shown that tagging efficiency was understood to be a result effective variable. *Id.* at 41.

Patent Owner refers to these preliminary findings in its Response, urging that Petitioner has not met its burden for claims 4 and 6 for these reasons in addition to its arguments for claim 1. *See* Resp. 61.

In reply, Petitioner urges the evidence showing lower tagging efficiency for lower quality DNA relates to "chemically damaged DNA[]—far different from cfDNA isolated from fresh blood cells." Reply 25–26

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[17] Fisher et al., *A Scalable, Fully Automated Process for Construction of Sequence-Ready Human Exome Targeted Capture Libraries*, 12:R1 GENOME BIOLOGY 1–15 (2011). Ex. 1031 ("Fisher").

(citing Ex. 1015, 2, 4; Ex. 1098 ¶¶ 96–98). Petitioner further asserts that it was "well-known . . . that significant quantities of cfDNA could be isolated from blood samples." *Id.* at 26 (citing Ex. 1002 ¶¶ 64–80).

Having considered these arguments on the full trial record, we find Petitioner's showing for the tagging efficiency limitations unpersuasive. As an initial matter, we note that Ground 2 in the instant Petition relies on nearly identical or similar arguments set forth by Petitioner in related proceedings, which we have previously rejected. *Compare, e.g.,* IPR2022-01115, Paper 2, 30–31, 35, 38, *with* Pet. 58–61. Thus, for the reasons stated in the decisions denying institution in those proceedings, we similarly find Petitioner's showing for the tagging efficiency limitations in this proceeding to be deficient. *See, e.g.*, IPR2022-01115, Paper 14, 12–19.

That is, although Meyer teaches that its particular protocol results in 40–60% tagging efficiency, Petitioner has not sufficiently explained why a person of ordinary skill in the art would have expected Meyer's tagging efficiency to apply to Schmitt's DCS method when used with Narayan's sequencing of cfDNA. For example, the Petition does not address the differences between Meyer's adapters and Schmitt's adapters and how those differences affect the tagging efficiency. Meyer's barcodes comprise "single self-hybridized palindromic oligonucleotides, carrying an *Srf* I restriction site in the middle (GCCCGGGC), a sequence tag at the 3' end and the reverse complementary tag sequence at the 5' end." Ex. 1005, Fig. 1. Schmitt, on the other hand, teaches a "hybrid method" of tagging that uses a combination of the sheared ends of the DNA fragments and a "shorter n-mer tag (such as 1 or 2 or 3 or 4 or more degenerate or semi degenerate bases) in the adaptor." Ex. 1083 ¶ 30.

Petitioner's argument that a "POSA would have expected Schmitt's sticky-end adapter-DNA litigation to be even more efficient than Meyer's blunt-end adapter-DNA ligation" is also unpersuasive. *See* Pet. 60–61. The KAPA data sheet discloses sticky-end adapter DNA ligation, but KAPA teaches that only 0.5 to 15% tagging efficiency is expected when the starting sample is 100 pg–100 ng of "high quality DNA." Ex. 1015, 4. Thus, the KAPA data suggests that the use of sticky-end adapters, such as what Schmitt discloses, does not in and of itself teach or suggest the tagging efficiency limitations.

Moreover, the record shows that tagging efficiency depends on a variety of other factors, including the quality of the input DNA. The KAPA data sheet states that tagging efficiency "can be significantly lower for DNA of lower quality." Ex. 1015, 4. Fisher also explains that "the efficiency of adaptor ligation varies between samples, probably because of variation in input DNA quality." Ex. 1031, 5–6.

Petitioner's argument that the DNA the KAPA data sheet refers to as "lower quality" is "from *formalin-fixed, paraffin-embedded* ('FFPE') samples (well known for having chemically damaged DNA)" is unavailing. *See* Reply 25–26 (citing Ex. 1098 ¶¶ 96–98). First, neither Petitioner, nor its declarant, address the similar teaching in Fisher. Second, even if the specific type of lower quality DNA in the KAPA data sheet is different, the salient point, i.e., that tagging efficiency depends on DNA quality, remains unrebutted. This is significant because Petitioner does not sufficiently address how the quality of the cfDNA of Narayan compares to the quality of the genomic DNA samples of Meyer and Schmitt or how that would affect the expected tagging efficiency. Petitioner's assertion that "significant quantities of cfDNA could be isolated from blood samples" (*see* Reply 26),

does not overcome this shortcoming. Even if we credit that assertion, the fact that a significant quantity of cfDNA could be obtained does not show that the quality of that DNA is similar such that a POSA using such would have expected to achieve a tagging efficiency similar to that in Meyer.

Finally, Petitioner has not shown "that adapter-DNA tagging efficiency is a *result-effective variable* that only requires routine skill to optimize." Pet. 60 (citing Ex. 1002 ¶¶ 348). Dr. Spellman states "it was understood that increasing the concentration of adapters was a routine technique that could be used to maximize ligation efficiency such as by using a molar excess of adapters relative to DNA fragments in the ligation reaction." Ex. 1002 ¶ 263. But, as we preliminarily found at the institution stage, the other evidence Petitioner relies upon suggests that achieving a particular level of tagging efficiency is not as simple as varying the concentration of adapters and is also affected by other factors, e.g., the amount of starting sample and the quality of the DNA. Dec. 41 (citing Ex. 1015, 4; Ex. 1031, 6). As explained above, Petitioner's Reply does not sufficiently address these issues, much less demonstrate that a POSA would have understood that the tagging efficiency recited in claims 4 and 6 could be achieved by increasing the adapter concentration through routine optimization.

For these reasons, Petitioner has not shown by a preponderance of the evidence that the tagging efficiency limitations would have been obvious over the asserted combination of Narayan, Schmitt, and Myer. In addition, claims 4 and 6 depend from claim 1 and Petitioner's Ground 2 relies on the same showing for element 1(b) in Ground 1. *See* Pet. 60 (incorporating "[t]he rationales for obviousness of claims 1 and 5"). As explained above,

that showing is deficient. Thus, Petitioner has not met its burden to show that claims 4 and 6 are unpatentable.

G. Ground 3: Obviousness over Narayan, Schmitt, and Craig

Petitioner asserts that claim 8 would have been obvious over Narayan, Schmitt, and Craig. Pet. 61–63. Claim 8 depends from claim 1 and Petitioner's Ground 3 relies on the same showing for element 1(b) in Ground 1. *See* Pet. 62 (incorporating "[t]he rationales for obviousness of claim 1). That is, Petitioner's contentions regarding element 1(b) do not rely on any teaching in Craig.

As explained above, Petitioner's showing for element 1(b) based on Narayan and Schmitt is deficient. Thus, Petitioner has not shown by a preponderance of the evidence that claims 8 is unpatentable.

H. Ground 4: Obviousness over Narayan, Schmitt, and Kivioja

Petitioner asserts that claims 15, 16, and 28 would have been obvious over Narayan, Schmitt, and Kivioja. Pet. 63–69. Claims 15 and 16 depend from claim 1 and Petitioner's Ground 4 relies on the same showing for element 1(b) in Ground 1. *See generally* Pet. 63–69. Claim 28 depends from claim 17 and Petitioner's Ground 4 relies on the same showing for element 17(d) as in Ground 1. *Id.* That is, Petitioner does not rely on any teaching in Kivioja for its contentions regarding elements 1(b) and 17(d). *Id.*

As explained above, Petitioner's showing for elements 1(b) and 17(d) based on Narayan and Schmitt is deficient. Thus, Petitioner has not shown by a preponderance of the evidence that claims 15, 16, and 28 are unpatentable.

IV. PATENT OWNER'S MOTION TO STRIKE

Patent Owner moves to strike the declarations of Dr. Satija (Ex. 1098) and Dr. Rajkovic (Ex. 1099). MTS 9. According to Patent Owner, Petitioner

"improperly attempt[s] to replace its original expert, Dr. Spellman" with testimony from these "replacement witnesses to backfill identified deficiencies" in the Petition and offer "perfunctory testimony . . . often conflicting with earlier deposition testimony" from Dr. Spellman. *Id.* at 1–3. Patent Owner further contends that Petitioner relies on their testimony to introduce new theories in the Reply, including a "new 'developmental research testing' motivation" for combining Narayan and Schmitt and new claim constructions. *See id.* at 4–8.

Patent Owner also moves to strike Exhibits 1093–1095 and 1102–1105, which it characterizes as "newly introduced exhibits that are primarily attempts to backfill content missing from the petition case, irrelevant . . . and/or not properly responsive to argument made in the" Response. *Id.* at 8–9. For these reasons, Patent Owner contends all of these exhibits "should be stricken or given no weight." *Id.* at 9.

Petitioner opposes Patent Owner's motion, urging that "neither the Reply nor Reply evidence raise[s] new theories for proving Petitioner's prima facie case," but rather are "directly responsive to arguments in the" Response. MTS Opp. 1. Petitioner explains it "encountered scheduling hurdles with Dr. Spellman during Petitioner's reply period." *Id.* According to Petitioner, "neither Dr. Satija nor Dr. Rajkovic 'replaced' Dr. Spellman," but rather "provide rebuttal opinions directly responding to arguments raised in the Quackenbush and Hagemann declarations." *Id.* at 2. Petitioner further contends that its Reply materials do not raise new theories, but are properly responsive to issues raised in the Reply and institution decision. *See* 3–10. Finally, Petitioner argues that Patent Owner is not prejudiced because it has been able to cross-examine Drs. Satija and Rajkovic and brief these issues in the Sur-Reply. *Id.* at 10.

Having considered the parties' arguments, Patent Owner's motion to strike is denied. We accept Petitioner's explanation that there were scheduling difficulties arising from Dr. Spellman's acceptance of a new position at an out-of-state academic institution that prevented his submission of declaration with the Reply. *See* MTS Opp. 1. Patent Owner's arguments that testimony offered by Drs. Satija and Rajkovic is "perfunctory" or conflicts with admissions in Dr. Spellman's earlier deposition testimony, at most, relate to the weight that testimony should be afforded, not its admissibility. Therefore, those arguments are not a sufficient basis for striking these declarations from the record.

Patent Owner's assertion that the Satija and Rajkovic Declarations, and other Reply materials, improperly introduce new theories requiring that those declarations and materials be stricken from the record is unpersuasive. *See* MTS 4–9. "In most cases, the Board is capable of identifying new issue or belatedly presented evidence when weighing the evidence at the close of trial, and disregarding any new issues or belated presented evidence that exceeds the proper scope of reply." Consolidated Trial Practice Guide, Nov. 2019, 80 ("TPG").[18] Accordingly, "striking the entirety or a portion of a party's brief," or in this case the entirety of both of Petitioner's Reply declarations and a number of related exhibits, is an "exceptional remedy." *Id.* Patent Owner has not shown that such a remedy is warranted on the circumstances of this case. We have identified those issues pertaining to our analysis that exceed the proper scope of reply and addressed them accordingly in our analysis of the merits above. We have also considered the

[18] available at https://www.uspto.gov/sites/default/files/documents/tpgnov.pdf

credibility of Drs. Satija and Rajkovic's testimony within the context of the record as a whole and afforded it appropriate weight in our analysis.

Accordingly, Patent Owner's motion to strike is denied.

V. PATENT OWNER'S MOTION TO EXCLUDE

Patent Owner moves to exclude four categories of exhibits, which it characterizes as follows: (1) "exhibits related to other proceedings" (Exhibits 1069, 1074, 1078, 1079, and 1085–1088); (2) "uncited exhibits" (Exhibits 1010, 1013, 1016, 1017, 1029, 1030, 1035–1037, 1042–1045, 1058, 1061, 1062, 1065, 1068, 1070, 1072, 1075–1077, 1080, 1081, and 1104); (3) "exhibits in support of untimely reply arguments" (Exhibits 1093–1095, 1102, 1103, and 1105); and (4) "replacement expert declarations" (Exhibits 1098 and 1099). *See* MTE 1–4. Petitioner opposes Patent Owner's motion. *See* MTE Opp. 1–15. We address each category below.

A. Exhibits 1069, 1074, 1078, 1079, and 1085–1088

Patent Owner objects to these exhibits under Federal Rules of Evidence (FRE) 106, 401, 402, and 403, asserting they should be excluded because they are "briefing and evidence filed in other cases and court statistics" and "cannot be used to prove any fact relevant to resolution of this case." MTE 1.

Petitioner explains that these exhibits pertain to particular issues in the Petition, i.e., "discuss[ion] of discretionary denial under 35 U.S.C. § 314(a)" and "Patent Owner's previous arguments regarding objective indicia," and therefore meet the threshold for relevancy. MTE Opp. 1–4.

We do not rely on any of these exhibits in rendering our decision on Petitioner's grounds. Therefore, we dismiss as moot Petitioner's motion to exclude Exhibits 1069, 1074, 1078, 1079, and 1085–1088.

B. Exhibits 1010, 1013, 1016, 1017, 1029, 1030, 1035–1037, 1042–1045, 1058, 1061, 1062, 1065, 1068, 1070, 1072, 1075–1077, 1080, 1081, and 1104

Patent Owner objects to these exhibits under FRE 401, 402, and 403, asserting they should be excluded because they "were not cited in the petition or in any other briefing." MTE 2.

Petitioner explains that these exhibits were cited by Dr. Spellman and Dr. Satija in their declarations and properly identified in the exhibit lists included with Petitioner's briefing. *See* MTE Opp. 5–6. According to Petitioner, while Petitioner "does not rely on these exhibits to meet claim elements or to prove its *prima facie* case," they are relevant to "the background knowledge of a POSA" and "general knowledge in the art." *Id.* at 5–6.

Patent Owner's argument that these exhibits should be excluded is unpersuasive. Here, Dr. Spellman and Dr. Satija rely on the identified exhibits in their declarations. As such, they are relevant to understanding the weight to be given the declarants' testimony. Accordingly, we deny Petitioner's motion to exclude Exhibits 1010, 1013, 1016, 1017, 1029, 1030, 1035–1037, 1042–1045, 1058, 1061, 1062, 1065, 1068, 1070, 1072, 1075–1077, 1080, 1081, and 1104.

C. Exhibits 1093–1095, 1102, 1103, and 1105

Patent Owner objects to these exhibits under FRE 401, 402, and 403, asserting they should be excluded because they are cited in support of untimely arguments in the Reply. MTE 3. More particularly, Patent Owner argues these exhibits could have been presented with the Petition and that Petitioner improperly relies on them to try to "fill[] a gap in the *prima facie* case that Patent Owner identified in its response to the petition." *Id.* at 3–4.

Petitioner asserts that "Patent Owner's argument regarding the scope of reply evidence is improper in a motion to exclude." MTE Opp. 8. In addition, Petitioner's motion fails on its merits because these exhibits properly respond to arguments raised by Patent Owner and its declarants in the Response. *See id.* at 9–10.

We agree with Petitioner. As explained above, we deny Patent Owner's motion to strike these exhibits and the related testimony of Drs. Satija and Rajkovic. The motion to exclude reraises the same arguments we have already found unpersuasive for the motion to strike. Accordingly, Patent Owner's motion to exclude exhibits 1093–1095, 1102, 1103, and 1105 is denied.

D. Exhibits 1098 and 1099

Patent Owner objects to these exhibits under FRE 401, 402, 403, 701, 702, and 703, asserting that the declarations of Drs. Satija and Rajkovic "should be excluded as based on legally erroneous claim constructions, representing the testimony of improper replacement witnesses, and as non-responsive under [37 C.F.R. §] 42.23." MTE 4–8. Patent Owner also asserts that Dr. Satija's declaration is "not 'the product of reliable principles' and methods 'reliably applied' to the facts of this case" because it "is predicated on erroneous legal standards for claim construction." *Id.* at 5.

Petitioner argues that "the majority of Patent Owner's Motion to Exclude is a re-briefing of its Motion to Strike" and should be rejected for the same reasons. MTE Opp. 13. Petitioner further asserts that Dr. Satija did not rely on an erroneous legal standard, but rather "[i]n responding to Dr. Quackenbush's opinions, Dr. Satija interpreted the claim terms based on their plain and ordinary meaning *in light of the specification* just as *Phillips*

[*v. AWH Corp.*, 415 F.3d 1303 (Fed. Cir. 2005) (en banc)] instructs." *Id.* at 12 (citing Ex. 1098 ¶¶ 24–37).

We agree with Petitioner that Patent Owner has not presented a sufficient basis for excluding the declarations of Drs. Satija and Rajkovic. As an initial matter, we agree that Patent Owner's motion to exclude largely reraises the same arguments in the motion to strike. Those arguments are unpersuasive for the reasons explained above.

Patent Owner's argument that Dr. Satija's testimony should be excluded under FRE 702 because he applied legally erroneous claim constructions is also unpersuasive. Dr. Satija offers testimony regarding his understanding of how the cited prior art relates to the challenged claims. *See, e.g.*, Ex. 1098 ¶¶ 24–45. While some of that testimony is unpersuasive, particularly as it relates to Petitioner's arguments that we have addressed and rejected in our analysis above, Patent Owner's arguments for exclusion go to the weight that should be accorded that testimony, not its admissibility. *See* PTAB Consolidated Trial Practice Guide (Nov. 2019) 79 ("A motion to exclude must explain why the evidence is not admissible (e.g., relevance or hearsay) but may not be used to challenge the sufficiency of the evidence to prove a particular fact."). As it relates to our findings and analysis above, we have afforded appropriate weight to the testimony of Drs. Satija and Rajkovic in view of the record as a whole.

Accordingly, Patent Owner's motion to exclude Exhibits 1098 and 1099 is denied.

VI. CONCLUSION

Petitioner has not shown by a preponderance of the evidence that any of claims 1–29 of the '306 patent are unpatentable. Moreover, Patent Owner's motions to strike and exclude are denied.

Claims	35 U.S.C. §	Reference(s)/ Basis	Claims Shown Unpatentable	Claims Not shown Unpatentable
1–3, 5, 7, 9–14, 17–27, 29	103	Narayan, Schmitt		1–3, 5, 7, 9–14, 17–27, 29
4, 6	103	Narayan, Schmitt, Meyer		4, 6
8	103	Narayan, Schmitt, Craig		8
15, 16, 28	103	Narayan, Schmitt, Kivioja		15, 16, 28
Overall Outcome				1–29

VII. ORDER

Accordingly, it is:

ORDERED that Petitioner has not shown that claims 1–29 of U.S. Patent 11,149,306 B2 are unpatentable;

FURTHER ORDERED that Patent Owner's Motion to Strike (Paper 29) is denied;

FURTHER ORDERED that Patent Owner's Motion to Exclude (Paper 32) is denied; and

FURTHER ORDERED that, because this is a Final Written Decision, parties to this proceeding seeking judicial review of our Decision must comply with the notice and service requirements of 37 C.F.R. § 90.2.

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NOTE: This order is nonprecedential.

United States Court of Appeals for the Federal Circuit

TWINSTRAND BIOSCIENCES, INC.,
Appellant

v.

GUARDANT HEALTH, INC.,
Appellee

2024-1626

Appeal from the United States Patent and Trademark Office, Patent Trial and Appeal Board in No. IPR2022-01400.

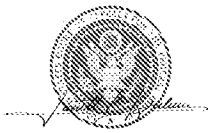
O R D E R

The parties having so agreed, it is ordered that:

(1) The proceeding is DISMISSED under Fed. R. App. P. 42 (b).

(2) Each side shall bear their own costs.

FOR THE COURT



Jarrett B. Perlow
Clerk of Court

June 10, 2024
Date

ISSUED AS A MANDATE: June 10, 2024