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

DETAILED ACTION

2. This office action is in response to an amendment filed August 19, 2015. Claims 1-21 were previously pending, with claims 4-6 and 8 withdrawn from consideration. Applicant amended claims 1, 17 and 21. Claims 1-3, 7 and 9-21 are under consideration.

3. Applicant's amendments overcame the rejection of claim 21 under 35 U.S.C. 102(a)(1) as being anticipated by Grothues et al. and the rejection of claim 21 under 35 U.S.C. 102(a)(1) as being anticipated by McCloskey et al. All other previously presented rejections are maintained for reasons given in the "Response to Arguments" section below.

4. This office action contains new grounds for rejection necessitated by amendment.

Information Disclosure Statement

5. The information disclosure statement (IDS) submitted on August 19, 2015 was filed after the mailing date of the non-final office action on May 20, 2015. The submission is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement is being considered by the examiner.

Response to Arguments

6. Applicant's arguments filed August 19, 2015 have been fully considered but they are not persuasive.

A) Regarding the rejection of claim 21 under 35 U.S.C. 101 Applicant argues the following:

"Solely for expediting prosecution, claim 21 has been amended to further recite oligonucleotide primers, reagents for amplification, instructions, and the limitations of the plurality of adaptors of instant claim 1. Instant claim 21 is drawn to a unique grouping of specific oligonucleotides. The plurality of adaptors claimed in the kit comprise an indexing primer binding site, an indexing site, an identifier site comprising at least 8 nucleotides, and a target sequencing primer binding site. The indexing site is unique amongst a subset of the adaptors and is an index for multiple polynucleotides, and the sequence of the identifier site is not conserved across all adaptor sequences. The instantly claimed plurality of adaptors cannot be equated to corresponding sequences occurring in nature. Furthermore, the amended claim now reads on a kit that cannot simply be interpreted as any composition of oligonucleotides. Accordingly, withdrawal of the rejection is respectfully requested."

However, all of these other added elements are products on nature themselves (such as primers) or are elements which are well-understood, routine and conventional elements used in the kits for methods of nucleic acid amplification and sequencing (see Bignell et al., US 2009/0233802A1, cited below). Therefore, the new limitations do not amount to "significantly more" than the judicial exceptions of adapters and primers.

The rejection is maintained.

B) Regarding the rejection of claims 1-3, 7 and 9-20 under 35 U.S.C. 103 as being unpatentable over Samuels et al. and McCloskey et al., Applicant argues the following:

"Samuels *et al.* do not teach (1) the inclusion of an identifier site comprising at least 8 nucleotides, wherein the identifier site is not conserved across all adaptor sequences; (2) the indexing site as unique amongst only a subset of the nucleic acid fragments; (3) a target sequence primer binding site; and (3) the detection and removal of duplicate sequencing reads.

McCloskey *et al.* disclose a method of identifying and removing duplicate reads through incorporating an adaptor comprising a batch stamp, a barcode, a primer-binding site for PCR, and extra bases that serve to minimize nonspecific annealing, wherein the barcodes comprise 7 random nucleotides (p. 763, Fig. 1; p. 764, para 2; p. 766, para 4-5).

McCloskey *et al.* does not teach the method wherein the identifier site (barcode) comprises at least 8 nucleotides or a target sequence primer binding site.

The Examiner alleges McCloskey *et al.* accounts for the deficiencies of Samuels *et al.* Applicant respectfully disagrees. Samuels *et al.* and McCloskey *et al.,* alone or in combination fail to teach an indexing site that is at least 8 nucleotides in length, as recited in the instant claims. McCloskey *et al.* only discloses barcodes that are 7 nucleotides in length, which limit the total number of adaptor sequences comprising unique barcodes that can be generated. The instant claims recite indexing sites of at least 8 nucleotides in length, which allows for a significantly greater population of adaptor sequences comprising unique barcodes that can be generated."

However, Samuels et al. specifically teach unique N-mers which contain barcodes 2-21 nucleotides in length (page 3, [0029]. Samuels et al. also specifically teach that barcodes contain 4-20 nucleotides which uniquely encode library members (page 15, [0209]) and further

specifically teach that the number of unique barcodes depends on the number of bases in a barcode, with 6, 7, 8 10 or 15 nucleotides resulting in unique identification of 4096, 16384, 65356, about one million and over than one billion libraries (page 15/16, [0217]). Therefore, since McCloskey et al. teach that barcodes can be used to filter out duplicate reads, and Samuels et al. teach the required lengths of barcodes, the rejection is maintained.

Claim Rejections - 35 USC § 112

7. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

8. Claim 21 is rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claim 21 was amended to read "A kit comprising a plurality of double-stranded adaptors…". However, there is no support in the specification or in the originally filed claims for the limitation of an adapter being double-stranded, therefore it represents new matter.

Claim Rejections - 35 USC § 101

9. 35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

10. Claim 21 is rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. Claim(s) 21 is directed to a kit comprising a plurality of adaptors, i.e., nucleic acid sequences, comprising an indexing primer binding site, an indexing site, an identifier site and a target sequencing binding site, the kit further comprising one or more oligonucleotide primers, reagents for amplification, and instructions for employing the kit; wherein the plurality of adaptors, one or more oligonucleotide primers, and reagents for amplification are separately contained.

No specific sequences of the adapters and oligonucleotide primers are claimed; therefore the claim is drawn to a plurality of oligonucleotides which have corresponding sequences occurring in nature. The claim(s) does/do not include additional elements that are sufficient to amount to significantly more than the judicial exception because the other elements of the kit are well-understood, routine and conventional in the art. Specifically, "reagents for amplification" are not specified, therefore could encompass water and salt solutions, for example. It is conventional in the art to include instructions for using a kit, as well as to partition components of a kit into separate containers (see, for example, US 2009/0233802 A1, [0103]-[0107]; US 5,384,242 A; issued January 1995; col. 11, lines 61-68; col. 12, lines 1-13). Therefore, the

additional elements recited in claim 21 do not amount to "significantly more" than the claimed judicial exceptions of nucleic acid sequences of adapters and primers.

In conclusion, claim 21 is not patent eligible under 35 U.S.C. 101.

Claim Rejections - 35 USC § 103

11. 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.

12. 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 of this title, 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.

13. Claims 1-3, 7 and 9-20 are rejected under 35 U.S.C. 103 as being unpatentable over Samuels et al. (US 2012/0220494 A1; filed February 16, 2012 and published August 30, 2012; previously cited) and McCloskey et al. (Biochem. Genet., vol. 45, pp. 761-767, 2007; previously cited).

A) Regarding claims 1 and 7, Samuels et al. teach a method for detecting a duplicate sequencing read from a population of sample sequencing reads comprising:

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

(i) an indexing primer binding site;

(ii) an indexing site;

(iii) an identifier site comprising at least 8 nucleotides; and

(iv) a target sequence primer binding site (page 14, [0205]; page 15, [0209], [0212], [0216]-[0217]; Fig. 6; page 16, [0231]-[0232]; page 17, [0232]-[0235]; page 16/17, [0245]; where the adaptors have an indexing primer binding site, an indexing site, an identifier site and a target sequence primer binding site; Samuels et al. teach identifier site between 2 and 21 nucleotides in length, or from 5-15 nucleotides (page 3, [0029]; page 15/16, [0217]). The indexing site and identifiers are both unique to subsets of polynucleotides are not conserved among all adaptor sequences, since they constitute random nucleotides);

b) amplifying the adapter-nucleic acid fragment ligated products (page 22, [0288]-[0289]; page 25, [0326]-[0327]);

c) generating a population of sequencing reads from amplified adapter-nucleic acid fragment ligated products (page 25, [0326]-[0328]; page 26, [0328]-[0337]; and

d) detecting the population of sequencing reads comprising a sequencing read with a duplicate identifier site and target sequence (page 26/27, [0328]; page 27, [0339]-[0341]; Fig. 43 and 44; where sequencing errors due to PCR are detected).

Regarding claim 3, Samuels et al. teach sequencing the identifier site with the indexing site (Fig. 43; page 27, [0339]-[0341]).

Regarding claim 9, Samuels et al. teach barcoding multiple samples (page 6, [0122]; page 14, [0202]).

Regarding claims 13 and 14, Samuels et al. teach DNA and RNA fragments, and genomic DNA (page 2, [0017]).

Regarding claims 17 and 18, Samuels et al. teach identifier site between 2 and 21 nucleotides in length, or from 5-15 nucleotides (page 3, [0029]; page 15/16, [0217]).

Regarding claim 19, Samuels et al. teach indexing primer site being a universal primer site (page 15, [0216]).

Regarding claim 20, Samuels et al. teach target sequence binding site being a universal binding site (page 15, [0216]).

B) Samuels et al. teach detection of PCR-introduced errors in sequencing, but do not specifically teach detecting duplicate sequencing reads.

C) Regarding claim 1, McCloskey et al. teach attaching an adaptor containing an indexing primer binding site, an indexing site, an identifier site and a target sequence binding site to genomic DNA by amplification, PCR-amplifying the resulting construct and sequencing (Fig. 1; page 762, paragraphs 3-5; page 763; page 764, paragraphs 1-4).

Regarding claims 1, 2 and 10-12, McCloskey et al. teach sequencing the PCR products containing the adapter sequences, grouping the sequencing reads based on the batch stamp and barcode and detecting and removing duplicate reads (page 766, fourth and fifth paragraphs).

Regarding claims 15 and 16, McCloskey et al. teach indexing site between six and eight bp (page 764, second paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to have used the batch-stamp and barcode-encoded oligonucleotides as suggested by McCloskey et al. in the method of Samuels et al. to eliminate PCR-originating artefacts. As stated by McCloskey et al. (Abstract):

"Polymerase chain reaction (PCR) has become the mainstay of DNA sequence analysis. Yet there is always uncertainty concerning the source of the template DNA that gave rise to a particular PCR product. The risks of contamination, biased amplification, and product redundancy are especially high when limited amounts of template DNA are used. We have developed and applied molecular encoding principles to solve this source-uncertainty problem for DNA sequences generated by standard PCR. Batch-stamps specify the date and sample identity, and barcodes detect template redundancy. Our approach thus enables classification of each PCR-derived sequence as valid, contaminant, or redundant, and provides a measure of sequence diversity. We recommend that batch-stamps and barcodes be used when amplifying irreplaceable DNAs and cDNAs available for forensic, clinical, single cell, and ancient DNA analyses."

14. Claim 21 is rejected under 35 U.S.C. 103 as being unpatentable over Samuels et al. (US 2012/0220494 A1; filed February 16, 2012 and published August 30, 2012; previously cited) and McCloskey et al. (Biochem. Genet., vol. 45, pp. 761-767, 2007; previously cited), Bignell et al. (US 2009/0233802 A1; published September 2009), Oakes (US 5,384,242 A; issued January 2005) and Stratagene Catalog (page 39, 1988).

A) Samuels et al. and McCloskey et al. teach the double-stranded adapters, but do not teach kits or the additional components recited in claim 21.

B) Regarding claim 21, Bignell et al. teach a kit comprising adapters, amplification primers, amplification reagents and sequencing primers (page 11, paragraphs [0104]-[0107]). Oakes teaches kits comprising primers, probes and amplification reagents in separate containers, as well as intructions for use of the kit.

Therefore it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to have packaged the components necessary for the method of Samuels et al. and McCloskey et al. into a kit as suggested by Bignell et al. and Oakes. The motivation for using kits is provided by Stratagene Catalog:

"Each kit provides two services: 1) a variety of different reagents have been assembled and pre-mixed specifically for a defined set of experiments. Thus one need not purchase gram quantities of 10 different reagents, each of which is needed in only microgram amounts, when beginning a series of experiments. When one considers all of the unused chemicals that typically accumulate in weighing rooms, desiccators, and freezers, one quickly realizes that it is actually far more expensive for a small number of users to prepare most buffer solutions from the basic reagents. Stratagene provides only the quantities you will actually need, premixed and tested. In actuality, the kit format saves money and resources for everyone by dramatically reducing waste. 2) The other service provided in a kit is quality control" (page 39, column 1).

15. No claims are allowed.

Conclusion

16. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). 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 date of this final action.

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

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

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

TERESA E STRZELECKA
Primary Examiner
Art Unit 1637

/TERESA E STRZELECKA/
Primary Examiner, Art Unit 1637
September 21, 2015

Notice of References Cited	Application/Control No. 14/540,917	Applicant(s)/Patent Under Reexamination AMORESE ET AL.	
	Examiner TERESA E. STRZELECKA	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-2009/0233802 A1	09-2009	Bignell; Helen	C12Q1/6874	506/2
*	B	US-5,384,242 A	01-1995	Oakes; Fred T.	C12Q1/6858	435/6.1
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Stratagene Catalog, page 39 (1988)
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14540917	AMORESE ET AL.
	Examiner	Art Unit
	TERESA E STRZELECKA	1637

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q1/6869 | C12Q2563/179 | C12Q2535/122 |C12Q2537/1376 | C12Q2563/159 | C12Q1/6806 |C12Q2521/319 | C12Q2521/507 | C12Q2525/15 |C12Q2525/155 | C12Q2525/161 | C12Q2525/185 |C12Q2525/191 | C12Q2525/197	5/13/15	/TS/

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
IDS (12/4/14; 3/21/15)	5/13/15	/TS/
WEST	5/13/15	/TS/
Google	5/13/15	/TS/
Inventor name search	5/13/15	/TS/
IDS (8/19/15)	9/21/15	/TS/

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14540917	AMORESE ET AL.
	Examiner	Art Unit
	TERESA E STRZELECKA	1637

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/14/2015	09/21/2015							
	1	✓	✓							
	2	✓	✓							
	3	✓	✓							
	4	N	N							
	5	N	N							
	6	N	N							
	7	✓	✓							
	8	N	N							
	9	✓	✓							
	10	✓	✓							
	11	✓	✓							
	12	✓	✓							
	13	✓	✓							
	14	✓	✓							
	15	✓	✓							
	16	✓	✓							
	17	✓	✓							
	18	✓	✓							
	19	✓	✓							
	20	✓	✓							
	21	✓	✓							

Electronic Acknowledgement Receipt

EFS ID:	24457246
Application Number:	14540917
International Application Number:	
Confirmation Number:	7076
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Customer Number:	58249
Filer:	Jeffrey R. Bolland/virginia melton
Filer Authorized By:	Jeffrey R. Bolland
Attorney Docket Number:	NUGN-001/01US
Receipt Date:	28-DEC-2015
Filing Date:	13-NOV-2014
Time Stamp:	15:12:51
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$2600
RAM confirmation Number	2261
Deposit Account	501283
Authorized User	COOLEY LLP
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 CFR 1.16 (National application filing, search, and examination fees) Charge any Additional Fees required under 37 CFR 1.17 (Patent application and reexamination processing fees)	

Charge any Additional Fees required under 37 CFR 1.19 (Document supply fees)

Charge any Additional Fees required under 37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	NUGN-001_01US_Cert_and_Req_for_Prioritized_Exam.pdf	135696 0dd147da63eaed0cacb097e357149b12ab182145	no	1
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	NUGN-001_01US_RCE_Transmittal.PDF	1350068 17c1beed1fd398e7fc15527e5b89883d989c2fd6	no	3
Warnings:					
Information:					
3	Response After Final Action	NUGN-001_01US_Resp_to_Final.pdf	149091 f35b95e3f3d84f7c33cc351950f09e597fb555e5	no	13
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	32536 c48a8781ae3fd724a6bc85cc9e6f824ed4b7958e	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1667391		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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:	14540917
Filing Date:	13-Nov-2014
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Filer:	Jeffrey R. Bolland/virginia melton
Attorney Docket Number:	NUGN-001/01US

Filed as Small 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	2817	1	2000	2000
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
Request for Continued Examination	2801	1	600	600
		Total in USD ($)		**2600**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

Filed *via* EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT/RESPONSE TO OFFICE ACTION

In response to the Final Office Action dated September 28, 2015, this paper is timely filed within the 3-month period for reply ending December 28, 2015. Please amend the above-identified patent application in the following manner:

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 6 of this paper.

REMARKS

I. Status

Claims 1-3 and 7-24 are pending; claims 4-6 have been cancelled; claims 21-24 are new; claim 8 has been withdrawn from consideration; and claims 1-3, 7, and 9-21 have been examined on the merits.

Claims 1, 10, 17, 18, and 21 have been amended. Support for the amendment of claims 1 and 21 can be found in the specification in at least the originally filed claim 17 and paragraphs [0029], [0030], and [0065]. Support for the amendment of claim 10 can be found in the specification in at least paragraph [0040]. Support for the amendment of claims 17 and 18 can be found in the specification in at least paragraphs [0030], [0091], and [0104]. Support for new claims 22 and 23 can be found in the specification in at least paragraphs [0030]. Support for new claim 24 can be found in the specification in at least paragraph [0040].

No new matter is introduced by way of amendment. Accordingly, entry thereof is respectfully requested. Upon entry of this amendment, claims 1-3, 7, and 9-22-24 are under examination.

II. Examiner Interview

Examiner Strzelecka is thanked for taking the time for the Applicant-initiated Examiner Interview held on December 22, 2015. Potential claim amendments were generally discussed, as well as potential arguments for addressing the 35 USC 101 rejection over claim 21.

III. 35 U.S.C. § 112(a)

Claim 21 is rejected as allegedly failing to comply with the written description requirement. With regard to the recitation of "A kit comprising a plurality of double-stranded adaptors…", the Examiner alleges that the specification fails to support the limitation of an adaptor being double-stranded. Office Action, p. 5. Applicant respectfully traverses this rejection.

Paragraph [0047] of the specification recites, "In some embodiments, the adaptor can be a double stranded DNA adaptor." In light of the specification explicitly disclosing the adaptor, in one embodiment, as double-stranded, withdrawal of the rejection is respectfully requested.

IV. 35 U.S.C. § 101

Claim 21 is rejected as allegedly directed to a judicial exception. The Examiner asserts that the claim is drawn to a "plurality of oligonucleotides which have corresponding sequences occurring in nature" and "the additional elements recited in claim 21 do not amount to 'significantly more' than the claimed judicial exceptions of nucleic acid sequences of adapters and primers." Office Action, p. 6. Applicant respectfully traverses this rejection.

Paragraph [0067] of the specification recites, "the identifier sequence is not logically present adjacent to naturally occurring sequence[s]," or in other words, that the combination of the identifier site and target site does not exist in nature. Indeed, if it did, the identifier site could not be used to distinguish between duplicate sequence reads. This runs counter to the Examiner's interpretation of the adaptors as being "sequences occurring in nature". The Office released guidance on 35 U.S.C. § 101 in December 2014 pointing to the patent eligible nature of claims comprising nucleic acids, wherein the nucleic acids comprise a heterologous nucleic acid

sequence. The specification clearly describes the identifier site as not being a part of a naturally occurring sequence. Given that the adaptors of claim 21 necessarily comprise the identifier site, said adaptors are clearly non-naturally occurring. Furthermore, the Examiner has not presented any evidence to support a conclusion that the claimed adaptors have a counterpart in nature. Accordingly, withdrawal of the rejection is respectfully requested.

V. 35 U.S.C. § 103

Claims 1-3, 7, and 9-20 are rejected as allegedly being unpatentable over Samuels *et al.* (US20120220494A1, filed 02/16/12 and published 08/30/12) and McCloskey *et al.* (2007. *Biochem. Genet.* 45:761-767). Office Action, p. 7. Applicant respectfully traverses this rejection.

The Examiner states "Samuels et al. teach a method for detecting a duplicate sequencing read from a population of sample sequence reads." Office Action at p. 7. However, the Examiner also states "Samuels et al. teach detection of PCR-introduced errors in sequencing, ***but do not specifically teach detecting duplicate sequencing reads***." Office Action, p. 8 (emphasis added). Samuels *et al.* discloses a method of providing barcode sequences to targets such as a nucleic acid sequences, wherein universal priming sites may be present in barcode libraries [205], [0216]-[0216]. The barcode sequences and universal priming sites may be attached to the nucleic acid sequences and amplified in a PCR reaction [0288]-[0289]. The amplification products may then be sequenced as a whole or in part [0326-0327]. Sequencing errors versus single nucleotide polymorphisms may be detected through the use of barcodes and sequencing of the amplification products [0339] – [0341].

Samuels *et al.* do not teach (1) the active step of "identifying a duplicate sequencing read as the sequencing read comprising the same identifier site and nucleic acid fragment as another

sequencing read in the population of sequencing reads;" (emphasis added); or (2) adaptors comprising distinct indexing sites and identifier sites. Basic disclosure of general barcodes is not enough to rise to the degree of arriving at the metes and bounds of the instantly claimed combination of an indexing site and an identifier site. Furthermore, Samuels *et al.*is simply not drawn to the detection of duplicate sequencing reads, rather Samuels *et al.* is drawn to the detection of PCR-introduced errors in methods of sequencing.

The Examiner cites paragraphs [0339] – [0341] in support of her assertion that Samuels *et al.* teaches detecting the population of sequencing reads comprising a sequencing read with a duplicate identifier site and target site. A review of paragraphs [0339] – [0341] reveals that the methods of Samuels *et al.* actually disclose a method quite distinct from the instant methods. The disclosed method is as follows. Two fragments are noted to exist in a sample, fragments A and B; and each of fragments A and B have two nucleotide positions of interest, X and Y. Fragments A and B have cytosines at position X, whereas fragment A has an adenine at position Y and fragment B has a guanine at position Y. Fragment A is labelled with barcode 1, and fragment B is labelled with barcode 2. After barcode labelling, each of the fragments are amplified in a single cycle of PCR. Fragment B plus corresponding barcode is replicated correctly, however Fragment A plus corresponding barcode features a base change at position X from cytosine to thymine in one of the strands. The fragments are then subjected to one additional round of PCR. Paragraph [0341] recites a method of comparing the fragments plus corresponding barcodes to identify sequences with the same barcode, but different bases within the fragments; which is contrary to the instant methods of identifying duplications. The methods of Samuels *et al.* are simply not drawn to identifying duplications.

McCloskey *et al.* disclose a method of identifying redundant sequences, based solely on two or more sequence reads sharing an identical "barcode" and "batch-stamp". P. 766, para 4-5. McCloskey *et al.* clearly fails to arrive at the instantly claimed method of "identifying a duplicate sequencing read as the sequencing read comprising the same identifier site and nucleic acid fragment…". (Emphasis added). The indexing site of the instant claims can be considered the "batch-stamp" and the identifier site of the instant can be considered "barcode" solely for the purposes of comparing the instant claims with the disclosure of McCloskey *et al.* However, the instant invention relies on the indexing site for batch identification of samples, and the sequence of the identifier site and the sequence of the fragment that is amplified are used in combination to discern duplication. This is very different than the method disclosed in McCloskey *et al.*

The instant methods are superior to McCloskey *et al.*, as McCloskey *et al.* teach the use of barcodes consisting of 7 nucleotides, which allows for 16,384 unique combinations of adaptors. P. 764, para. 2. This means that in just a single sample comprising 250,000 fragments attached to the "encoding oligonucleotide" of McCloskey *et al.*, there could be as many as 15 sequences that share the exact same barcode and batch-stamp. Operating within the teachings of McCloskey *et al.*, these 15 sequences would be falsely identified as duplicates. Furthermore, it becomes costly to generate 16,384 unique adaptors. Theoretically, the instant methods allow for identifier sites consisting of only 1 nucleotide, which allows for the use of just 4 unique adaptors per sample, because identifying two or more sequences as duplicates in the method of the present invention requires only that the combination of the identifier site and the fragment attached to the adaptor be identical. The lower complexity of adaptors comprising fewer nucleotides provides for cost-effective production of adaptors while maintaining the ability to correctly identify true duplications.

There are several other aspects of the claimed invention that clearly distinguish over McCloskey *et al*. For example, the methods of McCloskey *et al.* only work on single stranded polynucleotides when polymerase extension can be performed at non-thermophilic temperatures, because the oligo used as the primer requires secondary structure to keep it from interfering in the reaction. In contrast to the single stranded annealed introduction of McCloskey *et al.*, the present invention employs a double stranded ligated introduction to the reaction. Further, McCloskey describes the use of an oligo that contains a sequence specific portion for targeting of specific nucleic acid sequences. The adapter employed in the claimed invention does not have any target specific regions and is more economical because it can be used in combination with any target.

In another aspect of the claimed invention, new claims 22 and 23 recite "the sequence of the identifier site is not variable in a plurality of adaptors", which reads on a population of the plurality of adaptors sharing the same identifier site. In contrast, the methods of McCloskey *et al.* require each of the identifier sites or "barcodes" to be unique. Furthermore, McCloskey's "batch stamp" is used solely for the purose of detecting contaminants in a given reaction. In contrast, the indexing site of the present invention is used to separate out data from different samples after multiplex amplification (captured in new claim 24). This use of the indexing site in combination with the identifier site further highlights the deficiencies of McCloskey et al., in that it would be extremely cost prohibitive and difficult to design the many thousands of different primers that would be required to ensure that all primers are unique, as the methods of McCloskey et al. would require.

In sum, the teachings of McCloskey *et al.* fail to adequately address the deficiencies of Samuels *et al.* in arriving at a *prima facie* case for obviousness in the instant claims. Samuels *et*

al. is drawn to methods that run counter to the instantly claimed methods as discussed above, and McCloskey *et al.* utilizes a method that is very different than the presently claimed methods. Withdrawal of the rejection is respectfully requested.

Claim 21 is rejected as allegedly being unpatentable over Samuels *et al.* and McCloskey *et al.*, Bignell *et al.* (US2009/0233802A1; published September 2009), Oakes (US Patent 5,384,242A), and Stratagene Catalog (1998. p. 39). Office Action, p. 9. Withdrawal of the rejection is respectfully requested.

The Examiner asserts that the combination of Samuels *et al.* and McCloskey *et al.* arrives at the double-stranded adapters, and further presents the disclosures of Bignell *et al.,* Oakes, and Stratagene to arrive at a kit and the components thereof that are not disclosed by Samuels *et al.* and McCloskey *et al.* As previously presented, the disclosures of Samuels *et al.* and McCloskey *et al.* fail to arrive at the instantly claimed adaptors of the claimed kit. McCloskey *et al.* is necessarily limited to single-stranded adaptors, and Samuels *et al.* fail to disclose methods utilizing adaptors comprising distinct indexing sites and identifier sites as claimed Withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the foregoing, Applicant respectfully submits that no further impediments exist to the allowance of this application and, therefore, requests an indication of allowability. However, the Examiner is requested to call the undersigned if any questions or comments arise.

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

Dated: December 28, 2015

Respectfully submitted,
COOLEY LLP

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By: /Jeffrey R. Bolland/
Jeffrey R. Bolland, Ph.D.
Reg. No. 73,759

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

First Named Inventor:	AMORESE, Douglas	Nonprovisional Application Number (if known):	14/540,917
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ		

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

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

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

3. The applicable box is checked below:

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

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

---OR---

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

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

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

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

Signature /Jeffrey R. Bolland/	Date December 28, 2015
Name (Print/Typed) Jeffrey R. Bolland	Practitioner Registration Number 73759

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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PTO/SB/30EFS (07-14)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	14/540,917	Filing Date	2014-11-13	Docket Number (if applicable)	NUGN-001/01US	Art Unit	1637
First Named Inventor	Douglas AMORESE			Examiner Name	Teresa E. Strzelecka		

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, to any international application that does not comply with the requirements of 35 U.S.C. 371, 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 orde 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 501283

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (07-14)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Signature of Registered U.S. Patent Practitioner			
Signature	/Jeffrey R. Bolland/	Date (YYYY-MM-DD)	2015-12-28
Name	Jeffrey R. Bolland	Registration Number	73759

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 t 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 estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Tim will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions fo 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 informatio 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.

IN THE CLAIMS:

1. (Currently Amended) A method for detecting a duplicate sequencing read from a population of sample sequencing reads comprising:

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

        (i) an indexing primer binding site;

        (ii) an indexing site;

        (iii) an identifier site consisting of~~comprising at least~~ between 1 and 8 nucleotides; and

        (iv) a target sequence primer binding site;

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

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

    d) ~~detecting~~ identifying a duplicate sequencing read as the~~ population of~~ sequencing read~~s~~ comprising the same identifier site and nucleic acid fragment as another sequencing read in the population of sequencing reads~~a sequencing read with a duplicate identifier site and target sequence~~;

wherein the indexing site is unique amongst a subset of the plurality of nucleic acid fragments and is an index for multiple polynucleotides; and wherein the sequence of the identifier site is ~~not conserved across all adaptor sequences~~variable in a plurality of adaptors.

2. (Original) The method of claim 1, wherein the method further comprises removing from the population of sequence reads the sequencing read with a duplicate identifier site and target sequence.
3. (Original) The method of claim 1, wherein the identifier site is sequenced with the indexing site.
4. (Cancelled)
5. (Cancelled)
6. (Cancelled)
7. (Original) The method of claim 1, wherein the adaptor comprises from 5' to 3':
    (i) the indexing primer binding site;
    (ii) the indexing site;
    (iii) the identifier site; and
    (iv) the target sequence primer binding site.
8. (Withdrawn) The method of claim 1, wherein the adaptor comprises from 5' to 3':
    (i) the indexing primer binding site;
    (ii) the indexing site;
    (iii) the target sequence primer binding site; and
    (iv) the identifier site.
9. (Original) The method of claim 1, wherein the plurality of nucleic acid fragments is generated from more than one sample.
10. (Currently Amended) The method of claim 9, wherein the nucleic acid fragments from each sample [[has]]<u>have</u> the same indexing site<u>, and each sample has a unique indexing site</u>.

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

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

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

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

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

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

17. (Currently Amended) The method of claim 1, wherein the identifier site is ~~at least 10~~ 6 nucleotides in length.

18. (Currently Amended) The method of claim 1, wherein the identifier site is ~~about~~ 8 nucleotides in length.

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

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

21. (Currently Amended) A kit comprising a plurality of double-stranded adaptors, wherein each adaptor comprises:

(i) an indexing primer binding site;

(ii) an indexing site;

(iii) an identifier site ~~comprising at least~~ consisting of between 1 and 8 nucleotides; and

(iv) a target sequencing primer binding site;

wherein the kit further comprises one or more oligonucleotide primers, reagents for amplification, and instructions for employing the kit; wherein the plurality of adaptors, one or more oligonucleotide primers, and reagents for amplification are separately contained; wherein the indexing site is unique amongst a subset of the adaptors and is an index for multiple polynucleotides; and wherein the sequence of the identifier site is ~~not conserved across all adaptor sequences~~variable in a plurality of adaptors.

22. (New) The method of claim 1, wherein the sequence of the identifier site is not variable in a plurality of adaptors.

23. (New) The kit of claim 21, wherein the sequence of the identifier site is not variable in a plurality of adaptors.

24. (New) The method of claim 10, wherein the method further comprises separating fragments or sequencing reads based on indexing site.

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/540,917	Filing Date 11/13/2014	☐ 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 $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ 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

AMENDMENT	12/28/2015	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 21	Minus	** 21	= 0	X $80 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $420 =	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**

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ 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.
** 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 number found in the appropriate box in column 1.

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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.**

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Cooley

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TO:	Phone Number:	Fax Number:
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DATE: December 22, 2015

RE: **U.S. Application Serial No. 14/540,917**
Our Ref. No.: NUGN-001/01US (301735-2003)

COMMENTS:

Please see the attached claims in connection with U.S. Application Serial No. 14/540,917.

If you do not receive all of the pages or find that they are illegible, please call Sender's Name at Sender's Phone Number.

COOLEY LLP 1299 PENNSYLVANIA AVENUE, NW, SUITE 700 WASHINGTON, DC 20004-2400
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PAGE 1/5 * RCVD AT 12/22/2015 1:10:32 PM [Eastern Standard Time] * SVR:W-PTOFAX-001/45 * DNIS:2730789 * CSID: * DURATION (mm-ss):01-32

FOR DISCUSSION PURPOSES ONLY

IN THE CLAIMS:

1. (Currently Amended) A method for detecting a duplicate sequencing read from a population of sample sequencing reads comprising:

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

      (i) an indexing primer binding site;

      (ii) an indexing site;

      (iii) an identifier site consisting of~~comprising at least~~ between 1 and 8 nucleotides; and

      (iv) a target sequence primer binding site;

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

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

   d) ~~detecting~~ identifying a duplicate sequencing read as the ~~population of~~ sequencing reads comprising the same identifier site and nucleic acid fragment as another sequencing read in the population of sequencing reads~~a sequencing read with a duplicate identifier site and target sequence~~;

wherein the indexing site is unique amongst a subset of the plurality of nucleic acid fragments and is an index for multiple polynucleotides; and wherein the sequence of the identifier site is ~~not conserved across all adaptor sequences~~variable in a plurality of adaptors.

Cooley LLP DRAFT DATED: DECEMBER 17, 2015

PAGE 2/5 * RCVD AT 12/22/2015 1:10:32 PM [Eastern Standard Time] * SVR:W-PTOFAX-001/45 * DNIS:2730789 * CSID: * DURATION (mm-ss):01-32

Tecan Ex. 2021, Page 813 of 1314
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00026

2. (Original) The method of claim 1, wherein the method further comprises removing from the population of sequence reads the sequencing read with a duplicate identifier site and target sequence.
3. (Original) The method of claim 1, wherein the identifier site is sequenced with the indexing site.
4. (Withdrawn) The method of claim 1, wherein the identifier site is sequenced separately from the indexing site.
5. (Withdrawn) The method of claim 1, wherein the identifier site is sequenced with the target sequence.
6. (Withdrawn) The method of claim 1, wherein the identifier site is sequenced separately from the target sequence.
7. (Original) The method of claim 1, wherein the adaptor comprises from 5' to 3':
    (i) the indexing primer binding site;
    (ii) the indexing site;
    (iii) the identifier site; and
    (iv) the target sequence primer binding site.
8. (Withdrawn) The method of claim 1, wherein the adaptor comprises from 5' to 3':
    (i) the indexing primer binding site;
    (ii) the indexing site;
    (iii) the target sequence primer binding site; and
    (iv) the identifier site.
9. (Original) The method of claim 1, wherein the plurality of nucleic acid fragments is generated from more than one sample.

Cooley LLP DRAFT DATED: DECEMBER 17, 2015

PAGE 3/5 * RCVD AT 12/22/2015 1:10:32 PM [Eastern Standard Time] * SVR:W-PTOFAX-001/45 * DNIS:2730789 * CSID: * DURATION (mm-ss):01-32

10. (Currently Amended) The method of claim 9, wherein the nucleic acid fragments from each sample [[has]]have the same indexing site, and each sample has a unique indexing site.

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

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

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

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

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

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

17. (Currently Amended) The method of claim 1, wherein the identifier site is ~~at least 10~~ 6 nucleotides in length.

18. (Currently Amended) The method of claim 1, wherein the identifier site is ~~about~~ 8 nucleotides in length.

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

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

Cooley LLP DRAFT DATED: DECEMBER 17, 2015

PAGE 4/5 * RCVD AT 12/22/2015 1:10:32 PM [Eastern Standard Time] * SVR:W-PTOFAX-001/45 * DNIS:2730789 * CSID: * DURATION (mm-ss):01-32

21. (Currently Amended) A kit comprising a plurality of double-stranded adaptors, wherein each adaptor comprises:

(i) an indexing primer binding site;

(ii) an indexing site;

(iii) an identifier site ~~comprising at least~~ consisting of between 1 and 8 nucleotides; and

(iv) a target sequencing primer binding site;

wherein the kit further comprises one or more oligonucleotide primers, reagents for amplification, and instructions for employing the kit; wherein the plurality of adaptors, one or more oligonucleotide primers, and reagents for amplification are separately contained; wherein the indexing site is unique amongst a subset of the adaptors and is an index for multiple polynucleotides; and wherein the sequence of the identifier site is ~~not conserved across all adaptor sequences~~variable in a plurality of adaptors.

Cooley LLP DRAFT DATED: DECEMBER 17, 2015

22. (New) The method of claim 1, wherein the sequence of the identifier site is not variable in a plurality of adaptors.

23. (New) The kit of claim 21, wherein the sequence of the identifier site is not variable in a plurality of adaptors.

24. (New) The method of claim 10, wherein the method further comprises separating fragments or sequencing reads based on indexing site.

PAGE 5/5 * RCVD AT 12/22/2015 1:10:32 PM [Eastern Standard Time] * SVR:W-PTOFAX-001/45 * DNIS:2730789 * CSID: * DURATION (mm-ss):01-32

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

58249 7590 01/14/2016

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

EXAMINER
STRZELECKA, TERESA E

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
01/14/2016	ELECTRONIC

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

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

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

zpatdcdocketing@cooley.com

Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	14/540,917	AMORESE ET AL.	
	Examiner	Art Unit	
	TERESA E. STRZELECKA	1637	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Teresa Strzelecka*.

(2) *Jeffrey Bolland*.

(3) *Bonnie McLeod*.

(4) _____.

Date of Interview: *22 December 2015*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1*.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant's representaive submitted proposed claim amendments which were discussed during the interview.

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.

☒ Attachment

/TERESA E STRZELECKA/ Primary Examiner, Art Unit 1637	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

———

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

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COOLEY LLP
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1299 PENNSYLVANIA AVNEUE, NW
SUITE 700
WASHINGTON DC 20004

Doc Code: TRACK1.GRANT

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

1. THE REQUEST FILED 12/28/15 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, Office of Petitions. In his/her absence, calls may be directed to Brian W. Brown, (571)272-5338.

Cheryl Gibson-Baylor
/Cheryl Gibson-Baylor/
[*Signature*]

Petitions Paralegal Specialist
(Title)

UNITED STATES PATENT AND TRADEMARK OFFICE

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

58249 7590 03/03/2016

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

EXAMINER
STRZELECKA, TERESA E

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
03/03/2016	ELECTRONIC

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

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

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

zpatdcdocketing@cooley.com

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

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *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 *12/28/15*.

☐ 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) *1-3 and 7-24* is/are pending in the application.

5a) Of the above claim(s) *8* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-3,7 and 9-24* 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 _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

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

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

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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

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

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

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

Attachment(s)

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

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

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

4) ☐ Other: _____.

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

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

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 December 28, 2015 has been entered.

3. Claims 1-21 were previously pending, with claims 4-6 and 8 withdrawn from consideration. Applicant cancelled claims 4-6, amended claims 1, 10, 17, 18 and 21 and added new claims 22-24. Claims 1-3, 7 and 9-24 are under consideration.

4. Applicant's amendments overcame the previously presented rejections with the exception of the rejection of claim 21 under 35 U.S.C. 101. Arguments regarding this rejection are addressed in the "Response to Arguments" section below.

5. This office action contains new grounds for rejection necessitated by amendment.

Response to Arguments

6. Applicant's arguments filed December 28, 2015 have been fully considered but they are not persuasive.

Regarding the rejection of claim 21 under 35 U.S.C. 101, Applicant argues the following:

"Paragraph [0067] of the specification recites, "the identifier sequence is not logically present adjacent to naturally occurring sequence[s]," or in other words, that the combination of

the identifier site and target site does not exist in nature. Indeed, if it did, the identifier site could not be used to distinguish between duplicate sequence reads. This runs counter to the Examiner's interpretation of the adaptors as being "sequences occurring in nature". The Office released guidance on 35 U.S.C. § 101 in December 2014 pointing to the patent eligible nature of claims comprising nucleic acids, wherein the nucleic acids comprise a heterologous nucleic acid sequence. The specification clearly describes the identifier site as not being a part of a naturally occurring sequence. Given that the adaptors of claim 21 necessarily comprise the identifier site, said adaptors are clearly non-naturally occurring. Furthermore, the Examiner has not presented any evidence to support a conclusion that the claimed adaptors have a counterpart in nature. Accordingly, withdrawal of the rejection is respectfully requested."

First, even assuming that one would understand what "...not logically present adjacent to naturally occurring sequence…" means, almost any sequence is bound to be found in nature. The claim does not limit the length of the adaptors, therefore, for example, they can be 8 bp long. As detailed by Liu et al. (BMC Genomics, vol. 9:509, pp. 1-17, 2008), almost all possible 12mers are present in a human genome, and other genomes contain variable numbers of the different n-mers (Abstract; page 2, paragraphs 4-6; page 3, page 5). Further, considering the fact that in 2013 GenBank database contained 4.5×10^{11} bp of sequences (Benson et al., Nucl. Acids Res., vol. 41, D36-D42, 2013; see Table 1), and that there are 4^8, or 6.5×10^4 possible 8mer sequences, each one of them would be represented multiple times in the GenBank database, as would every possible 16mer, for example, of which there are 4.3×10^9 possible sequences.

Finally, Applicant's sequence with SEQ ID NO: 4, which has 58 bp without the 3' 8-bp random part was found in the GenBank database 106 times (see attached BLAST search result).

In conclusion, the claim is not patent eligible under 35 U.S.C. 101. The rejection is maintained.

Claim Rejections - 35 USC § 112

7. 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.

8. Claims 1-3, 7 and 9-24 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 pre-AIA the applicant regards as the invention.

A) Claims 1, 7, 9-20, 22 and 24 are indefinite in claim 1. Claim 1 is indefinite over the recitation of "…wherein the sequence of the identifier site is variable in a plurality of adaptors". It is not clear what the term "variable" means: does it mean sequence length variability or sequence content variability? In conclusion, the claims do not have clear metes and bounds.

B) Claim 22 is indefinite over the recitation of "…wherein the sequence of the identifier site is not variable in a plurality of adaptors". First, it is not clear what the term "variable" means: does it mean sequence length variability or sequence content variability? Further, claim

22 depends from claim 1, which requires that "…the sequence of the identifier site is variable in a plurality of adaptors." It is therefore not clear how a sequence identifier can be both variable and not variable in the same plurality of adaptors.

In conclusion, the claim does not have clear metes and bounds.

C) Claims 21 and 23 are indefinite in claim 21. Claim 21 is indefinite over the recitation of "…wherein the sequence of the identifier site is variable in a plurality of adaptors". It is not clear what the term "variable" means: does it mean sequence length variability or sequence content variability? In conclusion, the claims do not have clear metes and bounds.

D) Claim 23 is indefinite over the recitation of "…wherein the sequence of the identifier site is not variable in a plurality of adaptors". First, it is not clear what the term "variable" means: does it mean sequence length variability or sequence content variability? Further, claim 23 depends from claim 21, which requires that "…the sequence of the identifier site is variable in a plurality of adaptors." It is therefore not clear how a sequence identifier can be both variable and not variable in the same plurality of adaptors.

In conclusion, the claim does not have clear metes and bounds.

Claim Rejections - 35 USC § 101

9. 35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

10. Claims 21 and 23 are rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more.

Claim(s) 21 is directed to a kit comprising a plurality of adaptors, i.e., nucleic acid sequences, comprising an indexing primer binding site, an indexing site, an identifier site and a target sequencing binding site, the kit further comprising one or more oligonucleotide primers, reagents for amplification, and instructions for employing the kit; wherein the plurality of adaptors, one or more oligonucleotide primers, and reagents for amplification are separately contained.

No specific sequences of the adapters and oligonucleotide primers are claimed; therefore the claim is drawn to a plurality of oligonucleotides which have corresponding sequences occurring in nature. The claim does not limit the length of the adaptors, therefore, for example, they can be 8 bp long. As detailed by Liu et al. (BMC Genomics, vol. 9:509, pp. 1-17, 2008), almost all possible 12mers are present in a human genome, and other genomes contain variable numbers of the different n-mers (Abstract; page 2, paragraphs 4-6; page 3, page 5). Further, considering the fact that in 2013 GenBank database contained 4.5×10^{11} bp of sequences (Benson et al., Nucl. Acids Res., vol. 41, D36-D42, 2013; see Table 1), and that there are 4^8, or 6.5×10^4 possible 8mer sequences, each one of them would be represented multiple times in the GenBank database, as would every possible 16mer, for example, of which there are 4.3×10^9 possible sequences.

Finally, Applicant's sequence with SEQ ID NO: 4, which has 58 bp without the 3' 8bp random part was found in the GenBank database 106 times (see attached BLAST search result).

The claim(s) does/do not include additional elements that are sufficient to amount to significantly more than the judicial exception because the other elements of the kit are well-understood, routine and conventional in the art. Specifically, "reagents for amplification" are not specified, therefore could encompass water and salt solutions, for example. It is conventional in the art to include instructions for using a kit, as well as to partition components of a kit into separate containers (see, for example, US 2009/0233802 A1, [0103]-[0107]; US 5,384,242 A; issued January 1995; col. 11, lines 61-68; col. 12, lines 1-13). Therefore, the additional elements recited in claim 21 do not amount to "significantly more" than the claimed judicial exceptions of nucleic acid sequences of adapters and primers.

In conclusion, claim 21 is not patent eligible under 35 U.S.C. 101.

Claim Rejections - 35 USC § 102

10. 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)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale or otherwise available to the public before the effective filing date of the claimed invention.

11. Claims 21 and 23 are rejected under 35 U.S.C. 102(a)(1) as being anticipated by Casbon et al. (US 2012/0071331 A1).

Regarding claims 21 and 23, Casbon et al. teach a kit comprising in separate containers reaction reagents comprising adapters containing indexing sites, variable identifier sites and primer sites, primers, polymerases, ligases, etc. (page 3, [0021]; page 13, [0091]-[0093]; page 1,

[0005]-[0006]; page 2, [0016]-[0017]; page 3, [0023]; page 7, [0045]-[0046]; page 8, [0051]-[0055]; page 9, [0057]-[0061]).

12. No claims are allowed.

Conclusion

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

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

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

TERESA E STRZELECKA
Primary Examiner
Art Unit 1637

/TERESA E STRZELECKA/
Primary Examiner, Art Unit 1637
February 25, 2016

Notice of References Cited	Application/Control No. 14/540,917	Applicant(s)/Patent Under Reexamination AMORESE ET AL.	
	Examiner TERESA E. STRZELECKA	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-2012/0071331 A1	03-2012	Casbon; James	C12Q1/6855	506/4
	B	US-				
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Liu, Z. et al., BMC Genomics, vol. 9:509, pp. 1-17 (2008)
	V	Benson, D.A. et al., Nucl. Acids Res., vol. 41, D36-D42 (2013)
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14540917	AMORESE ET AL.
	Examiner	Art Unit
	TERESA E STRZELECKA	1637

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/14/2015	09/21/2015	02/25/2016						
	1	✓	✓	✓						
	2	✓	✓	✓						
	3	✓	✓	✓						
	4	N	N	-						
	5	N	N	-						
	6	N	N	-						
	7	✓	✓	✓						
	8	N	N	N						
	9	✓	✓	✓						
	10	✓	✓	✓						
	11	✓	✓	✓						
	12	✓	✓	✓						
	13	✓	✓	✓						
	14	✓	✓	✓						
	15	✓	✓	✓						
	16	✓	✓	✓						
	17	✓	✓	✓						
	18	✓	✓	✓						
	19	✓	✓	✓						
	20	✓	✓	✓						
	21	✓	✓	✓						
	22			✓						
	23			✓						
	24			✓						

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BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - CTN8H65N014**
Formatting options
Download
Blast report description

SEQID4

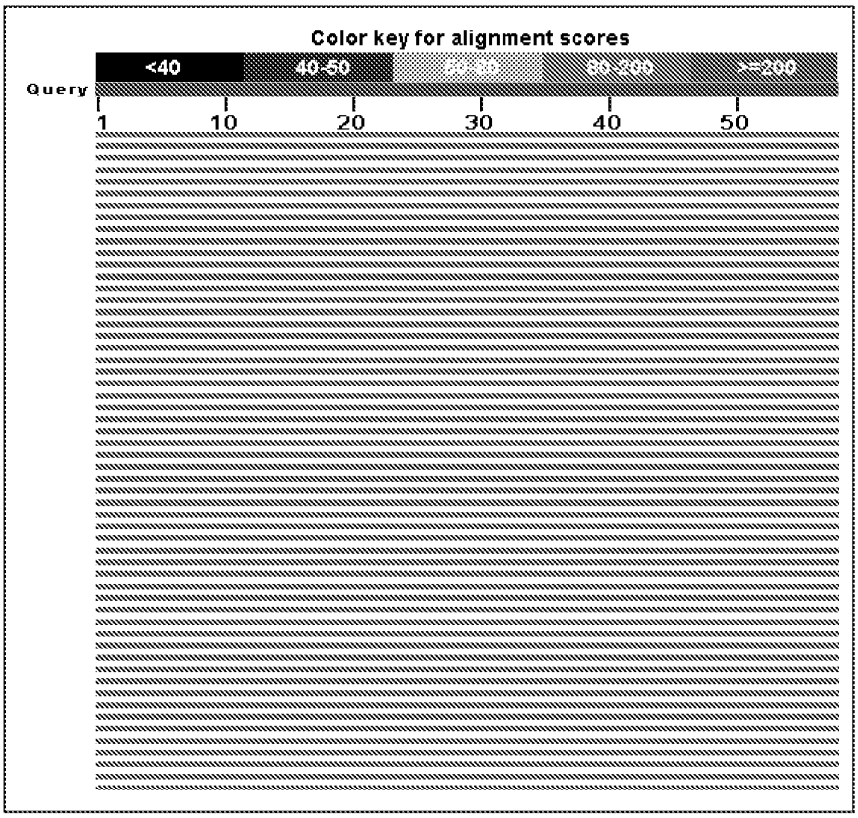
RID CTN8H65N014 (Expires on 02-25 04:54 am)

Query ID lcl|Query_78557
Description None
Molecule type nucleic acid
Query Length 58

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.3.1+

Graphic Summary

Distribution of 106 Blast Hits on the Query Sequence



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Clupea harengus uncharacterized LOC105905600	105	105	100%	4e-20	100%	XR_001162831.1

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Description	Max score	Total score	Query cover	E value	Ident	Accession
(LOC105905600), transcript variant X11, ncRNA						
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X10, ncRNA	105	105	100%	4e-20	100%	XR_001162630.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X9, ncRNA	105	105	100%	4e-20	100%	XR_001162629.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X8, ncRNA	105	105	100%	4e-20	100%	XR_001162628.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X7, ncRNA	105	105	100%	4e-20	100%	XR_001162627.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X6, ncRNA	105	105	100%	4e-20	100%	XR_001162626.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X5, ncRNA	105	105	100%	4e-20	100%	XR_001162625.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X4, ncRNA	105	105	100%	4e-20	100%	XR_001162624.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X3, ncRNA	105	105	100%	4e-20	100%	XR_001162623.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X2, ncRNA	105	105	100%	4e-20	100%	XR_001162622.1
PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X1, ncRNA	105	105	100%	4e-20	100%	XR_001162621.1
PREDICTED: Clupea harengus uncharacterized LOC105901894 (LOC105901894), transcript variant X2, ncRNA	105	105	100%	4e-20	100%	XR_001162457.1
PREDICTED: Clupea harengus uncharacterized LOC105901894 (LOC105901894), transcript variant X1, ncRNA	105	105	100%	4e-20	100%	XR_001162456.1
PREDICTED: Clupea harengus uncharacterized LOC105898768 (LOC105898768), ncRNA	105	105	100%	4e-20	100%	XR_001162326.1
PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X4, ncRNA	105	105	100%	4e-20	100%	XR_001162263.1
PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X3, ncRNA	105	105	100%	4e-20	100%	XR_001162262.1

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Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X2, ncRNA	105	105	100%	4e-20	100%	XR_001162261.1
PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X1, ncRNA	105	105	100%	4e-20	100%	XR_001162260.1
PREDICTED: Clupea harengus uncharacterized LOC105895935 (LOC105895935), transcript variant X3, ncRNA	105	105	100%	4e-20	100%	XR_001162196.1
PREDICTED: Clupea harengus uncharacterized LOC105895935 (LOC105895935), transcript variant X2, ncRNA	105	105	100%	4e-20	100%	XR_001162195.1
PREDICTED: Clupea harengus uncharacterized LOC105895935 (LOC105895935), transcript variant X1, ncRNA	105	105	100%	4e-20	100%	XR_001162194.1
Dicrocoelium dendriticum genome assembly D_dendriticum_Leon_v1_0_4, scaffold DDEL_contig0063667	105	105	100%	4e-20	100%	LK829630.1
Nippostrongylus brasiliensis genome assembly N_brasiliensis_RM07_v1_5_4, scaffold NBR_scaffold0003529	105	105	100%	4e-20	100%	LM437148.1
Nippostrongylus brasiliensis genome assembly N_brasiliensis_RM07_v1_5_4, scaffold NBR_scaffold0003502	105	105	100%	4e-20	100%	LM437114.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002276	105	105	100%	4e-20	100%	LN850047.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000045	105	105	100%	4e-20	100%	LN848550.1
Cyprinus carpio genome assembly common carp genome, scaffold 000003581	105	105	100%	4e-20	100%	LN848496.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001750	105	105	100%	4e-20	100%	LN848313.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001003	105	105	100%	4e-20	100%	LN848263.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001567	105	105	100%	4e-20	100%	LN848209.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000916	105	105	100%	4e-20	100%	LN848136.1
Cyprinus carpio genome assembly common carp genome, scaffold 000003735	105	105	100%	4e-20	100%	LN847769.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001344	105	105	100%	4e-20	100%	LN846822.1
	105	186	100%	4e-20	100%	LN846727.1

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Cyprinus carpio genome assembly common carp genome, scaffold 000000040						
Cyprinus carpio genome assembly common carp genome, scaffold 000001121	105	105	100%	4e-20	100%	LN596723.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000158	105	105	100%	4e-20	100%	LN596719.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002540	105	105	100%	4e-20	100%	LN596606.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002987	105	105	100%	4e-20	100%	LN596327.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001762	105	105	100%	4e-20	100%	LN596274.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001964	105	211	100%	4e-20	100%	LN596222.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000564	105	105	100%	4e-20	100%	LN596142.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000654	105	105	100%	4e-20	100%	LN595796.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002386	105	105	100%	4e-20	100%	LN595666.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002381	105	105	100%	4e-20	100%	LN595475.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000064	105	105	100%	4e-20	100%	LN595230.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001650	105	105	100%	4e-20	100%	LN595223.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000702	105	105	100%	4e-20	100%	LN595171.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002009	105	105	100%	4e-20	100%	LN595112.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001123	105	105	100%	4e-20	100%	LN595103.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002070	105	105	100%	4e-20	100%	LN594987.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000549	105	105	100%	4e-20	100%	LN594921.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002464	105	105	100%	4e-20	100%	LN594859.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000619	105	105	100%	4e-20	100%	LN594833.1

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Cyprinus carpio genome assembly common carp genome, scaffold 000000122	105	314	100%	4e-20	100%	LN584771.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001987	105	105	100%	4e-20	100%	LN584742.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002509	105	105	100%	4e-20	100%	LN584739.1
Cyprinus carpio genome assembly common carp genome, scaffold 000003052	105	105	100%	4e-20	100%	LN584651.1
Cyprinus carpio genome assembly common carp genome, scaffold 000004095	105	105	100%	4e-20	100%	LN584602.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000925	105	105	100%	4e-20	100%	LN584463.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001670	105	105	100%	4e-20	100%	LN584450.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002477	105	105	100%	4e-20	100%	LN584374.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002610	105	105	100%	4e-20	100%	LN584146.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001475	105	105	100%	4e-20	100%	LN584027.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001023	105	105	100%	4e-20	100%	LN583855.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001156	105	105	100%	4e-20	100%	LN583842.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000879	105	105	100%	4e-20	100%	LN583720.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000579	105	105	100%	4e-20	100%	LN583390.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001592	105	105	100%	4e-20	100%	LN583384.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001168	105	105	100%	4e-20	100%	LN583298.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001951	105	105	100%	4e-20	100%	LN582971.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000038	105	105	100%	4e-20	100%	LN582912.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000741	105	105	100%	4e-20	100%	LN582861.1
	105	105	100%	4e-20	100%	LN582771.1

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Cyprinus carpio genome assembly common carp genome, scaffold 000002239						
Cyprinus carpio genome assembly common carp genome, scaffold 000001653	105	105	100%	4e-20	100%	LN592793.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001717	105	105	100%	4e-20	100%	LN592632.1
Cyprinus carpio genome assembly common carp genome, scaffold 000006258	105	105	100%	4e-20	100%	LN592578.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002277	105	105	100%	4e-20	100%	LN592402.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001359	105	105	100%	4e-20	100%	LN592387.1
Cyprinus carpio genome assembly common carp genome, scaffold 000008356	105	105	100%	4e-20	100%	LN592384.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000021	105	211	100%	4e-20	100%	LN592208.1
Cyprinus carpio genome assembly common carp genome, scaffold 000000540	105	105	100%	4e-20	100%	LN592089.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001009	105	105	100%	4e-20	100%	LN591795.1
Cyprinus carpio genome assembly common carp genome, scaffold 000002927	105	105	100%	4e-20	100%	LN591675.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001264	105	105	100%	4e-20	100%	LN591563.1
Cyprinus carpio genome assembly common carp genome, scaffold 000007887	105	105	100%	4e-20	100%	LN591511.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001448	105	105	100%	4e-20	100%	LN591505.1
Cyprinus carpio genome assembly common carp genome, scaffold 000029065	105	105	100%	4e-20	100%	LN591261.1
Cyprinus carpio genome assembly common carp genome, scaffold 000029034	105	105	100%	4e-20	100%	LN591245.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028985	105	211	100%	4e-20	100%	LN591218.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028978	105	105	100%	4e-20	100%	LN591212.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028950	105	105	100%	4e-20	100%	LN591198.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028897	105	105	100%	4e-20	100%	LN591168.1

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Cyprinus carpio genome assembly common carp genome, scaffold 000028849	105	105	100%	4e-20	100%	LN591137.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028846	105	105	100%	4e-20	100%	LN591136.1
Cyprinus carpio genome assembly common carp genome, scaffold 000028815	105	105	100%	4e-20	100%	LN591118.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001451	105	105	100%	4e-20	100%	LN591656.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001346	105	105	100%	4e-20	100%	LN591651.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001187	105	105	100%	4e-20	100%	LN591636.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001040	105	105	100%	4e-20	100%	LN590987.1
Cyprinus carpio genome assembly common carp genome, scaffold 000001016	105	105	100%	4e-20	100%	LN590978.1

Alignments

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X11, ncRNA
Sequence ID: **ref|XR_001162631.1|** Length: 124 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X10, ncRNA
Sequence ID: **ref|XR_001162630.1|** Length: 133 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X9, ncRNA
Sequence ID: **ref|XR_001162629.1|** Length: 131 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame

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105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X8, ncRNA

Sequence ID: **ref|XR_001162628.1|** Length: 141 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X7, ncRNA

Sequence ID: **ref|XR_001162627.1|** Length: 137 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X6, ncRNA

Sequence ID: **ref|XR_001162626.1|** Length: 140 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X5, ncRNA

Sequence ID: **ref|XR_001162625.1|** Length: 139 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
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Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X4, ncRNA

Sequence ID: **ref|XR_001162624.1|** Length: 133 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame

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105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X3, ncRNA

Sequence ID: **ref|XR_001162623.1|** Length: 144 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X2, ncRNA

Sequence ID: **ref|XR_001162622.1|** Length: 124 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105905600 (LOC105905600), transcript variant X1, ncRNA

Sequence ID: **ref|XR_001162621.1|** Length: 129 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

PREDICTED: Clupea harengus uncharacterized LOC105901894 (LOC105901894), transcript variant X2, ncRNA

Sequence ID: **ref|XR_001162457.1|** Length: 79 Number of Matches: 1
Range 1: 14 to 71

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  14
```

PREDICTED: Clupea harengus uncharacterized LOC105901894 (LOC105901894), transcript variant X1, ncRNA

Sequence ID: **ref|XR_001162456.1|** Length: 84 Number of Matches: 1
Range 1: 19 to 76

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Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  19
```

PREDICTED: Clupea harengus uncharacterized LOC105898768 (LOC105898768), ncRNA

Sequence ID: **ref|XR_001162326.1|** Length: 96 Number of Matches: 1
Range 1: 26 to 83

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  83  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  26
```

PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X4, ncRNA

Sequence ID: **ref|XR_001162263.1|** Length: 158 Number of Matches: 1
Range 1: 37 to 94

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  94
```

PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X3, ncRNA

Sequence ID: **ref|XR_001162262.1|** Length: 177 Number of Matches: 1
Range 1: 56 to 113

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1    AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  113
```

PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X2, ncRNA

Sequence ID: **ref|XR_001162261.1|** Length: 166 Number of Matches: 1
Range 1: 45 to 102

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1    AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  45   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  102
```

PREDICTED: Clupea harengus uncharacterized LOC105897580 (LOC105897580), transcript variant X1, ncRNA

Sequence ID: **ref|XR_001162260.1|** Length: 161 Number of Matches: 1
Range 1: 40 to 97

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Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  40  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  97
```

PREDICTED: Clupea harengus uncharacterized LOC105895935 (LOC105895935), transcript variant X3, ncRNA
Sequence ID: **ref|XR_001162196.1|** Length: 111 Number of Matches: 1
Range 1: 44 to 101

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1    AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  101  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  44
```

PREDICTED: Clupea harengus uncharacterized LOC105895935 (LOC105895935), transcript variant X2, ncRNA
Sequence ID: **ref|XR_001162195.1|** Length: 104 Number of Matches: 1
Range 1: 37 to 94

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  37
```

PREDICTED: Clupea harengus uncharacterized LOC105895935 (LOC105895935), transcript variant X1, ncRNA
Sequence ID: **ref|XR_001162194.1|** Length: 107 Number of Matches: 1
Range 1: 40 to 97

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1   AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  40
```

Dicrocoelium dendriticum genome assembly D_dendriticum_Leon_v1_0_4, scaffold DDEL_contig0063667
Sequence ID: **emb|LK829830.1|** Length: 603 Number of Matches: 1
Range 1: 540 to 597

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1    AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  597  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  540
```

Nippostrongylus brasiliensis genome assembly N_brasiliensis_RM07_v1_5_4 ,scaffold NBR_scaffold0003529
Sequence ID: **emb|LM437148.1|** Length: 19176 Number of Matches: 1
Range 1: 19117 to 19174

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Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1      AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19174  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  19117
```

Nippostrongylus brasiliensis genome assembly N_brasiliensis_RM07_v1_5_4 ,scaffold NBR_scaffold0003502

Sequence ID: **emb|LM437114.1|** Length: 19368 Number of Matches: 1
Range 1: 3936 to 3993

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1     AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3993  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  3936
```

Cyprinus carpio genome assembly common carp genome ,scaffold 000002276

Sequence ID: **emb|LN600047.1|** Length: 341394 Number of Matches: 1
Range 1: 248502 to 248559

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1       AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248559  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  248502
```

Cyprinus carpio genome assembly common carp genome ,scaffold 000000045

Sequence ID: **emb|LN598550.1|** Length: 947182 Number of Matches: 1
Range 1: 57307 to 57364

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1      AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57307  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  57364
```

Cyprinus carpio genome assembly common carp genome ,scaffold 000003581

Sequence ID: **emb|LN598496.1|** Length: 28110 Number of Matches: 1
Range 1: 22731 to 22788

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1      AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  22731  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  22788
```

Cyprinus carpio genome assembly common carp genome ,scaffold 000001750

Sequence ID: **emb|LN598313.1|** Length: 345239 Number of Matches: 1
Range 1: 3317 to 3374

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Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Minus	

Features:

```
Query  1     AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3374  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  3317
```

Cyprinus carpio genome assembly common carp genome ,scaffold 000001003

Sequence ID: **emb|LN598283.1|** Length: 1505843 Number of Matches: 1

Range 1: 257631 to 257688

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1       AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257631  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  257688
```

Cyprinus carpio genome assembly common carp genome ,scaffold 000001567

Sequence ID: **emb|LN598209.1|** Length: 1073906 Number of Matches: 1

Range 1: 116306 to 116363

Score	Expect	Identities	Gaps	Strand	Frame
105 bits(116)	4e-20()	58/58(100%)	0/58(0%)	Plus/Plus	

Features:

```
Query  1       AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  58
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  116306  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT  116363
```


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PALM INTRANET

Day : Tuesday
Date: 2/23/2016

Time: 15:45:15

Inventor Name Search

Enter the first few letters of the Inventor's Last Name.
Additionally, enter the first few letters of the Inventor's First name.

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Amorese	Douglas	Search

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Day : Tuesday
Date: 2/23/2016

Time: 15:45:15

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Day : Tuesday
Date: 2/23/2016

Time: 15:45:15

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Additionally, enter the first few letters of the Inventor's First name.

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Schroeder	Ben	Search

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WEST Search History for Application 14540917

Creation Date: 2016022415:01

Prior Art Searches

Query	DB	Hits	Op.	Plur.	Thes.	Date
sequencing and reads near3 (duplicat$7 or redundan$8) and (barcod$8 or index$8) near3 random and (duplicat$7 or redundan$8) near3 remov$8	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015
sequencing and reads near3 (duplicat$7 or redundan$8) and (barcod$8 or index$8) near3 (random$8 or degener$8)	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015
sequencing and reads near3 (duplicat$7 or redundan$8) and (barcod$8 or index$8) near3 (random$8 or degener$8)	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
sequencing and duplicate near3 remov$8 and ((C12Q1/6869 | C12Q2563/179 | C12Q2535/122 | C12Q2537/1376 | C12Q2563/159 | C12Q1/6806 | C12Q2521/319 | C12Q2521/507 | C12Q2525/15 | C12Q2525/155 | C12Q2525/161 | C12Q2525/185 | C12Q2525/191 | C12Q2525/197 | C12Q2525/313 | C12Q2537/159 | C12Q2537/165 | C12Q2549/10 | C12Q2565/514 | C12Q1/6874 | C12Q1/6876 | C12Q1/6879 | C12Q1/689 | C12Q2600/156 | C12Q2600/166 | C12N15/1065 | C12N15/1093 | C12N15/1089 | G06F19/18).CPC.)	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015
(sequencing and duplicate near3 remov$8 and ((C12Q1/6869 | C12Q2563/179 | C12Q2535/122 | C12Q2537/1376 | C12Q2563/159 | C12Q1/6806 | C12Q2521/319 | C12Q2521/507 | C12Q2525/15 | C12Q2525/155 | C12Q2525/161 | C12Q2525/185 | C12Q2525/191 | C12Q2525/197 | C12Q2525/313 | C12Q2537/159 | C12Q2537/165 | C12Q2549/10 | C12Q2565/514 | C12Q1/6874 | C12Q1/6876 | C12Q1/6879 | C12Q1/689 | C12Q2600/156 | C12Q2600/166 | C12N15/1065 | C12N15/1093 | C12N15/1089 | G06F19/18).CPC.)) and random$8 near3	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015

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(barcod$8 or index$8)						
sequencing and duplicate near3 remov$8 and ((C12Q1/6869 | C12Q2563/179 | C12Q2535/122 | C12Q2537/1376 | C12Q2563/159 | C12Q1/6806 | C12Q2521/319 | C12Q2521/507 | C12Q2525/15 | C12Q2525/155 | C12Q2525/161 | C12Q2525/185 | C12Q2525/191 | C12Q2525/197 | C12Q2525/313 | C12Q2537/159 | C12Q2537/165 | C12Q2549/10 | C12Q2565/514 | C12Q1/6874 | C12Q1/6876 | C12Q1/6879 | C12Q1/689 | C12Q2600/156 | C12Q2600/166 | C12N15/1065 | C12N15/1093 | C12N15/1089 | G06F19/18).CPC.)	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
(sequencing and duplicate near3 remov$8 and ((C12Q1/6869 | C12Q2563/179 | C12Q2535/122 | C12Q2537/1376 | C12Q2563/159 | C12Q1/6806 | C12Q2521/319 | C12Q2521/507 | C12Q2525/15 | C12Q2525/155 | C12Q2525/161 | C12Q2525/185 | C12Q2525/191 | C12Q2525/197 | C12Q2525/313 | C12Q2537/159 | C12Q2537/165 | C12Q2549/10 | C12Q2565/514 | C12Q1/6874 | C12Q1/6876 | C12Q1/6879 | C12Q1/689 | C12Q2600/156 | C12Q2600/166 | C12N15/1065 | C12N15/1093 | C12N15/1089 | G06F19/18).CPC.)) and random$8 near3 (barcod$8 or index$8)	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
sequencing and duplicate near3 remov$8 and ((C12Q1/6869 | C12Q2563/179 | C12Q2535/122 | C12Q2537/1376 | C12Q2563/159 | C12Q1/6806 | C12Q2521/319 | C12Q2521/507 | C12Q2525/15 | C12Q2525/155 | C12Q2525/161 | C12Q2525/185 | C12Q2525/191 | C12Q2525/197 | C12Q2525/313 | C12Q2537/159 | C12Q2537/165 | C12Q2549/10 | C12Q2565/514 | C12Q1/6874 | C12Q1/6876 | C12Q1/6879 | C12Q1/689 | C12Q2600/156 | C12Q2600/166 | C12N15/1065 | C12N15/1093 | C12N15/1089 | G06F19/18).CPC.)	DWPI	n/a	ADJ	YES	ASSIGNEE	05-13-2015
20110269647	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015
(Amorese.in. or Scolnick.in. or Schroeder.in. or Nugen.asn.) and sequencing and duplicate	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015

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(Amorese.in. or Scolnick.in. or Schroeder.in. or Nugen.asn.) and sequencing and duplicate	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
US-6251639-$.DID. OR US-5750341-$.DID. OR US-6306597-$.DID. OR US-5969119-$.DID. OR US-7244559-$.DID. OR US-7335762-$.DID. OR US-7211390-$.DID. OR US-7244567-$.DID.	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
US-7264929-$.DID. OR US-7323305-$.DID. OR US-7501245-$.DID. OR US-7491498-$.DID. OR US-7276720-$.DID. OR US-7462452-$.DID. OR US-7476504-$.DID. OR US-7405281-$.DID. OR US-7170050-$.DID. OR US-7462468-$.DID. OR US-7476503-$.DID.	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
US-7315019-$.DID. OR US-7302146-$.DID. OR US-7313318-$.DID. OR US-6449562-$.DID. OR US-6287766-$.DID. OR US-7361468-$.DID. OR US-6225109-$.DID. OR US-6110709-$.DID.	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
US-20130231253-$.DID. OR US-20060024711-$.DID. OR US-20090061439-$.DID. OR US-20080087826-$.DID. OR US-20060286566-$.DID. OR US-20060024678-$.DID. OR US-20080213770-$.DID. OR US-20080103058-$.DID. OR US-20090029385-$.DID. OR US-20090068655-$.DID.	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015
US-20090024331-$.DID. OR US-20080206764-$.DID. OR US-20090026082-$.DID.	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015
((US-20130231253-$.DID. OR US-20060024711-$.DID. OR US-20090061439-$.DID. OR US-20080087826-$.DID. OR US-20060286566-$.DID. OR US-20060024678-$.DID. OR US-20080213770-$.DID. OR US-20080103058-$.DID. OR US-20090029385-$.DID. OR US-20090068655-$.DID.) or (US-20090024331-$.DID. OR US-20080206764-$.DID. OR US-20090026082-$.DID.)) and sequencing and duplicate	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015

((US-20130231253-$.DID. OR US-20060024711-$.DID. OR US-20090061439-$.DID. OR US-20080087826-$.DID. OR US-20060286566-$.DID. OR US-20060024678-$.DID. OR US-20080213770-$.DID. OR US-20080103058-$.DID. OR US-20090029385-$.DID. OR US-20090068655-$.DID.) or (US-20090024331-$.DID. OR US-20080206764-$.DID. OR US-20090026082-$.DID.)) and sequencing and random near3 (barcod$8 or index$8)	PGPB	n/a	ADJ	YES	ASSIGNEE	05-13-2015
((US-6251639-$.DID. OR US-5750341-$.DID. OR US-6306597-$.DID. OR US-5969119-$.DID. OR US-7244559-$.DID. OR US-7335762-$.DID. OR US-7211390-$.DID. OR US-7244567-$.DID.) or (US-7264929-$.DID. OR US-7323305-$.DID. OR US-7501245-$.DID. OR US-7491498-$.DID. OR US-7276720-$.DID. OR US-7462452-$.DID. OR US-7476504-$.DID. OR US-7405281-$.DID. OR US-7170050-$.DID. OR US-7462468-$.DID. OR US-7476503-$.DID.) or (US-7315019-$.DID. OR US-7302146-$.DID. OR US-7313318-$.DID. OR US-6449562-$.DID. OR US-6287766-$.DID. OR US-7361468-$.DID. OR US-6225109-$.DID. OR US-6110709-$.DID.)) and sequencing and duplicate	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015
((US-6251639-$.DID. OR US-5750341-$.DID. OR US-6306597-$.DID. OR US-5969119-$.DID. OR US-7244559-$.DID. OR US-7335762-$.DID. OR US-7211390-$.DID. OR US-7244567-$.DID.) or (US-7264929-$.DID. OR US-7323305-$.DID. OR US-7501245-$.DID. OR US-7491498-$.DID. OR US-7276720-$.DID. OR US-7462452-$.DID. OR US-7476504-$.DID. OR US-7405281-$.DID. OR US-7170050-$.DID. OR US-7462468-$.DID. OR US-7476503-$.DID.) or (US-7315019-$.DID. OR US-7302146-$.DID. OR US-7313318-$.DID. OR US-6449562-$.DID. OR US-6287766-$.DID. OR	USPT	n/a	ADJ	YES	ASSIGNEE	05-13-2015

US-7361468-$.DID. OR US-6225109-$.DID. OR US-6110709-$.DID.)) and sequencing and random near3 (barcod$8 or index$8)						
sequencing and reads near3 (duplicat$7 or redundan$8) and (barcod$8 or index$8) near3 (random$8 or degener$8)	USPT	10	ADJ	YES	ASSIGNEE	02-24-2016
sequencing and reads near3 (duplicat$7 or redundan$8) and (barcod$8 or index$8) near3 (random$8 or degener$8)	PGPB	33	ADJ	YES	ASSIGNEE	02-24-2016
sequencing and duplicate near3 remov$8	USPT	314	ADJ	YES	ASSIGNEE	02-24-2016
(sequencing and duplicate near3 remov$8) and duplicate near3 (barcod$7 or index$7 or tag$7)	USPT	9	ADJ	YES	ASSIGNEE	02-24-2016
sequencing and duplicate near3 read and duplicate near3 remov$8	USPT	9	ADJ	YES	ASSIGNEE	02-24-2016
sequencing and duplicate near3 read and duplicate near3 remov$8	PGPB	58	ADJ	YES	ASSIGNEE	02-24-2016
(sequencing and duplicate near3 read and duplicate near3 remov$8) and (barcod$5 or index$7 or tag$7)	PGPB	52	ADJ	YES	ASSIGNEE	02-24-2016
sequencing and duplicate near3 read and duplicate near3 remov$8	DWPI	2	ADJ	YES	ASSIGNEE	02-24-2016
WO-2014171898-A2.did.	EPAB	0	ADJ	YES	ASSIGNEE	02-24-2016

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14540917	AMORESE ET AL.
	Examiner	Art Unit
	TERESA E STRZELECKA	1637

CPC- SEARCHED		
Symbol	Date	Examiner
C12Q1/6869 | C12Q2563/179 | C12Q2535/122 |C12Q2537/1376 | C12Q2563/159 | C12Q1/6806 |C12Q2521/319 | C12Q2521/507 | C12Q2525/15 |C12Q2525/155 | C12Q2525/161 | C12Q2525/185 |C12Q2525/191 | C12Q2525/197	5/13/15	/TS/

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
IDS (12/4/14; 3/21/15)	5/13/15	/TS/
WEST	5/13/15	/TS/
Google	5/13/15	/TS/
Inventor name search	5/13/15	/TS/
IDS (8/19/15)	9/21/15	/TS/
Inventor name search	2/23/16	/TS/
Google	2/23/16	/TS/
BLAST	2/23/16	/TS/
WEST	2/24/16	/TS/

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

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How PCR **duplicates** arise in **next-generation sequencing**
www.cureffi.org/.../how-pcr-**duplicates**-arise-in-**next-generation-sequencing**/
Dec 11, 2012 ... This will let you have the fraction of your final **reads** that are PCR **duplicates** can be as low as 4%. Higher rates of PCR **duplicates** e.g. 30% ...

What Is Pcr **Duplicates** Of Ngs? - BioStar
https://www.biostars.org/p/15818/
Is it **duplicates** when preparing libraries, or produced during bridge amplification step of Illumina ... Or, only 1 out of 1000000 of our **reads** is really valuable?? This is ... What'S The Meaning Of "Bridge Pcr" In The **Next Generation Sequencing**.

Read **Duplicates** - BioStar
https://www.biostars.org/p/47229/
I am doing differential gene expression analysis by **next-generation sequencing**. NGS generates read **duplicates** and there are several programs available for the ... An artificial site tends to show heavy imbalances by strand, with most **reads**

[PDF] **Next-Generation Sequencing** Technologies - Navin lab
www.navinlab.com/bioinfo/**Next-Gen-Sequencing**_NAVIN.pdf
Next-Generation Sequencing ... **Generations** of **Sequencing** Technologies ..... PCR **duplicates** usually constitute about 10% of the total sequence **reads**.

Next Generation Sequencing (NGS)/Alignment - Wikibooks, open ...
https://en.wikibooks.org/.../**Next_Generation_Sequencing**.../Alignment
Next-generation sequencing generally produces short **reads** or short read pairs, ... A related error would be PCR **duplicates**, where the same read pair occurs ...
Introduction · Short reads · Alignment · Software packages

Removing **duplicates** is it really necessary? - SEQanswers
seqanswers.com/forums/showthread.php?t=6854
If you define **duplicates** as **reads** with identical sequence you may underestimate the ... of the **reads** avoids the influence of library **sequencing** error rate ... to PCR amplification bias is to reduce their **generation** in the first place.

How important are those NGS metrics? - Integrated DNA Technologies
https://www.idtdna.com/.../how-important-are-those-ngs-metrics
Jun 12, 2014 ... **Duplicate reads**, especially in paired-end sequencing, are assumed to be .... for targeted **next generation sequencing** of defined gene families..

FastUniq: A Fast De Novo **Duplicates** Removal Tool for Paired Short ...
www.ncbi.nlm.nih.gov/pmc/articles/PMC3527383/
Dec 20, 2012 ... The presence of **duplicates** introduced by PCR amplification is a major issue in paired short **reads** from **next-generation sequencing** platforms.

[PDF] Bias from removing read duplication in ultra-deep **sequencing** ...
bioinformatics.oxfordjournals.org/content/.../bioinformatics.btt771.full.pdf
Jan 2, 2014 ... read duplication is non-negligible, and removing **duplicate reads** ... **reads** from **next-generation sequencing** (NGS) experiments (Mardis, 2011).

Duplicate reads removal - OMICtools
omictools.com/**duplicate-reads**-removal-category
Duplicate read removal software tools | High-throughput . ... amplification is a major issue in paired short **reads** from **next-generation sequencing** platforms.

Next Gen Sequencing - Power your science on Google Cloud

cloud.google.com/genomics
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

Filed *via* EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT/RESPONSE TO OFFICE ACTION

This paper is filed in response to the Office Action dated March 3, 2016. Please amend the above-identified patent application in the following manner:

Amendments to the Claims begin on page 2 of this paper.

Remarks/Arguments begin on page 6 of this paper.

Electronic Patent Application Fee Transmittal

Application Number:	14540917
Filing Date:	13-Nov-2014
Title of Invention:	COMPOSITIONS AND METHODS FOR IDENTIFICATION OF A DUPLICATE SEQUENCING READ
First Named Inventor/Applicant Name:	Douglas AMORESE
Filer:	Jeffrey R. Bolland
Attorney Docket Number:	NUGN-001/01US

Filed as Small 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($)
Miscellaneous:				
Submission- Information Disclosure Stmt	2806	1	90	90
		Total in USD ($)		**90**

CLAIMS:

1. (Currently Amended) A method for detecting a duplicate sequencing read from a population of sample sequencing reads comprising:

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

        (i) an indexing primer binding site;

        (ii) an indexing site;

        (iii) an identifier site consisting of between 1 and 8 nucleotides; and

        (iv) a target sequence primer binding site;

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

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

    d) identifying a duplicate sequencing read as the sequencing read comprising the same identifier site and nucleic acid fragment as another sequencing read in the population of sequencing reads;

    wherein the indexing site is unique amongst a subset of the plurality of nucleic acid fragments and is an index for multiple polynucleotides; and wherein the sequence of the identifier site is variable in sequence content in a plurality of adaptors.

2. (Original) The method of claim 1, wherein the method further comprises removing from the population of sequence reads the sequencing read with a duplicate identifier site and target sequence.

3. (Original) The method of claim 1, wherein the identifier site is sequenced with the indexing site.

4. (Cancelled)

5. (Cancelled)

6. (Cancelled)

7. (Original) The method of claim 1, wherein the adaptor comprises from 5' to 3':

    (i) the indexing primer binding site;

    (ii) the indexing site;

    (iii) the identifier site; and

    (iv) the target sequence primer binding site.

8. (Cancelled)

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

10. (Previously Presented) The method of claim 9, wherein the nucleic acid fragments from each sample have the same indexing site, and each sample has a unique indexing site.

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

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

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

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

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

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

17. (Previously Presented) The method of claim 1, wherein the identifier site is 6 nucleotides in length.

18. (Previously Presented) The method of claim 1, wherein the identifier site is 8 nucleotides in length.

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

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

21. (Cancelled)

22. (Cancelled)

23. (Cancelled)

24. (Previously Presented) The method of claim 10, wherein the method further comprises separating fragments or sequencing reads based on indexing site.

25. (New) A method for detecting a duplicate sequencing read from a population of sample sequencing reads comprising:

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

(i) an indexing primer binding site;

(ii) an indexing site;

(iii) an identifier site consisting of between 1 and 8 nucleotides; and

(iv) a target sequence primer binding site;

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

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

d) identifying a duplicate sequencing read as the sequencing read comprising the same identifier site and nucleic acid fragment as another sequencing read in the population of sequencing reads;

wherein the indexing site is unique amongst a subset of the plurality of nucleic acid fragments and is an index for multiple polynucleotides; and wherein the sequence of the identifier site is not variable in sequence content in a plurality of adaptors.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. §1.97(c)

In accordance with the duty of disclosure set forth in 37 C.F.R. §1.56, Applicant(s) hereby submits the following information in conformance with 37 C.F.R. §§1.97 and 1.98.

☒ Pursuant to 37 C.F.R. §1.98, a copy of each non-US patent document cited in the attached Form PTO/SB/08 is enclosed.

☐ No copies of the publications listed on the attached Form PTO/SB/08 are being provided pursuant to 37 C.F.R. §1.98(d) because the publications were previously cited by or submitted to the Office in prior Application Serial No. _____ to which the above-identified application claims priority under 35 U.S.C. §120.

☒ No copies of any U.S. patents or U.S. patent application publications listed on the attached Form PTO/SB/08 are being provided pursuant to 37 C.F.R. §1.98.

☐ Publications listed on the attached Form PTO/SB/08 were cited in a foreign search or examination report corresponding to _____ application serial no. _____ and mailed on _____

☐ Enclosed is a copy of a non-English publication(s) _____. Pursuant to §609 of the M.P.E.P., Applicant submits the attached foreign search or examination report, which cites such non-English language publication(s).

☐ Enclosed is a copy of a non-English publication(s) _____ English language publication _____ (copy enclosed) claims priority from this non-English publication.

☐ Enclosed is an explanation of non-English publication(s) _____ for which an English translation is not available.

☐ Enclosed is an English translation of non-English publication(s) _____ cited in the attached Form PTO/SB/08.

☐ Enclosed is a copy of pending patent Application Serial No. _____

This Information Disclosure Statement is filed after the period specified in 37 C.F.R. § 1.97(b), but before the mailing of any of the following:

☒ a final action under 37 C.F.R. §1.113;

☒ a notice of allowance under 37 C.F.R. §1.311; or

☒ an action that otherwise closes prosecution in this application.

In accordance with 37 C.F.R. §1.97(c) also enclosed is:

☐ Fee under 37 C.F.R. §1.17(p) in the amount of $180.00;

☒ Fee under 37 C.F.R. §1.17(p) in the amount of $90.00;

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☐ Statement as specified in 37 C.F.R. §1.97(e):

☐ Each item of information contained in the Information Disclosure Statement cited herein was first cited in a communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing date of the Information Disclosure Statement; or

☐ No item of information contained in the Information Disclosure Statement submitted herewith was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the undersigned, having made a reasonable inquiry, no item of information contained in the Information Disclosure Statement was known to any individual designated in 37 C.F.R. §1.56(c) more than three months prior to the filing date of the Information Disclosure Statement.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Form PTO/SB/08 to the undersigned.

Dated: May 31, 2016

Respectfully submitted,
COOLEY LLP

COOLEY LLP
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By: /Jeffrey R. Bolland/
Jeffrey R. Bolland, Ph.D.
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(74) Agents: **ANDRES, Charles, Jr.** et al.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).

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

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(54) Title: MASSIVELY PARALLEL SINGLE CELL ANALYSIS

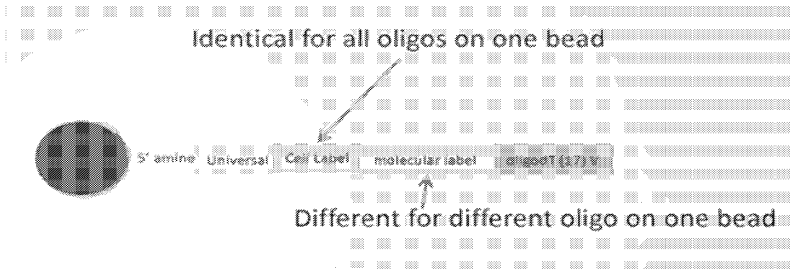

FIG. 1

(57) Abstract: The disclosure provides for methods, compositions, and kits for multiplex nucleic acid analysis of single cells. The methods, compositions and systems may be used for massively parallel single cell sequencing. The methods, compositions and systems may be used to analyze thousands of cells concurrently. The thousands of cells may comprise a mixed population of cells (e.g., cells of different types or subtypes, different sizes).

WO 2015/031691 A1

MASSIVELY PARALLEL SINGLE CELL ANALYSIS

CROSS REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 62/012,237, filed on June 13, 2014, U.S. Provisional Application No. 61/952,036, filed on March 12, 2014, and U.S. Provisional Application No. 61/871,232, filed on August 28, 2013, each of which applications are incorporated herein by reference in their entirety.

BACKGROUND

[0002] Multicellular masses, such as tissues and tumors, may comprise a heterogeneous cellular milieu. These complex cellular environments may often display multiple phenoytpes, which may be indicative of multiple genotypes. Distilling multicellular complexity down to single cell variability is an important facet of understanding multicellular heterogeneity. This understanding may be important in the development of therapeutic regimens to combat diseases with multiple resistance genotypes.

SUMMARY OF THE INVENTION

[0003] One aspect provided is a method, comprising obtaining a sample comprising a plurality of cells; labeling at least a portion of two or more polynucleotide molecules, complements thereof, or reaction products therefrom, from a first cell of the plurality and a second cell of the plurality with a first same cell label specific to the first cell and a second same cell label specific to the second cell; and a molecular label specific to each of the two or more polynucleotide molecules, complements thereof, or reaction products therefrom, wherein each molecular label of the two or more polynucleotide molecules, complements thereof, or reaction products therefrom, from the first cell are unique with respect to each other, and wherein each molecular label of the two or more polynucleotide molecules, complements thereof, or reaction products therefrom, from the second cell are unique with respect to each other. In some embodiments, the method further comprises sequencing the at least a portion of two or more polynucleotide molecules, complements thereof, or reaction products therefrom. In some embodiments, the method further comprises analyzing sequence data from the sequencing to identify a number of individual molecules of the polynucleotides in a specific one of the cells. In some embodiments, the cells are cancer cells. In some embodiments, the cells are infected with viral polynucleotides. In some embodiments, the cells are bacteria or fungi. In some embodiments, the sequencing comprises sequencing with

read lengths of at least 100 bases. In some embodiments, the sequencing comprises sequencing with read lengths of at least 500 bases. In some embodiments, the polynucleotide molecules are mRNAs or micro RNAs, and the complements thereof and reaction products thereof are complements of and reaction products therefrom the mRNAs or micro RNAs. In some embodiments, the molecular labels are on a bead. In some embodiments, the label specific to an individual cell is on a bead. In some embodiments, the label specific to an individual cell and the molecular labels are on beads. In some embodiments, the method is performed at least in part in an emulsion. In some embodiments, the method is performed at least in part in a well or microwell of an array. In some embodiments, the presence of a polynucleotide that is associated with a disease or condition is detected. In some embodiments, the disease or condition is a cancer. In some embodiments, at least a portion of a microRNA, complement thereof, or reaction product therefrom is detected. In some embodiments, the disease or condition is a viral infection.In some embodiments, the viral infection is from an enveloped virus. In some embodiments, the viral infection is from a non-enveloped virus. In some embodiments, the virus contains viral DNA that is double stranded. In some embodiments, the virus contains viral DNA that is single stranded. In some embodiments, the virus is selected from the group consisting of a pox virus, a herpes virus, a vericella zoster virus, a cytomegalovirus, an Epstein-Barr virus, a hepadnavirus, a papovavirus, polyomavirus, and any combination thereof. In some embodiments, the first cell is from a person not having a disease or condition and the second cell is from a person having the disease or condition. In some embodiments, the persons are different. In some embodiments, the persons are the same but cells are taken at different time points. In some embodiments, the first cell is from a person having the disease or condition and the second cell is from the same person. In some embodiments, the cells in the sample comprise cells from a tissue or organ. In some embodiments, the cells in the sample comprise cells from a thymus, white blood cells, red blood cells, liver cells, spleen cells, lung cells, heart cells, brain cells, skin cells, pancreas cells, stomach cells, cells from the oral cavity, cells from the nasal cavity, colon cells, small intestine cells, kidney cells, cells from a gland, brain cells, neural cells, glial cells, eye cells, reproductive organ cells, bladder cells, gamete cells, human cells, fetal cells, amniotic cells, or any combination thereof.

[0004] One aspect provided is a solid support comprising a plurality of oligonucleotides each comprising a cellular label and a molecular label, wherein each cellular label of the plurality of oligonucleotides are the same, and each molecular label of the plurality of oligonucleotides are different; and wherein the solid support is a bead,the cellular label is specific to the solid

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support, the solid support, when placed at the center of a three dimensional Cartesian coordinate system, has oligonucleotides extending into at least seven of eight octants, or any combination thereof. In some embodiments, the plurality of oligonucleotides further comprises at least one of a sample label; a universal label; and a target nucleic acid binding region. In some embodiments, the solid support comprises the target nucleic acid binding region, wherein the target nucleic acid binding region comprises a sequence selected from the group consisting of a gene-specific sequence, an oligo-dT sequence, a random multimer, and any combination thereof. In some embodiments, the solid support further comprises a target nucleic acid or complement thereof. In some embodiments, the solid support comprises a plurality of target nucleic acids or complements thereof comprising from about 0.01% to about 100% of transcripts of a transcriptome of an organism or complements thereof, or from about 0.01% to about 100% of genes of a genome of an organism or complements thereof. In some embodiments, the cellular labels of the plurality of oligonucleotides comprise a first random sequence connected to a second random sequence by a first label linking sequence; and the molecular labels of the plurality of oligonucleotides comprise random sequences. In some embodiments, the solid support is selected from the group consisting of a polydimethylsiloxane (PDMS) solid support, a polystyrene solid support, a glass solid support, a polypropylene solid support, an agarose solid support, a gelatin solid support, a magnetic solid support, a pluronic solid support, and any combination thereof. In some embodiments, the plurality of oligonucleotides comprise a linker comprising a linker functional group, and the solid support comprises a solid support functional group; wherein the solid support functional group and linker functional group connect to each other. In some embodiments, the linker functional group and the solid support functional group are individually selected from the group consisting of C6, biotin, streptavidin, primary amine(s), aldehyde(s), ketone(s), and any combination thereof. In some embodiments, molecular labels of the plurality of oligonucleotides comprise at least 15 nucleotides.

[0005] One aspect provided is a kit comprising any of the solid supports described herein, and instructions for use. In some embodiments, the kit further comprises a well. In some embodiments, the well is comprised in an array. In some embodiments, the well is a microwell. In some embodiments, the kit further comprises a buffer. In some embodiments, the kit is contained in a package. In some embodiments, the package is a box. In some embodiments, the package or box has a volume of 2 cubic feet or less. In some embodiments, the package or box has a volume of 1 cubic foot or less.

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[0006] One aspect provided is an emulsion comprising any of the solid supports described herein.

[0007] One aspect provided is a composition comprising a well and any of the solid supports described herein.

[0008] One aspect provided is a composition comprising a cell and any of the solid supports described herein.

[0009] In some embodiments, the emulsion or composition further comprises a cell. In some embodiments, the cell is a single cell. In some embodiments, the well is a microwell. In some embodiments, the microwell has a volume ranging from about 1,000 μm^3 to about 120,000 μm^3.

[0010] One aspect provided is a method, comprising contacting a sample with any solid support disclosed herein, hybridizing a target nucleic acid from the sample to an oligonucleotide of the plurality of oligonucleotides. In some embodiments, the method further comprises amplifying the target nucleic acid or complement thereof. In some embodiments, the method further comprises sequencing the target nucleic acid or complement thereof, wherein the sequencing comprises sequencing the molecular label of the oligonucleotide to which the target nucleic acid or complement thereof is bound. In some embodiments, the method further comprises determining an amount of the target nucleic acid or complement thereof, wherein the determining comprises quantifying levels of the target nucleic acid or complement thereof; counting a number of sequences comprising the same molecular label; or a combination thereof. In some embodiments, the method does not comprise aligning any same molecular labels or any same cellular labels. In some embodiments, the amplifying comprises reverse transcribing the target nucleic acid. In some embodiments, the amplifying employs a method selected from the group consisting of: PCR, nested PCR, quantitative PCR, real time PCR, digital PCR, and any combination thereof. In some embodiments, the amplifying is performed directly on the solid support; on a template transcribed from the solid support; or a combination thereof. In some embodiments, the sample comprises a cell. In some embodiments, the cell is a single cell. In some embodiments, the contacting occurs in a well. In some embodiments, the well is a microwell and is contained in an array of microwells.

[0011] One aspect provided is a device, comprising a plurality of microwells, wherein each microwell of the plurality of microwells has a volume ranging from about 1,000 μm^3 to about 120,000 μm^3. In some embodiments, each microwell of the plurality of microwells has a volume of about 20,000 μm^3. In some embodiments, the plurality of microwells comprises

from about 96 to about 200,000 microwells. In some embodiments, the microwells are comprised in a layer of a material. In some embodiments, at least about 10 % of the microwells further comprise a cell. In some embodiments, the device further comprises any of the solid supports described herein.

[0012] One aspect provided is an apparatus comprising any of the devices described herein, and a liquid handler. In some embodiments, the liquid handler delivers liquid to the plurality of microwells in about one second. In some embodiments, the liquid handler delivers liquid to the plurality of microwells from a single input port. In some embodiments, the apparatus further comprises a magnet. In some embodiments, the apparatus further comprises at least one of: an inlet port, an outlet port, a pump, a valve, a vent, a reservoir, a sample collection chamber, a temperature control apparatus, or any combination thereof. In some embodiments, the apparatus comprises the sample collection chamber, wherein the sample collection chamber is removable from the apparatus. In some embodiments, the apparatus further comprises an optical imager. In some embodiments, the optical imager produces an output signal which is used to control the liquid handler. In some embodiments, the apparatus further comprises a thermal cycling mechanism configured to perform a polymerase chain reaction (PCR) amplification of oligonucleotides.

[0013] One aspect provided is a method of producing a clinical diagnostic test result, comprising producing the clinical diagnostic test result with any device or apparatus described herein; any solid support described herein; any method described herein; or any combination thereof. In some embodiments, the clinical diagnostic test result is transmitted via a communication medium.

[0014] One aspect provided is a method of making any of the solid supports described herein, comprising attaching to a solid support: a first polynucleotide comprising a first portion of the cellular label, and a first linker; andcontacting a second polynucleotide comprising a second portion of the cellular label, a sequence complementary to the first liker, and the molecular label. In some embodiments, the third polynucleotide further comprises a target nucleic acid binding region.

[0015] In some embodiments, an emulsion, microwell, or well contains only one cell. In some embodiments, from 1 to 2,000,000 emulsions, microwells, or wells each contain only one cell. In some embodiments, the method comprises distributing at most one cell into each emulsion, microwell, or well. In some embodiments, a single solid support and a single cell are distributed to an emulsion, microwell, or well. In some embodiments, from 1 to 2,000,000 emulsions, microwells, or wells each have distributed thereto one cell and one solid support.

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In some embodiments, the method comprises distributing at most one solid support per emulsion, microwell, or well. In some embodiments, the method comprises distributing one solid support and one cell to each of from 1 to 2,000,000 microwells, emulsions, or wells. In some embodiments, cell distribution is random or non-random. In some embodiments, cell distribution is stochastic. In some embodiments, a cell is distributed by a cell sorter. In some embodiments, a cell is distributed by contacting one or more wells, microwells, or emulsions with a dilute solution of cells diluted so that at most one cell is distributed to the one or more wells, microwells, or emulsions.

[0016] In some embodiments, the target specific regions, target specific regions of the plurality of oligonucleotides, or the target specific region of the two or more polynucleotide molecules, comprise sequences complementary to two or more targets of a target panel. In some embodiments, the two or more targets of the target panel are biomarkers. In some embodiments, the biomarkers are biomarkers for a disease or condition. In some embodiments, the disease or condition is a cancer, an infection, a viral infection, an inflammatory disease, a neurodegenerative disease, a fungal disease, a bacterial infection, or any combination thereof. In some embodiments, the panel comprises from: 2-50,000, 2-40,000, 2-30,000, 2-20,000, 2-10,000, 2-9000, 2-8,000, 2-7,000, 2-6,000, 2-5,000, 2-1,000, 2-800, 2-700, 2-600, 2-500, 2-400, 2-300, 2-200, 2-100, 2-75, 2-50, 2-40, 2-30, 2-20, 2-10, or 2-5 biomarkers.

INCORPORATION BY REFERENCE

[0017] 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

[0018] 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 of which:

[0019] **FIG. 1** depicts an exemplary solid support conjugated with an exemplary oligonucleotide.

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[0020] FIG. 2A-C depicts an exemplary workflow for synthesizing oligonucleotide coupled beads using split-pool synthesis.

[0021] FIG. 3 depicts an exemplary oligonucleotide coupled bead.

[0022] FIG. 4 illustrates an exemplary embodiment of a microwell array.

[0023] FIG. 5 depicts an exemplary distribution of solid supports in a microwell array.

[0024] FIG. 6A-C show exemplary distribution cells onto microwell arrays. FIG. 6A shows the distribution of K562 cells (large cell size). FIG. 6B shows the distribution of Ramos cells (small cell size). FIG. 6C shows the distribution of Ramos cells and oligonucleotide coupled beads onto microwell arrays, with solid arrows pointing to the Ramos cells and dashed arrows pointing to the oligonucleotide coupled beads.

[0025] FIG. 7 shows exemplary statistics of the microwell volume, solid support volume, and amount of biological material obtained from lysis.

[0026] FIG. 8A-C illustrates an exemplary embodiment of bead cap sealing. FIG. 8A-B show images of a microarray well with cells and oligonucleotide beads distributed into wells of a microarray well and with larger sephadex beads used to seal the wells. Dotted arrows point to the cells, dashed arrows point to the oligonucleotide coupled beads and the solid arrows point to the sephadex beads. FIG. 8C depicts a schematic of the cell and oligonucleotide bead (*e.g.*, oligobead) deposited within a well with a sephadex bead used to seal the well.

[0027] FIG. 9 depicts a bar graph comparing amplification efficiency of GAPDH and RPL19 amplified from microwells and tubes. The grey bars represent data from the microwell. The white bars represent data from the tube.

[0028] FIG. 10 depicts an agarose gel comparing amplification specificity of three different genes directly on a solid support.

[0029] FIG. 11A-I show graphical representations of the sequencing results.

[0030] FIG. 12A-C show a histogram of the sequencing results for the K562-only sample, Ramos-only sample, and K562 + Ramos mixture sample, respectively.

[0031] FIG. 12D-E shows a graph of the copy number for genes listed in Table 3 for the Ramos-only cell sample and K562-only cell sample, respectively.

[0032] FIG. 12F-I show the copy number for individual genes.

[0033] FIG. 12J-M show graphs of the number of unique molecules per gene (y-axis) for the beads with the 100 unique barcode combinations.

[0034] FIG. 12N-O show enlarged graphs of two beads that depict the general pattern of gene expression profiles for the two cell types.

[0035] FIG. 12P shows a scatter plot of results based on principal component analysis of gene expression profile of 768 beads with > 30 molecules per bead from the K562+Ramos mixture sample.

[0036] FIG. 12Q-R show histograms of the copy number per amplicon per bead for the K562-like cells (beads on the left of the first principal component based on FIG. 12P) and Ramos-like cells (beads on the right of the first principal component based on FIG. 12P), respectively.

[0037] FIG. 12S-T show the copy number per bead or single cell of the individual genes for the K562-like cells (beads on the left of the first principal component based on FIG. 12P) and Ramos-like cells (beads on the right of the first principal component based on FIG. 12P), respectively.

[0038] FIG. 13A depicts general gene expression patterns for the mouse and Ramos cells.

[0039] FIG. 13B-C show scatter plots of results based on principal component analysis of gene expression profile of the high density sample and low density sample, respectively.

[0040] FIG. 13D-E depict graphs of the read per barcode (bc) combination (y-axis) versus the unique barcode combination, sorted by the total number of molecules per bc combination (x-axis) for Ramos-like cells and mouse-like cells from the high density sample, respectively.

[0041] FIG. 13F-G depict graphs of the number of molecules per barcode (bc) combination (y-axis) versus the unique barcode combination, sorted by the total number of molecules per bc combination (x-axis) for Ramos-like cells and mouse-like cells from the high density sample, respectively.

[0042] FIG. 13H-I depict graphs of the read per barcode (bc) combination (y-axis) versus the unique barcode combination, sorted by the total number of molecules per barcode combination (x-axis) for Ramos-like cells and mouse-like cells from the low density sample, respectively.

[0043] FIG. 13J-K depict graphs of the number of molecules per barcode combination (y-axis) versus the unique barcode combination, sorted by the total number of molecules per barcode combination (x-axis) for Ramos-like cells and mouse-like cells from the low density sample, respectively.

[0044] FIG. 14 shows a graph depicting the genes on the X-axis and the log10 of the number of reads.

[0045] FIG. 15A shows a graph of the distribution of genes detected per three-part cell label (*e.g.*, cell barcode). **FIG. 15B** shows a graph of the distribution of unique molecules detected per bead (expressing the gene panel).

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[0046] FIG. 16 depicts the cell clusters based on the genes associated with a cell barcode.

[0047] FIG. 17A-D show the analysis of monocyte specific markers. **FIG. 17E** shows the cell cluster depicted in FIG. 16.

[0048] FIG. 18A-B show the analysis of the T cell specific markers. **FIG. 18C** shows the cell cluster depicted in FIG. 16.

[0049] FIG. 19A-B show the analysis of the CD8+ T cell specific markers. **FIG. 19C** shows the cell cluster depicted in FIG. 16.

[0050] FIG. 20A shows the analysis of CD4+ T cell specific markers. **FIG. 20B** shows the cell cluster depicted in FIG. 16.

[0051] FIG. 21A-D show the analysis of Natural Killer (NK) cell specific markers. **FIG. 20E** shows the cell cluster depicted in FIG. 16.

[0052] FIG. 22A-E show the analysis of B cell specific markers. **FIG. 22F** shows the cell cluster depicted in FIG. 16.

[0053] FIG. 23A-F show the analysis of Toll-like receptors. Toll-like receptors are mainly expressed by monocytes and some B cells. FIG. 23G shows the cell cluster depicted in FIG. 16.

[0054] FIG. 24 depicts a graph of the genes versus the log10 of the number of reads.

[0055] FIG. 25A-D shows graphs of the molecular barcode versus the number of reads or log10 of the number of reads for two genes.

[0056] FIG. 26A shows a graph of the number of genes in the panel expressed per cell barcode versus the number of unique cell barcodes/single cell. **FIG. 26B** shows a histogram of the number of unique molecules detected per bead versus frequency of the number of cells per unique cell barcode carrying a given number of molecules. **FIG. 26C** shows a histogram of the number of unique GAPDH molecules detected per bead versus frequency of the number of cells/unique cell barcode carrying a given number of molecules.

[0057] FIG. 27 shows a scatterplot of the 856 cells.

[0058] FIG. 28 shows a heat map of expression of the top 100 (in terms of the total number of molecules detected).

[0059] FIG. 29 shows a workflow for Example 12.

[0060] FIG. 30 shows a workflow for Example 13.

[0061] FIG. 31A-C. Clustering of single cells in controlled mixtures containing two distinct cell types. A. Clustering of a 1:1 mixture of K562 and Ramos cells by principal component analysis of the expression of 12 genes. The biplot shows two distinct clusters, with one cluster expressing Ramos specific genes and the other expressing K562 specific genes. B.

Principal component analysis of a mixture containing a small percentage of Ramos cells in a background of primary B cells from a healthy individual using a panel of 111 genes. The color of each data point indicates the total number of unique transcript molecules detected across the entire gene panel. A set of 18 cells (circled) out of 1198 cells displays a distinct gene expression profile and with much higher transcription levels. C. Heatmap showing expression level of each gene in the top 100 cells in the sample of FIG. 31B, ranked by the total number of transcript molecules detected in the gene panel. Genes are ordered via hierarchical clustering in terms of correlation. The top 18 cells, indicated by the horizontal red bar, expressed preferentially a set of genes known to be associated with follicular lymphoma, as indicated by the vertical red bar.

[0062] FIG. 31D. PCA analysis of primary B cells with spiked in Ramos cells. Color of each data point (single cell) indicates the log of the number of transcript molecules each cell carries for the particular gene. Top 7 rows: Genes that are preferentially expressed by the subset of 18 cells that are likely Ramos cells. First row genes (from left to right) include GAPDH, TCL1A, MKI67 and BCL6. Second row genes (from left to right) include MYC, CCND3, CD81 and GNAI2. Third row of genes (from left to right) include IGBP1, CD20, BLNK and DOCK8. Fourth row of genes (from left to right) include IRF4, CD22, IGHM and AURKB. Fifth row of genes (from left to right) include CD38, CD10, LEF1 and AICDA. Sixth row of genes (from left to right) include CD40, CD27, IL4R and PRKCD. Seventh row of genes (from left to right) include RGS1, MCL1, CD79a and HLA-DRA. Last row: Genes that are expressed preferentially by a subset of primary B cells but not especially enriched in those 18 cells. Genes in the last row (from left to right) include IL6, CD23a, CCR7 and CXCR5.

[0063] FIG. 32 Expression of GAPDH. Color indicates natural log of the number of unique transcript molecules observed per cell.

[0064] FIG. 33A-F shows the principal component analysis (PCA) for monocyte associated genes. FIG. 33A shows the PCA for CD16. FIG. 33B shows the PCA for CCRvarA. FIG. 33C shows the PCA for CD14. FIG. 33D shows the PCA for S100A12. FIG. 33E shows the PCA for CD209. FIG. 33F shows the PCA for IFNGR1.

[0065] FIG. 34A-B shows the principal component analysis (PCA) for pan-T cell markers (CD3). FIG. 34A shows the PCA for CD3D and FIG. 34B shows the PCA for CD3E.

[0066] FIG. 35A-E shows the principal component analysis (PCA) for CD8 T cell associated genes. FIG. 35A shows the PCA for CD8A. FIG. 35B shows the PCA for EOMES. FIG. 35C

shows the PCA for CD8B. FIG. 35D shows the PCA for PRF1. FIG. 35E shows the PCA for RUNX3.

[0067] FIG. 36A-C shows the principal component analysis (PCA) for CD4 T cell associated genes. FIG. 36A shows the PCA for CD4. FIG. 36B shows the PCA for CCR7. FIG. 36C shows the PCA for CD62L.

[0068] FIG. 37A-F shows the principal component analysis (PCA) for B cell associated genes. FIG. 37A shows the PCA for CD20. FIG. 37B shows the PCA for IGHD. FIG. 37C shows the PCA for PAX5. FIG. 37D shows the PCA for TCL1A. FIG. 37E shows the PCA for IGHM. FIG. 37F shows the PCA for CD24.

[0069] FIG. 38A-C shows the principal component analysis (PCA) for Natural Killer cell associated genes. FIG. 38A shows the PCA for KIR2DS5. FIG. 38B shows the PCA for CD16. FIG. 38C shows the PCA for CD62L.

[0070] FIG. 39 Simultaneous identification of major cell types in a human PBMC sample (632 cells) by PCA analysis of 81 genes assayed by CytoSeq Cells with highly correlated expression profile are coded with similar color.

[0071] FIG. 40A-B Correlation analysis of single cell gene expression profile of PBMC sample. 40A. A matrix showing the pairwise correlation coefficient across 632 cells in the sample. The cells are ordered such that those with highly correlated gene expression profile are grouped together. 40B. Heatmap showing the expression of each gene by each cell. The cells (columns) are ordered in the same manner as the correlation matrix above. The genes (rows) are ordered such that genes that share highly similar expression pattern across the cells are grouped together. The cell type of each cluster of cells may be identified by the group of genes the cells co-expressed. Within each major cell cluster, there is substantial degree of heterogeneity in terms of gene expression.

[0072] FIG. 41 data represents that of 731 cells from a replicate experiment of PBMC sample from the same donor. Cells with similar gene expression profile (based on hierarchical clustering using correlation coefficient) are plotted with similar color.

[0073] FIG. 42 shows a heat map demonstrating the correlation in gene expression profile between genes.

[0074] FIG. 43 Description of CytoSeq. A. Experimental procedure for CytoSeq. B. Structure of oligonucleotides attached to beads.

[0075] FIG. 44 Dissecting sub-populations of CD3+ T cells. A. PCA of Donor 1 unstimulated sample reveals two major branches of cells. The expression level (log of unique transcript molecule) of a particular gene within each cell is indicated with color. Helper T cell

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associated cytokine and effector genes are enriched in cells in the lower branch, while cytotoxic T cell associated genes are enriched in the upper branch. Shown here are representative genes. First row shows helper T cell related genes and include (from left to right) CD4, SELL and CCR7. Second row shows cytotoxic T cell related genes and include (from left to right) CD8A, NKG2D and EOMES. B. PCA of Donor 1 anti-CD3/anti-CD28 stimulated sample showing enrichment of expression of indicated genes to one of the two main branches representing helper and cytotoxic T cells. These genes are present at low amounts in the unstimulated sample. First two rows show genes that are known to be associated with activated T cells and include (from left to right) in the first row IRF4, CD69 and MYC and in the second row GAPDH, TNF and IFNG. The third row shows genes that are known to be associated with activated helper T cells and include (from left to right) IL2, LTA and CD40LG. The fourth row shows genes that are known to be associated with activated cytotoxic T cells and include (from left to right) CCL4, CCL3 and GZMB. C. Number of cells that contribute to the overall expression level of genes that exhibit large fold-changes when comparing stimulated over unstimulated samples in aggregate data. For several cytokines (red arrows), the contribution from only a small number of cells is responsible for large overall gene expression change in the entire population.

[0076] FIG. 45. PCA plots of T cell samples that have undergone stimulation with anti-CD28/anti-CD3 beads in the two donors, and the corresponding unstimulated samples, with emphasis on the expression of genes that clearly show preferential expression in either helper or cytotoxic subsets in the unstimulated samples. The color of each data point (single cell) indicates log(number of unique transcript molecule) per cell for the indicated gene. For each pair of stimulated and unstimulated graphs in each donor, the color range is adjusted to be the same. A. Genes that are known to be associated with both helper and cytotoxic T cells. B. Genes that are known to be associated with cytotoxic T cells. C. Genes that are known to be associated with helper T cells.

[0077] FIG. 46A-D PCA plots of T cell samples that have undergone stimulation with anti-CD28/anti-CD3 beads in the two donors, and the corresponding unstimulated samples, with emphasis on the expression of genes that are expressed in the stimulated samples but at low or undetectable level in the unstimulated samples. The color of each data point (single cell) indicates log(number of unique transcript molecule) per cell for the indicated gene. For each pair of stimulated and unstimulated graphs in each donor, the color range is adjusted to be the same. 46A and 46D. Genes that are expressed by both branches of cells upon activation. 46B. Genes that are expressed preferentially by cells in the upper branch upon activation. These

genes are known to be associated with activated cytotoxic T cells. 46C. Genes that are expressed preferentially by cells in the lower branch upon activation. These genes are known to be associated with activated helper T cells.

[0078] FIG. 47 Clustering of data from Donor 1's unstimulated CD3+ T cells shows separations of CD4 and CD8 cells, as well as a group of cells that express Granzyme K and Granzyme A but little CD8. Top: Heatmap showing correlation between each pair of cells. Cells that are highly correlated are grouped together. Bottom: Heatmap showing the level of expression of each gene of each cell. Cells and genes are ordered via bidirectional hierarchical clustering.

[0079] FIG. 48. Similar to FIG. 47, but showing data from anti-CD3/anti-CD28 stimulated CD3+ T cell sample of Donor 1. Top: Heatmap showing correlation between each pair of cells. Cells that are highly correlated are grouped together. Bottom: Heatmap showing the level of expression of each gene of each cell. Cells and genes are ordered via bidirectional hierarchical clustering.

[0080] FIG. 49A-C In donor 1, large overall fold change was observed for various cytokines in the antiCD28/antiCD3 stimulated sample, as compared to the unstimulated one. A-B: The large fold changes of these cytokines were mostly contributed by only a few single cells (dots that are enclosed with squares or circles). A number of these cytokines were contributed by the same small number of cells. C: The co-expression patterns of these cytokines coincide with the signature cytokine combination for the Th2 and Th17 subsets of helper T cells.

[0081] FIG. 50A-B. Dissecting sub-populations of CD8+ T cells. A. Clustering of CytoSeq data defines two major groups of CD8+ cells – one group expresses genes shared by central memory/naïve cells, and the other group expresses genes shared by effector memory/effector cells. Shown here is data of Donor 2's unstimulated sample. Top: Heatmap showing correlation between each pair of cells. Bottom: Heatmap showing the level of expression of each gene in each cell. Cells and genes are ordered via bidirectional hierarchical clustering. B. Identification of rare antigen specific T cell by expression of gamma interferon (IFNG) in CD8+ T cells from two donors after stimulation with CMV peptide pool. Each cell is plotted on the 2D principal component space. Cells expressing IFNG (circled) are usually among those with the most total detected transcripts in the panel (indicated by the color). In donor 2, the top expressing cell (square) does not produce IFNG but expresses cytokines IL6 and IL1B. Number next to each circle indicates the rank in descending order the number of total unique transcript molecules detected for that cell.

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[0082] FIG. 51. Similar to Figure 50A except the data here represents that of Donor 2 CMV stimulated sample. A. Clustering of CytoSeq data defines two major groups of CD8+ cells – one group expresses genes shared by central memory/naïve cells, and the other group expresses genes shared by effector memory/effector cells. Shown here is data of Donor 2's unstimulated sample. Top: Heatmap showing correlation between each pair of cells. Bottom: Heatmap showing the level of expression of each gene in each cell. Cells and genes are ordered via bidirectional hierarchical clustering.

[0083] FIG. 52. Data are plotted in principal component space. Color indicates log(number of unique transcript molecule detected) for the particular gene. A. Genes that appear to be expressed by larger proportion of cells upon stimulation by CMV peptide pool. B. Genes that are enriched in one branch of cells. These genes are also known to be associated with naïve and central memory CD8+ T cells. C. Genes that are enriched in the other branch of cells. These genes are known to be associated with effector and effector memory CD8+ T cells. D. Granzyme K expressing cells occupy a region between the naïve/central memory and effector/effector memory cells on the PC space. E. HLA-DRA expressing cells constitute a special subset. F. Genes that are expressed in both branches of cells.

[0084] FIG. 53. Same as FIG. 50B, except the data represents those of the unstimulated controls. None of the cells in Donor 1's sample expressed IFNG, while one cell in Donor 2's sample expressed IFNG yet with overall low expression across the entire gene panel (rank 1069). Color scale is adjusted to match that of the respective graph for the stimulated sample.

[0085] FIG. 54. Heatmaps showing the heterogeneous expression of the gene panel in cells that express gamma interferon (IFNG) in CMV stimulated CD8+ T cells of Donors 1 and 2. Also shown is the cell that carries most total transcripts detected in Donor 2. This particular cell does not express IFNG but expresses strongly IL6, IL1B and CCL4. The cells and genes are ordered by bidirectional hierarchical clustering based on correlation. Cell ID refers to the rank in total number of detected transcripts of the gene panel, and are indicated in the PCA plots in FIG. 50.

[0086] FIG. 55. Amplification scheme. The first PCR amplifies molecules attached to the bead using a gene specific primer and a primer against the universal Illumina sequencing primer 1 sequence. The second PCR amplifies the first PCR products using a nested gene specific primer flanked by Illumina sequencing primer 2 sequence, and a primer against the universal Illumina sequencing primer 1 sequence. The third PCR adds P5 and P7 and sample index to turn PCR products into Illumina sequencing library. 150bp x 2 sequencing reveals

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the cell label and molecule label on read 1, the gene on read 2, and the sample index on index 1 read.

[0087] **FIG. 56** depicts a schematic of a workflow for analyzing molecules from a sample.

[0088] **FIG. 57** depicts a schematic of a workflow for analyzing molecules from a sample.

[0089] **FIG. 58A-B** depict agarose gels of PCR products.

[0090] **FIG. 59** depicts a plot of sequencing reads for a plurality of genes.

[0091] **FIG. 60A-D** depicts plots of the reads observed per label detected (RPLD) for Lys, Phe, Thr, and Dap spike-in controls, respectively. FIG. 60E depicts a plot of Reads versus Input.

[0092] **FIG. 61** depicts a plot of the reads observed per label detected (RPLD) for various genes.

[0093] **FIG. 62** depicts a plot of the reads observed per label detected (RPLD) for various genes.

[0094] **FIG. 63** depicts a plot of total reads (labels) versus rpld for various genes.

[0095] **FIG. 64** depicts a plot of RPKM for undetected genes.

[0096] **FIG. 65** depicts a schematic for the synthesis of molecular barcodes.

[0097] **FIG. 66A-C** depict schematics for the synthesis of molecular barcodes.

[0098] **FIG. 67** shows a schematic of a workflow for stochastically labeling nucleic acids.

[0099] **FIG. 68** is a schematic of a workflow for stochastically labeling nucleic acids.

[00100] **FIG. 69** illustrates a mechanical fixture within which microwell array substrates may be clamped, thereby forming a reaction chamber or well into which samples and reagents may be pipetted for performing multiplexed, single cell stochastic labeling / molecular indexing experiments. Upper: exploded view showing the upper and lower parts of the fixture and an elastomeric gasket for forming a leak-proof seal with the microwell array substrate. Lower: exploded side-view of the fixture.

[00101] **FIG. 70** illustrates a mechanical fixture which creates two reaction chambers or wells when a microwell array substrate is clamped within the fixture.

[00102] **FIG. 71** illustrates two examples of elastomeric (*e.g.*, polydimethylsiloxane) gaskets for use with the mechanical fixtures illustrated in FIGS. 69 and 70. The elastomeric gaskets provide for a leak-proof seal with the microwell array substrate to create a reagent well around the microwell array. The gaskets may contain one (upper), two (lower), or more openings for creating reagent wells.

[00103] **FIG. 72** depicts one embodiment of a cartridge within which a microwell array is packaged. Left: An exploded view of the cartridge illustrating (from bottom to top) the

microwell array substrate, a gasket that defines the flow cell or array chamber, a reagent and/or waste reservoir component for defining compartments to contain pre-loaded assay reagents or store spent reagents, and a cover for sealing the reagent and waste reservoirs and defining the sample inlet and outlet ports. Right: An assembled view of one embodiment of the cartridge design illustrating relief for bringing an external magnet into close proximity with the microwell array.

[00104] FIG. 73 depicts one embodiment of a cartridge designed to include onboard assay reagents with the packaged microwell array.

[00105] FIG. 74 provides a schematic illustration of an instrument system for performing multiplexed, single cell stochastic labeling / molecular indexing assay. The instrument system may provide a variety of control and analysis capabilities, and may be packaged as individual modules or as a fully integrated system. Microwell arrays may be integrated with flow cells that are either a fixed component of the system or are removable, or may be packaged within removable cartridges that further comprise pre-loaded assay reagent reservoirs and other functionality.

[00106] FIG. 75 illustrates one embodiment of the process steps to be performed by an automated system for performing multiplexed, single cell stochastic labeling / molecular indexing assays.

[00107] FIG. 76 illustrates one embodiment of a computer system or processor for providing instrument control and data analysis capabilities for the assay system presently disclosed.

[00108] FIG. 77 shows a block diagram illustrating one example of a computer system architecture that can be used in connection with example embodiments of the assay systems of the present disclosure.

[00109] FIG. 78 depicts a diagram showing a network with a plurality of computer systems, cell phones, personal data assistants, and Network Attached Storage (NAS), that can be used with example embodiments of the assay systems of the present disclosure.

[00110] FIG. 79 depicts a block diagram of a multiprocessor computer system that can be used with example embodiments of the assay systems of the present disclosure.

[00111] FIG. 80 depicts a diagram of analysis of a test sample and communication of test result obtained from the test sample via a communication media.

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DETAILED DESCRIPTION

[00112] Disclosed herein are methods, kits, and compositions for analyzing molecules in a plurality of samples. Generally, the methods, kits, and compositions comprise (a) stochastically labeling molecules in two or more samples with molecular barcodes to produce labeled molecules; and (b) detecting the labeled molecules. The molecular barcodes may comprise one or more target specific regions, label regions, sample index regions, universal PCR regions, adaptors, linkers, or a combination thereof. The labeled molecules may comprise a) a molecule region; b) a sample index region; and c) a label region. The molecule region may comprise at least a portion of the molecule from the molecular barcode was originally attached to. The molecule region may comprise a fragment of the molecule from the molecular barcode was originally attached to. The sample index region may be used to determine the source of the molecule region. The sample index region may be used to determine from which sample the molecule region originated from. The sample index region may be used to differentiate molecule regions from two or more different samples. The label region may be used to confer a unique identity to identical molecule regions originating from the same source. The label region may be used to confer a unique identity to identical molecule regions originating from the same sample.

[00113] The method for analyzing molecules in a plurality of samples may comprise: a) producing a plurality of sample-tagged nucleic acids by: i) contacting a first sample comprising a plurality of nucleic acids with a plurality of first sample tags to produce a plurality of first sample-tagged nucleic acids; and ii) contacting a second sample comprising a plurality of nucleic acids with a plurality of second sample tags to produce a plurality of second sample-tagged nucleic acids, wherein the plurality of second sample tags are different from the first sample tags; b) contacting the plurality of sample-tagged nucleic acids with a plurality of molecular identifier labels to produce a plurality of labeled nucleic acids; and c) detecting at least a portion of the labeled nucleic acids, thereby determining a count of a plurality of nucleic acids in a plurality of samples. The plurality of samples may comprise a single cell.

[00114] Alternatively, the method for analyzing molecules in a plurality of samples may comprise: a) producing a plurality of labeled nucleic acids comprising: i) contacting a first sample with a first plurality of sample tags, wherein the first plurality of sample tags comprises identical nucleic acid sequences; ii) contacting the first sample with a first plurality of molecular identifier labels may comprise different nucleic acid sequences, wherein contacting the first sample with the first plurality of sample tags or first plurality of molecular

identifier labels occurs simultaneously or sequentially to produce a plurality of first-labeled nucleic acids; iii) contacting a second sample with a second plurality of sample tags, wherein the second plurality of sample tags may comprise identical nucleic acid sequences; iv) contacting the second sample with a second plurality of molecular identifier labels may comprise different nucleic acid sequences, wherein contacting the second sample with the second plurality of sample tags or second plurality of molecular identifier labels occurs simultaneously or sequentially to produce a plurality of second-labeled nucleic acids, wherein the plurality of labeled nucleic acids may comprise the plurality of first-labeled nucleic acids and the second-labeled nucleic acids; and b) determining a number of different labeled nucleic acids, thereby determining a count of a plurality of nucleic acids in a plurality of samples.

[00115] The method for analyzing molecules in a plurality of samples may comprise: a) contacting a plurality of samples may comprise two or more different nucleic acids with a plurality of sample tags and a plurality of molecular identifier labels to produce a plurality of labeled nucleic acids, wherein: i) the plurality of labeled nucleic acids may comprise two or more nucleic acids attached to two or more sample tags and two or more molecular identifier labels; ii) the sample tags attached to nucleic acids from a first sample of the plurality of samples are different from the sample tags attached to nucleic acid molecules from a second sample of the plurality of samples; and iii) two or more identical nucleic acids in the same sample are attached to two or more different molecular identifier labels; and b) detecting at least a portion of the labeled nucleic acids, thereby determining a count of two or more different nucleic acids in the plurality of samples.

[00116] FIG. 56 depicts an exemplary workflow for the quantification of RNA molecules in a sample. As shown in Step 1 of FIG. 56, RNA molecules (110) may be reverse transcribed to produce cDNA molecules (105) by the stochastic hybridization of a set of molecular identifier labels (115) to the polyA tail region of the RNA molecules. The molecular identifier labels (115) may comprise an oligodT region (120), label region (125), and universal PCR region (130). The set of molecular identifier labels may contain 960 different types of label regions. As shown in Step 2 of FIG. 56, the labeled cDNA molecules (170) may be purified to remove excess molecular identifier labels (115). Purification may comprise Ampure bead purification. As shown in Step 3 of FIG. 56, the labeled cDNA molecules (170) may be amplified to produce a labeled amplicon (180). Amplification may comprise multiplex PCR amplification. Amplification may comprise a multiplex PCR amplification with 96 multiplex primers in a single reaction volume. Amplification may

comprise a custom primer (135) and a universal primer (140). The custom primer (135) may hybridize to a region within the cDNA (105) portion of the labeled cDNA molecule (170). The universal primer (140) may hybridize to the universal PCR region (130) of the labeled cDNA molecule (170). As shown in Step 4, the labeled amplicons (180) may be further amplified by nested PCR. The nested PCR may comprise multiplex PCR with 96 multiplex primers in a single reaction volume. Nested PCR may comprise a custom primer (145) and a universal primer (140). The custom primer (135) may hybridize to a region within the cDNA (105) portion of the labeled amplicon (180). The universal primer (140) may hybridize to the universal PCR region (130) of the labeled amplicon (180). As shown in Step 5, one or more adaptors (150, 155) may be attached to the labeled amplicon (180) to produce an adaptor-labeled amplicon (190). The one or more adaptors may be attached to the labeled amplicon (180) via ligation. As shown in Step 6, the one or more adaptors (150, 155) may be used to conduct one or more additional assays on the adaptor-labeled amplicon (190). The one or more adaptors (150, 155) may be hybridized to one or more primers (160, 165). The one or more primers (160, 165) be PCR amplification primers. The one or more primers (160, 165) may be sequencing primers. The one or more adaptors (150, 155) may be used for further amplification of the adaptor-labeled amplicons. The one or more adaptors (150, 155) may be used for sequencing the adaptor-labeled amplicon.

[00117] FIG. 57 depicts an exemplary schematic of a workflow for analyzing nucleic acids from two or more samples. As shown in FIG. 57, a method for analyzing nucleic acids from two or more samples may comprise selecting two or more genes for analysis and designing custom primers based on the selected genes (210). The method may further comprise supplementing one or more samples comprising nucleic acids (*e.g.*, RNA) with one or more spike-in controls (220). The nucleic acids in the sample may be amplified by multiplex RT-PCR (230) with molecular barcodes (or sample tags or molecular identifier labels) and the custom primers to produce labeled amplicons. The labeled amplicons may further treated with one or more sequencing adaptors to produce adaptor labeled amplicons (240). The adaptor labeled amplicons can be analyzed (250). As shown in FIG. 57, analysis of the labeled amplicons (250) may comprise one or more of (1) detection of a universal PCR primer seq, polyA and/or molecular barcode (or sample tag, molecular identifier label); (2) map read on the end of the adaptor labeled amplicons (*e.g.*, 96 genes and spike-in controls) that is not attached to the adaptor and/or barcode (*e.g.*, molecular barcode, sample tag, molecular identifier label); and (3) count and/or summarize the number of different adaptor labeled amplicons.

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[00118] FIG. 67 shows a schematic of a workflow for stochastically labeling nucleic acids with molecular barcodes (1220). As shown in step 1 of FIG. 67, RNA molecules may be stochastically labeled with a set of molecular barcodes (1220). The molecular barcodes (1220) may comprise a target binding region (1221), label region (1222), sample index region (1223) and universal PCR region (1224). In some instances, the target binding region comprises an oligodT sequence that hybridizes to a polyA sequence in the RNA molecules. The label region (1222) may contain a unique sequence that may be used to distinguish two or more different molecular barcodes. When the molecular barcode hybridizes to an RNA molecule, the label region may be used to confer a unique identity to identical RNA molecules. The sample index region (1223) may be identical for a set of molecular barcodes. The sample index region (1223) may be used to distinguish labeled nucleic acids from different samples. The universal PCR region (1224) may serve as a primer binding site for amplification of the labeled molecules. Once the RNA molecules are labeled with the molecular barcodes, the RNA molecules may be reverse transcribed to produce labeled cDNA molecules (1230) containing a cDNA copy of the RNA molecule (1210) and the molecular barcode (1220).

[00119] As shown in Step 2 of FIG. 67, excess oligos (*e.g.*, molecular barcodes) may be removed by Ampure bead purification. As shown in Step 3 of FIG. 67, the labeled cDNA molecules may be amplified by multiplex PCR. Multiplex PCR of the labeled cDNA molecules may be performed by using a first set of forward primers (F1, 1235 in FIG. 67) and universal primers (1240) in a single reaction volume to produce labeled amplicons (1245). As shown in Step 4 of FIG. 67, the labeled amplicons may be further amplified by multiplex PCR using nested primers. Nested primer amplification of the labeled amplicons may be performed by using a second set of forward primers (F2, 1250 in FIG. 67) and universal primers (1240) in a single reaction volume to produce labeled nested PCR amplicons. In some instances, the F2 primers (1250) contain an adaptor (1251) and a target binding region (1252). The target binding region (1252) of the F2 primers may hybridize to the labeled amplicons and may prime amplification of the labeled amplicons. The adaptor (1251) and the universal PCR region (1224) of the nested PCR amplicons may be used in the sequencing of the labeled nested PCR amplicons. The amplicons may be sequenced by MiSeq. Alternatively, the amplicons may be sequenced by HiSeq.

[00120] FIG. 68 shows a schematic of a workflow for stochastically labeling nucleic acids. As shown in Step 1 of FIG. 68, RNA molecules (1305) may be stochastically labeled with a set molecular barcodes (1320). The molecular barcodes may comprise a target binding region

(1321), label region (1322), and universal PCR region (1323). Once the molecular barcodes are attached to the RNA molecules, the RNA molecules (1305) may be reverse transcribed to produce labeled cDNA molecules (1325) comprising a cDNA copy of the RNA molecule (1310) and the molecular barcode (1320). As shown in Step 2 of FIG. 68, the labeled cDNA molecules may be purified by Ampure bead purification to remove excess oligos (*e.g.*, molecular barcodes). As shown in Step 3 of FIG. 68, the labeled amplicons may be amplified by multiplex PCR. Multiplex PCR of the labeled cDNA molecules may be performed by using a first set of forward primers (F1, 1330 in FIG. 68) and universal primers (1335) in a single reaction volume to produce labeled amplicons (1360). As shown in Step 4 of FIG. 67, the labeled amplicons may be further amplified by multiplex PCR using nested primers. Nested primer amplification of the labeled amplicons may be performed by using a second set of forward primers (F2, 1340 in FIG. 68) and sample index primers (1350) in a single reaction volume to produce labeled nested PCR amplicons. In some instances, the F2 primers (1340) contain an adaptor (1341) and a target binding region (1342). The target binding region (1342) of the F2 primers may hybridize to the labeled amplicons and may prime amplification of the labeled amplicons. The sample index primers (1350) may comprise a universal primer region (1351), sample index region (1352), and adaptor region (1353). As shown in Step 4 of FIG. 68, the universal primer region (1351) of the sample index primer may hybridize to the universal PCR region of the labeled amplicons. The sample index region (1352) of the sample index primer may be used to distinguish two or more samples. The adaptor regions (1341, 1353) may be used to sequence the labeled nested PCR amplicons. The amplicons may be sequenced by MiSeq. Alternatively, the amplicons may be sequenced by HiSeq.

[00121] Further disclosed herein are methods of producing one or more libraries. The one or more libraries may comprise a plurality of labeled molecules. The one or more libraries may comprise a plurality of labeled amplicons. The one or more libraries may comprise a plurality of enriched molecules or a derivative thereof (*e.g.*, labeled molecules, labeled amplicons). Generally, the method of producing one or more libraries comprises (a) stochastically labeling a plurality of molecules from two or more samples to produce a plurality of labeled molecules, wherein the labeled molecules comprise a molecule region, a sample index region, and label region; and (b) producing one or more libraries from the plurality of labeled molecules, wherein (i) the one or more libraries comprise two or more different labeled molecules, (ii) the two or more different labeled molecules differ by the molecule region, sample index region, label region, or a combination thereof.

[00122] The method for producing one or more libraries may comprise: a) producing a plurality of sample-tagged nucleic acids by: i) contacting a first sample comprising a plurality of nucleic acids with a plurality of first sample tags to produce a plurality of first sample-tagged nucleic acids; and ii) contacting a second sample comprising a plurality of nucleic acids with a plurality of second sample tags to produce a plurality of second sample-tagged nucleic acids, wherein the plurality of first sample tags are different from the second sample tags; and b) contacting the plurality of sample-tagged nucleic acids with a plurality of molecular identifier labels to produce a plurality of labeled nucleic acids, thereby producing a labeled nucleic acid library.

[00123] The contacting to a sample can be random or non-random. For example, the contacting of a sample with sample tags can be a random or non-random contacting. In some embodiments, the sample is contacted with sample tags randomly. In some embodiments, the sample is contacted with sample tags non-randomly. The contacting to a plurality of nucleic acids can be random or non-random. For example, the contacting of a plurality of nucleic acids with sample tags can be a random or non-random contacting. In some embodiments, the plurality of nucleic acids is contacted with sample tags randomly. In some embodiments, the plurality of nucleic acids is contacted with sample tags non-randomly.

[00124] Further disclosed herein are methods of producing one or more sets of labeled beads. The method of producing the one or more sets of labeled beads may comprise attaching one or more nucleic acids to one or more beads, thereby producing one or more sets of labeled beads. The one or more nucleic acids may comprise one or more molecular barcodes. The one or more nucleic acids may comprise one or more sample tags. The one or more nucleic acids may comprise one or more molecular identifier labels. The one or more nucleic acids may comprise a) a primer region; b) a sample index region; and c) a linker or adaptor region. The one or more nucleic acids may comprise a) a primer region; b) a label region; and c) a linker or adaptor region. The one or more nucleic acids may comprise a) a sample index region; and b) a label region. The one or more nucleic acids may further comprise a primer region. The one or more nucleic acids may further comprise a target specific region. The one or more nucleic acids may further comprise a linker region. The one or more nucleic acids may further comprise an adaptor region. The one or more nucleic acids may further comprise a sample index region. The one or more nucleic acids may further comprise a label region.

[00125] Further disclosed herein are methods for selecting one or more custom primers. The method of selecting a custom primer for analyzing molecules in a plurality of samples may

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comprise: a) a first pass, wherein primers chosen may comprise: i) no more than three sequential guanines, no more than three sequential cytosines, no more than four sequential adenines, and no more than four sequential thymines; ii) at least 3, 4, 5, or 6 nucleotides that are guanines or cytosines; and iii) a sequence that does not easily form a hairpin structure; b) a second pass, comprising: i) a first round of choosing a plurality of sequences that have high coverage of all transcripts; and ii) one or more subsequent rounds, selecting a sequence that has the highest coverage of remaining transcripts and a complementary score with other chosen sequences no more than 4; and c) adding sequences to a picked set until coverage saturates or total number of customer primers is less than or equal to about 96.

[00126] Further disclosed herein are kits for use in analyzing two or more molecules from two or more samples. The kit may comprise (a) a first container comprising a first set of molecular barcodes, wherein (i) a molecular barcode of the first set of molecular barcodes comprise a sample index region and a label region; (ii) the sample index region of two or more barcodes of the first set of molecular barcodes are the same; and (iii) the label region of two or more barcodes of the first set of molecular barcodes are different; and (b) a second container comprising a second set of molecular barcodes, wherein (i) a molecular barcode of the second set of molecular barcodes comprise a sample index region and a label region; (ii) the sample index region of two or more barcodes of the second set of molecular barcodes are the same; (iii) the label region of two or more barcodes of the second set of molecular barcodes are different; (iv) the sample index region of the barcodes of the second set of molecular barcodes are different from the sample index region of the barcodes of the first set of molecular barcodes; and (v) the label region of two or more barcodes of the second set of molecular barcodes are identical to the label region of two or more barcodes of the first set of molecular barcodes.

[00127] Alternatively, the kit comprises: a) a plurality of beads, wherein one or more beads of the plurality of beads may comprise at least one of a plurality of nucleic acids, wherein at least one of a plurality nucleic acids may comprise: i) at least one primer sequence, wherein the primer sequence of at least one of the plurality of nucleic acids is the same for the plurality of beads; ii) a bead-specific sequence, wherein the bead-specific sequence of any one of the plurality of nucleic acids is the same, and wherein the bead-specific sequence is different for any one of the plurality of beads; and iii) a stochastic sequence, wherein the stochastic sequence is different for any one of the plurality of nucleic acids; b) a primer may comprise a sequence complementary to the primer sequence; and c) one or more amplification agents suitable for nucleic acid amplification.

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[00128] Alternatively, the kit comprises: a) a first container comprising a first set of sample tags, wherein(i) a sample tag of the first set of sample tags comprises a sample index region; and (ii) the sample index regions of the sample tags of the first set of sample tags are at least about 80% identical; and b) a second container comprising a first set of molecular identifier labels, wherein (i) a molecular identifier label of the first set of molecular identifier labels comprises a label region; and (ii) at least about 30% of the label regions of the total molecular identifier labels of the first set of molecular identifier labels are different

[00129] Before the present methods, kits and compositions are described in greater detail, it is to be understood that this invention is not limited to particular method, kit or composition described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims. Examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.,* amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[00130] Methods, kits and compositions are provided for stochastic labeling of nucleic acids in a plurality of samples or in a complex nucleic acid preparation. These methods, kits and compositions find use in unraveling mechanisms of cellular response, differentiation or signal transduction and in performing a wide variety of clinical measurements. These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the methods, kits and compositions as more fully described below.

[00131] The methods disclosed herein comprise attaching one or more molecular barcodes, sample tags, and/or molecular identifier labels to two or more molecules from two or more samples. The molecular barcodes, sample tags and/or molecular identifier labels may comprise one or more oligonucleotides. In some instances, attachment of molecular barcodes, sample tags, and/or molecular identifier labels to the molecules comprises stochastic labeling of the molecules. Methods for stochastically labeling molecules may be found, for example, in U.S. Serial Numbers 12/969,581 and 13/327,526. Generally, the stochastic labeling

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method comprises the random attachment of a plurality of the tag and label oligonucleotides to one or more molecules. The molecular barcodes, sample tags, and/or molecular identifier labels are provided in excess of the one or more molecules to be labeled. In stochastic labeling, each individual molecule to be labeled has an individual probability of attaching to the plurality of the molecular barcodes, sample tags, and/or molecular identifier labels. The probability of each individual molecule to be labeled attaching to a particular molecular barcodes, sample tags, and/or molecular identifier labels may be about the same as any other individual molecule to be labeled. Accordingly, in some instances, the probability of any of the molecules in a sample finding any of the tags and labels is assumed to be equal, an assumption that may be used in mathematical calculations to estimate the number of molecules in the sample. In some circumstances the probability of attaching may be manipulated by, for example electing tags and labels with different properties that would increase or decrease the binding efficiency of that molecular barcodes, sample tags, and/or molecular identifier labels with an individual molecule. The tags and labels may also be varied in numbers to alter the probability that a particular molecular barcodes, sample tags, and/or molecular identifier labels will find a binding partner during the stochastic labeling. For example, one label is overrepresented in a pool of labels, thereby increasing the chances that the overrepresented label finds at least one binding partner.

[00132] The methods disclosed herein may further comprise combining two or more samples. The methods disclosed herein may further comprise combining one or more molecules from two or more samples. For example, the methods disclosed herein comprise combining a first sample and a second sample. The two or more samples may be combined after conducting one or more stochastic labeling procedures. The two or more samples may be combined after attachment of one or more sets of molecular barcodes to two or more molecules from the two or more samples. The two or more samples may be combined after attachment of one or more sets of sample tags to two or more molecules from the two or more samples. The two or more samples may be combined after attachment of one or more sets of molecular identifier labels to two or more molecules from the two or more samples. For example, the first and second samples are combined prior to contact with the plurality of molecular identifier labels.

[00133] Alternatively, the two or more samples may be combined prior to conducting one or more stochastic labeling procedures. The two or more samples may be combined prior to attachment of one or more sets of molecular barcodes to two or more molecules from the two or more samples. The two or more samples may be combined prior to attachment of one or

more sets of sample tags to two or more molecules from the two or more samples. The two or more samples may be combined prior to attachment of one or more sets of molecular identifier labels to two or more molecules from the two or more samples.

[00134] The two or more samples may be combined after conducting one or more assays on two or more molecules or derivatives thereof (*e.g.*, labeled molecules, amplicons) from the two or more samples. The one or more assays may comprise one or more amplification reactions. The one or more assays may comprise one or more enrichment assays. The one or more assays may comprise one or more detection assays. For example, the first and second samples are combined after detecting the labeled nucleic acids.

[00135] The two or more samples may be combined prior to conducting one or more assays on two or more molecules or derivatives thereof (*e.g.*, labeled molecules, amplicons) from the two or more samples. The one or more assays may comprise one or more amplification reactions. The one or more assays may comprise one or more enrichment assays. The one or more assays may comprise one or more detection assays. For example, the first and second samples are combined prior to detecting the labeled nucleic acids.

Supports

[00136] The present disclosure comprises compositions and methods for multiplex sequence analysis from single cells. The methods and compositions of the present disclosure provide for the use of solid supports. In some instances, the methods, kits, and compositions disclosed herein comprise a support.

[00137] The terms "support", "solid support", "semi-solid support", and "substrate" may be used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. A support may refer to any surface that is transferable from solution to solution or forms a structure for conducting oligonucleotide-based assays. The support or substrate may be a solid support. Alternatively, the support is a non-solid support. A support may refer to an insoluble, semi-soluble, or insoluble material. A support may be referred to as "functionalized" when it includes a linker, a scaffold, a building block, or other reactive moiety attached thereto, whereas a solid support may be "nonfunctionalized" when it lack such a reactive moiety attached thereto. The support may be employed free in solution, such as in a microtiter well format; in a flow-through format, such as in a column; or in a dipstick.

[00138] The support or substrate may comprise a membrane, paper, plastic, coated surface, flat surface, glass, slide, chip, or any combination thereof. In many embodiments, at least one surface of the support may be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example,

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wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) may take the form of resins, gels, microspheres, or other geometric configurations. Alternatively, the solid support(s) comprises silica chips, microparticles, nanoparticles, plates, and arrays. Solid supports may include beads (*e.g.*, silica gel, controlled pore glass, magnetic beads, Dynabeads, Wang resin; Merrifield resin, Sephadex/Sepharose beads, cellulose beads, polystyrene beads etc.), capillaries, flat supports such as glass fiber filters, glass surfaces, metal surfaces (steel, gold silver, aluminum, silicon and copper), glass supports, plastic supports, silicon supports, chips, filters, membranes, microwell plates, slides, or the like. plastic materials including multiwell plates or membranes (*e.g.*, formed of polyethylene, polypropylene, polyamide, polyvinylidenedifluoride), wafers, combs, pins or needles (*e.g.*, arrays of pins suitable for combinatorial synthesis or analysis) or beads in an array of pits or nanoliter wells of flat surfaces such as wafers (*e.g.,* silicon wafers), wafers with pits with or without filter bottoms.

[00139] 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, 5,624,711, 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. Additional exemplary substrates are disclosed in U.S. Patent No. 5,744,305 and US Patent Pub. Nos. 20090149340 and 20080038559.

[00140] The attachment of the labeled nucleic acids to the support may comprise amine-thiol crosslinking, maleimide crosslinking, N-hydroxysuccinimide or N-hydroxysulfosuccinimide, Zenon or SiteClick. Attaching the labeled nucleic acids to the support may comprise attaching biotin to the plurality of labeled nucleic acids and coating the one or more beads with streptavadin.

[00141] In some instances, a solid support may comprise a molecular scaffold. Exemplary molecular scaffolds may include antibodies, antigens, affinity reagents, polypeptides, nucleic acids, cellular organelles, and the like. Molecular scaffolds may be linked together (*e.g.*, a

solid support may comprise a plurality of connected molecular scaffolds). Molecular scaffolds may be linked together by an amino acid linker, a nucleic acid linker, a small molecule linkage (*e.g.*, biotin and avidin), and/or a matrix linkage (*e.g.*, PEG or glycerol). Linkages may be non-covalent. Linkages may be covalent. In some instances, molecular scaffolds may not be linked. A plurality of individual molecular scaffolds may be used in the methods of the disclosure.

[00142] In some instances a support may comprise a nanoparticle. The nanoparticle may be a nickel, gold, silver, carbon, copper, silicate, platinum cobalt, zinc oxide, silicon dioxide crystalline, and/or silver nanoparticle. Alternatively, or additionally, the nanoparticle may be a gold nanoparticle embedded in a porous manganese oxide. The nanoparticle may be an iron nanoparticle. The nanoparticle may be a nanotetrapod studded with nanoparticles of carbon.

[00143] A support may comprise a polymer. A polymer may comprise a matrix. A matrix may further comprise one or more beads. A polymer may comprise PEG, glycerol, polysaccharide, or a combination thereof. A polymer may be a plastic, rubber, nylon, silicone, neoprene, and/or polystyrene. A polymer may be a natural polymer. Examples of natural polymers include, but are not limited to, shellac, amber, wool, silk, cellulose, and natural rubber. A polymer may be a synthetic polymer. Examples of synthetic polymers include, but are not limited to, synthetic rubber, phenol formaldehyde resin (or Bakelite), neoprene, nylon, polyvinyl chloride (PVC or vinyl), polystyrene, polyethylene, polypropylene, polyacrylonitrile, PVB, and silicone.

[00144] A support may be a semi-solid support. A support may comprise a gel (*e.g.*, a hydrogel). The terms "hydrogel", "gel" and the like, are used interchangeably herein and may refer to a material which is not a readily flowable liquid and not a solid but a gel which gel is comprised of from 0.5% or more and preferably less than 40% by weight of gel forming solute material and from 95% or less and preferably more than 55% water. The gels of the invention may be formed by the use of a solute which is preferably a synthetic solute (but could be a natural solute, *e.g.*, for forming gelatin) which forms interconnected cells which binds to, entrap, absorb and/or otherwise hold water and thereby create a gel in combination with water, where water includes bound and unbound water. The gel may be the basic structure of the hydrogel patch of the invention will include additional components beyond the gel forming solute material and water such as an enzyme and a salt which components are further described herein. The gel may be a polymer gel.

[00145] A solid support may comprise a structured nanostructure. For example, the structured nanostructure may comprise capture containers (*e.g.*, a miniaturized honeycomb)

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which may comprise the oligonucleotides to capture the cell and/or contents of the cell. In some instances, structured nanostructures may not need the addition of exogenous reagents.

[00146] In some instances, the support comprises a bead. A bead may encompass any type of solid or hollow sphere, ball, bearing, cylinder, or other similar configuration composed of plastic, ceramic, metal, or polymeric material onto which a nucleic acid may be immobilized (*e.g.*, covalently or non-covalently). A bead may comprise nylon string or strings. A bead may be spherical in shape. A bead may be non-spherical in shape. Beads may be unpolished or, if polished, the polished bead may be roughened before treating, (*e.g.*, with an alkylating agent). A bead may comprise a discrete particle that may be spherical (*e.g.*, microspheres) or have an irregular shape. Beads may comprise a variety of materials including, but not limited to, paramagnetic materials, ceramic, plastic, glass, polystyrene, methylstyrene, acrylic polymers, titanium, latex, sepharose, cellulose, nylon and the like. A bead may be attached to or embedded into one or more supports. A bead may be attached to a gel or hydrogel. A bead may be embedded into a gel or hydrogel. A bead may be attached to a matrix. A bead may be embedded into a matrix. A bead may be attached to a polymer. A bead may be embedded into a polymer. The spatial position of a bead within the support (*e.g.*, gel, matrix, scaffold, or polymer) may be identified using the oligonucleotide present on the bead which serves as a location address. Examples of beads include, but are not limited to, streptavidin beads, agarose beads, magnetic beads, Dynabeads®, MACS® microbeads, antibody conjugated beads (*e.g.*, anti-immunoglobulin microbead), protein A conjugated beads, protein G conjugated beads, protein A/G conjugated beads, protein L conjugated beads, oligodT conjugated beads, silica beads, silica-like beads, anti-biotin microbead, anti-fluorochrome microbead, and BcMag™ Carboxy-Terminated Magnetic Beads. The diameter of the beads may be about 5μm, 10μm, 20μm, 25μm, 30μm, 35μm, 40μm, 45μm or 50μm. A bead may refer to any three dimensional structure that may provide an increased surface area for immobilization of biological particles and macromolecules, such as DNA and RNA.

[00147] A support may be porous. A support may be permeable or semi-permeable. A support may be solid. A support may be semi-solid. A support may be malleable. A support may be flexible. In some instances, a support may be molded into a shape. For example, a support may be placed over an object and the support may take the shape of the object. In some instances, the support is placed over an organ and takes the shape of the organ. In some instances, the support is produced by 3D-printing.

[00148] The support (*e.g.*, beads, nanoparticles) may be at least about 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 100, 500, 1000, or 2000 or more micrometers in diameter. The solid

supports (*e.g.*, beads) may be at most about 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 100, 500, 1000, or 2000 or more micrometers in diameter. The diameter of the bead may be about 20 microns.

[00149] In some instances, a solid support comprises a dendrimer. A dendrimer may be smaller than a bead. A dendrimer may be subcellular. A dendrimer may be less than 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 micron in diameter. A dendrimer may be less than 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, or 0.01 micron in diameterA dendrimer may comprise three major portions, a core, an inner shell, and an outer shell. A dendrimer may be synthesized to have different functionality in each of these portions. The different functionality of the portions of the dendrimer may control properties such as solubility, thermal stability, and attachment of compounds for particular applications. A dendrimer may be synthetically processed. A dendrimer may be synthesized by divergent synthesis. Divergent synthesis may comprise assembling a dendrimer from a multifunctional core, which is extended outward by a series of reactions. Divergent synthesis may comprise a series of Michael reactions. Alternatively, a dendrimer may be synthesized by convergent synthesis. Convergent synthesis may comprise building dendrimers from small molecules that end up at the surface of the sphere, and reactions may proceed inward and are eventually attached to a core. Dendrimers may also be prepared by click chemistry. Click chemistry may comprise Diels-Alder reactions, thiol-yne reactions, azide-alkyne reactions, or a combination thereof. Examples of dendrimers include, but are not limited to, poly(amidoamine) (PAMAM) dendrimer, PEG-core denderimer, phosphorous dendrimer, polypropylenimine dendrimer, and polylysine dendrimer. A dendrimer may be a chiral dendrimer. Alternatively, a dendrimer may be an achiral dendrimer.

[00150] A solid support may comprise a portion of a dendrimer. The portion of the dendrimer may comprise a dendron. A dendron may comprise monodisperse wedge-shaped dendrimer sections with multiple terminal groups and a single reaction function at the focal point. A solid support may comprise a polyester dendrom. Examples of dendrons include, but are not limited to, polyester-8-hydroxyl-1-acetylene bis-MPA dendron, polyester-16-hydroxyl-1-acetylene bis-MPA dendron, polyester-32-hydroxyl-1-acetylene bis-MPA dendron, polyester-8-hydroxyl-1-carboxyl bis-MPA dendron, polyester-16-hydroxyl-1-carboxyl bis-MPA dendron, and polyester-32-hydroxyl-1-carboxyl bis-MPA dendron.

[00151] A solid support may comprise a hyberbranched polymer. A hyperbranched polymer may comprise polydisperse dendritic macromolecules that possess dendrimer-like properties. Often, hyberbranched polymers are prepared in a single synthetic polymerization step. The

hyperbranched polymer may be based on 2,2-bis(hydroxymethyl)propanoic acid (bis-MPA) monomer. Examples of hyperbranched polymers include, but are not limited to, hyperbranched bis-MPA polyester-16-hydroxyl, hyperbranched bis-MPA polyester-32-hydroxyl, and hyperbranched bis-MPA polyester-64-hydroxyl.

[00152] The solid support may be an array or microarray. The solid support may comprise discrete regions. The solid support may be an addressable array. In some instances, the array comprises a plurality of probes fixed onto a solid surface. The plurality of probes enables hybridization of the labeled-molecule and/or labeled-amplicon to the solid surface. The plurality of probes comprises a sequence that is complementary to at least a portion of the labeled-molecule and/or labeled-amplicon. In some instances, the plurality of probes comprises a sequence that is complementary to at least a portion of the sample tag, molecular identifier label, nucleic acid, or a combination thereof. In other instances, the plurality of probes comprises a sequence that is complementary to the junction formed by the attachment of the sample tag or molecular identifier label to the nucleic acid.

[00153] The array may comprise one or more probes. The probes may be in a variety of formats. The array may comprise a probe comprising a sequence that is complementary to at least a portion of the target nucleic acid and a sequence that is complementary to the unique identifier region of a sample tag or molecular identifier label, wherein the sample tag or molecular identifier label comprises an oligonucleotide. The sequence that is complementary to at least a portion of the target nucleic acid may be attached to the array. The sequence that is complementary to the unique identifier region may be attached to the array. The array may comprise a first probe comprising a sequence that is complementary to at least a portion of the target nucleic acid and a second probe that is complementary to the unique identifier region. There are various ways in which a stochastically labeled nucleic acid may hybridize to the arrays. For example, the junction of the unique identifier region and the target nucleic acid of the stochastically labeled nucleic acid may hybridize to the probe on the array. There may be a gap in the regions of the stochastically labeled nucleic acid that may hybridize to the probe on the array. Different regions of the stochastically labeled nucleic acid may hybridize to two or more probes on the array. Thus, the array probes may be in many different formats. The array probes may comprise a sequence that is complementary to a unique identifier region, a sequence that is complementary to the target nucleic acid, or a combination thereof. Hybridization of the stochastically labeled nucleic acid to the array may occur by a variety of ways. For example, two or more nucleotides of the stochastically labeled nucleic acid may hybridize to one or more probes on the array. The two or more

nucleotides of the stochastically labeled nucleic acid that hybridize to the probes may be consecutive nucleotides, non-consecutive nucleotides, or a combination thereof. The stochastically labeled nucleic acid that is hybridized to the probe may be detected by any method known in the art. For example, the stochastically labeled nucleic acids may be directly detected. Directly detecting the stochastically labeled nucleic acid may comprise detection of a fluorophore, hapten, or detectable label. The stochastically labeled molecules may be indirectly detected. Indirect detection of the stochastically labeled nucleic acid may comprise ligation or other enzymatic or non-enzymatic methods.

[00154] The array may be in a variety of formats. For example, the array may be in a 16-, 32-, 48-, 64-, 80-, 96-, 112-, 128-, 144-, 160-, 176-, 192-, 208-, 224-, 240-, 256-, 272-, 288-, 304-, 320-, 336-, 352-, 368-, 384-, or 400-format. Alternatively, the array is in an 8x60K, 4x180K, 2x400K, 1x1M format. In other instances, the array is in an 8x15K, 4x44K, 2x105K, 1x244K format.

[00155] The array may comprise a single array. The single array may be on a single substrate. Alternatively, the array is on multiple substrates. The array may comprise multiple formats. The array may comprise a plurality of arrays. The plurality of arrays may comprise two or more arrays. For example, the plurality of arrays may comprise at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 arrays. In some instances, at least two arrays of the plurality of arrays are identical. Alternatively, at least two arrays of the plurality of arrays are different.

[00156] In some instances, the array comprises symmetrical chambered areas. For example, the array comprises 0.5 x 0.5 millimeters (mm), 1 x 1 mm, 1.5 x 1.5 mm, 2 x 2 mm, 2.5 x 2.5 mm, 3 x 3 mm, 3.5 x 3.5 mm, 4 x 4 mm, 4.5 x 4.5 mm, 5 x 5 mm, 5.5 x 5.5 mm, 6 x 6 mm, 6.5 x 6.5 mm, 7 x 7 mm, 7.5 x 7.5 mm, 8 x 8 mm, 8.5 x 8.5 mm, 9 x 9 mm, 9.5 x 9.5 mm, 10 x 10 mm, 10.5 x 10.5 mm, 11 x 11 mm, 11.5 x 11.5 mm, 12 x 12 mm, 12.5 x 12.5 mm, 13 x 13 mm, 13.5 x 13.5 mm, 14 x 14 mm, 14.5 x 14.5 mm, 15 x 15 mm, 15.5 x 15.5 mm, 16 x 16 mm, 16.5 x 16.5 mm, 17 x 17 mm, 17.5 x 17.5 mm, 18 x 18 mm, 18.5 x 18.5 mm, 19 x 19 mm, 19.5 x 19.5 mm, or 20 x 20 mm chambered areas. In some instances, the array comprises 6.5 x 6.5 mm chambered areas. Alternatively, the array comprises asymmetrical chambered areas. For example, the array comprises 6.5 x 0.5 mm, 6.5 x 1 mm, 6.5 x 1.5 mm, 6.5 x 2 mm, 6.5 x 2.5 mm, 6.5 x 3 mm, 6.5 x 3.5 mm, 6.5 x 4 mm, 6.5 x 4.5 mm, 6.5 x 5 mm, 6.5 x 5.5 mm, 6.5 x 6 mm, 6.5 x 6.5 mm, 6.5 x 7 mm, 6.5 x 7.5 mm, 6.5 x 8 mm, 6.5 x 8.5 mm, 6.5 x 9 mm, 6.5 x 9.5 mm, 6.5 x 10 mm, 6.5 x 10.5 mm, 6.5 x 11 mm, 6.5 x 11.5 mm, 6.5 x 12 mm, 6.5 x 12.5 mm, 6.5 x 13 mm, 6.5 x 13.5 mm, 6.5 x 14 mm, 6.5 x 14.5 mm, 6.5 x

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15 mm, 6.5 x 15.5 mm, 6.5 x 16 mm, 6.5 x 16.5 mm, 6.5 x 17 mm, 6.5 x 17.5 mm, 6.5 x 18 mm, 6.5 x 18.5 mm, 6.5 x 19 mm, 6.5 x 19.5 mm, or 6.5 x 20 mm chambered areas.

[00157] The array may comprise at least about 1 micron (μm), 2 μm, 3 μm, 4 μm, 5 μm, 6 μm, 7 μm, 8 μm, 9 μm, 10 μm, 15 μm, 20 μm, 25 μm, 30 μm, 35 μm, 40 μm, 45 μm, 50 μm, 55 μm, 60 μm, 65 μm, 70 μm, 75 μm, 80 μm, 85 μm, 90 μm, 95 μm, 100 μm, 125 μm, 150 μm, 175 μm, 200 μm , 225 μm, 250 μm, 275 μm, 300 μm, 325 μm, 350 μm, 375 μm, 400 μm , 425 μm, 450 μm, 475 μm, or 500 μm spots. In some instances, the array comprises 70 μm spots.

[00158] The array may comprise at least about 1 μm, 2 μm, 3 μm, 4 μm, 5 μm, 6 μm, 7 μm, 8 μm, 9 μm, 10 μm, 15 μm, 20 μm, 25 μm, 30 μm, 35 μm, 40 μm, 45 μm, 50 μm, 55 μm, 60 μm, 65 μm, 70 μm, 75 μm, 80 μm, 85 μm, 90 μm, 95 μm, 100 μm, 125 μm, 150 μm, 175 μm, 200 μm , 225 μm, 250 μm, 275 μm, 300 μm, 325 μm, 350 μm, 375 μm, 400 μm , 425 μm, 450 μm, 475 μm, 500 μm, 525 μm, 550 μm, 575 μm, 600 μm , 625 μm, 650 μm, 675 μm, 700 μm, 725 μm, 750 μm, 775 μm, 800 μm , 825 μm, 850 μm, 875 μm, 900 μm, 925 μm, 950 μm, 975 μm, 1000 μm feature pitch. In some instances, the array comprises 161 μm feature pitch.

[00159] The array may comprise one or more probes. In some instances, the array comprises at least about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 probes. Alternatively, the array comprises at least about 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, or 3000 probes. The array may comprise at least about 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, or 10000 probes. In some instances, the array comprises at least about 960 probes. Alternatively, the array comprises at least about 2780 probes. The probes may be specific for the plurality of oligonucleotide tags. The probes may be specific for at least a portion of the plurality of oligonucleotide tags. The probes may be specific for at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97% or 100% of the total number of the plurality of oligonucleotide tags. Alternatively, the probes are specific for at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97% or 100% of the total number of different oligonucleotide tags of the plurality of oligonucleotide tags. The probes may be oligonucleotides. The oligonucleotides may be at least about 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 nucleotides long. In other instances, the probes are non-specific probes. For example, the probes may be specific for a detectable label that is attached to the labeled-molecule. The probe may be streptavidin.

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[00160] The array may be a printed array. In some instances, the printed array comprises one or more oligonucleotides attached to a substrate. For example, the printed array comprises 5' amine modified oligonucleotides attached to an epoxy silane substrate.

[00161] Alternatively, the array comprises a slide with one or more wells. The slide may comprise at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 wells. Alternatively, the slide comprises at least about 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 650, 700, 750, 800, 850, 900, 950, or 1000 wells. In some instances, the slide comprises 16 wells. Alternatively, the slide comprises 96 wells. In other instances, the slide comprises at least about 80, 160, 240, 320, 400, 480, 560, 640, 720, 800, 880, or 960 wells.

[00162] In some instances, the solid support is an Affymetrix 3K tag array, Arrayjet non-contact printed array, or Applied Microarrays Inc (AMI) array. Alternatively, the support comprises a contact printer, impact printer, dot printer, or pin printer.

[00163] The solid support may comprise the use of beads that self-assemble in microwells. For example, the solid support comprises Illumina's BeadArray Technology. Alternatively, the solid support comprises Abbott Molecular's Bead Array technology, and Applied Microarray's FlexiPlexTM system.

[00164] In other instances, the solid support is a plate. Examples of plates include, but are not limited to, MSD multi-array plates, MSD Multi-Spot® plates, microplate, ProteOn microplate, AlphaPlate, DELFIA plate, IsoPlate, and LumaPlate.

[00165] The method may further comprise attaching at least one of a plurality of labeled nucleic acids to a support. The support may comprise a plurality of beads. The support may comprise an array. The support may comprise a glass slide.

[00166] The glass slide may comprise one or more wells. The one or more wells may be etched on the glass slide. The one or more wells may comprise at least 960 wells. The glass slide may comprise one or more probes. The one or more probes may be printed onto the glass slide. The one or more wells may further comprise one or more probes. The one or more probes may be printed within the one or more wells. The one or more probes may comprise 960 nucleic acids.

[00167] The methods and kits disclosed herein may further comprise distributing the plurality of first sample tags, the plurality of second sample tags, the plurality of molecular identifier labels, or any combination thereof in a microwell plate. The methods and kits disclosed herein may further comprise distributing one or more beads in the microwell plate. The methods and kits disclosed herein may further comprise distributing the plurality of

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samples in a plurality of wells of a microwell plate. The one or more of the plurality of samples may comprise a plurality of cells. One or more of the plurality of samples may comprise a plurality of nucleic acids. The method may further comprise distributing one or fewer cells to the plurality of wells. The plurality of cells may be lysed in the microwell plate. The method may further comprise synthesizing cDNA in the microwell plate. Synthesizing cDNA may comprise reverse transcription of mRNA. The microwell plate may comprise a microwell plate fabricated on PDMS by soft lithography, etched on a silicon wafer, etched on a glass slide, patterned photoresist on a glass slide, or a combination thereof. The microwell may comprise a hole on a microcapillary plate. The microwell plate may comprise a water-in-oil emulsion. The microwell plate may comprise at least one or more wells. The microwell plate may comprise at least about 6 wells, 12 wells, 48 wells, 96 wells, 384 wells, 960 wells or 1000 wells.

[00168] The methods and kits may further comprise a chip. The microwell plate may be attached to the chip. The chip may comprise at least about 6 wells, 12 wells, 48 wells, 96 wells, 384 wells, 960 wells, 1000 wells, 2000 wells, 3000 wells, 4000 wells, 5000 wells, 6000 wells, 7000 wells, 8000 wells, 9000 wells, 10,000 wells, 20,000 wells, 30,000 wells, 40,000 wells, 50,000 wells, 60,000 wells, 70,000 wells, 80, 000 wells, 90,000 wells, 100,000 wells, 200,000 wells, 500,000 wells, or a million wells. The wells may comprise an area of at least about 300 microns^2, 400 microns^2, 500 microns^2, 600 microns^2, 700 microns^2, 800 microns^2, 900 microns^2, 1000 microns^2, 1100 microns^2, 1200 microns^2, 1300 microns^2, 1400 microns^2, 1500 microns^2. The method may further comprise distributing between about 10,000 and 30,000 samples on the chip.

Functionalized surfaces and oligonucleotides

[00169] The bead may comprise a functionalized surface. A functionalized surface may refer to the surface of the solid support comprising a functional group. A functional group may be a group capable of forming an attachment with another functional group. For example, a functional group may be biotin, which may form an attachment with streptavidin, another functional group. Exemplary functional groups may include, but are not limited to, aldehydes, ketones, carboxy groups, amino groups, biotin, streptavidin, nucleic acids, small molecules (*e.g.*, for click chemistry), homo- and hetero-bifunctional reagents (*e.g.*, N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB), dimaleimide, dithio-bis-nitrobenzoic acid (DTNB), N-succinimidyl-S-acetyl-thioacetate (SATA), N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl 4-(N-mafeimidomethyl)-cyclohexane-1-carboxylate (SMCC) and 6-

hydrazinonicotimide (HYNIC), and antibodies. In some instances the functional group is a carboxy group (*e.g.*, COOH).

[00170] Oligonucleotides (*e.g.*, nucleic acids) may be attached to functionalized solid supports. The immobilized oligonucleotides on solid supports or similar structures may serve as nucleic acid probes, and hybridization assays may be conducted wherein specific target nucleic acids may be detected in complex biological samples.

[00171] The solid support (*e.g.*, beads) may be functionalized for the immobilization of oligonucleotides. An oligonucleotide may be conjugated to a solid support through a covalent amide bond formed between the solid support and the oligonucleotide.

[00172] A support may be conjugated to at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 or more oligonucleotides. A support may be conjugated to at least about 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000 or 10000000, 100000000, 500000000, 1000000000 or more oligonucleotides. A support may be conjugated to at least about 100000, 200000, 300000, 400000, 500000, 600000, 700000, 800000, 900000, 1000000, 2000000, 3000000, 4000000, 5000000, 6000000, 7000000, 8000000, 9000000 or 10000000, 100000000, 500000000, 1000000000 or more oligonucleotides. A support may be conjugated to at least 1 million oligonucleotides. A support may be conjugated to at least 10 million oligonucleotides. A support may be conjugated to at least 25 million oligonucleotides. A support may be conjugated to at least 50 million oligonucleotides. A support may be conjugated to at least 100 million oligonucleotides. A support may be conjugated to at least 250 million oligonucleotides. A support may be conjugated to at least 500 million oligonucleotides. A support may be conjugated to at least 750 million oligonucleotides. A support may be conjugated to at least about 1, 2, 3 4, 5, 6, 7, 8, 9, 10, 11, 12 13, 14, or 15 billion oligonucleotides. A support may be conjugated to at least 1 billion oligonucleotides. A support may be conjugated to at least 5 billion oligonucleotides.

[00173] The oligonucleotides may be attached to the support (*e.g.*, beads, polymers, gels) via a linker. Conjugation may comprise covalent or non-covalent attachment. Conjugation may introduce a variable spacer between the beads and the nucleic acids. The linker between the support and the oligonucleotide may be cleavable (*e.g.*, photocleavable linkage, acid labile linker, heat sensitive linker, and enzymatically cleavable linker).

[00174] Cross-linking agents for use for conjugating molecules to supports may include agents capable of reacting with a functional group present on a surface of the solid support

and with a functional group present in the molecule. Reagents capable of such reactivity may include aldehydes, ketones, carboxy groups, amino groups, biotin, streptavidin, nucleic acids, small molecules (*e.g.*, for click chemistry), homo- and hetero-bifunctional reagents (*e.g.*, N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB), dimaleimide, dithio-bis-nitrobenzoic acid (DTNB), N-succinimidyl-S-acetyl-thioacetate (SATA), N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl 4-(N-mafeimidomethyl)-cyclohexane-1-carboxylate (SMCC) and 6-hydrazinonicotimide (HYNIC).

[00175] A bead may be functionalized with a carboxy functional group and an oligonucleotide may be functionalized with an amino functional group.

[00176] A support may be smooth. Alternatively, or additionally, a support may comprise divets, ridges, or wells. A support may comprise a microwell array. A microwell array may be functionalized with functional groups that facilitate the attachment of oligonucleotides. The functional groups on the microwell array may be different for different positions on the microwell array. The functional groups on the microwell array may be the same for all regions of the microwell array.

Assay System Components

Microwell arrays

[00177] As described above, microwell arrays are used to entrap single cells and beads (one bead per cell) within a small reaction chamber of defined volume. Each bead comprises a library of oligonucleotide probes for use in stochastic labeling and digital counting of the entire complement of cellular mRNA molecules, which are released upon lysis of the cell. In one embodiment of the present disclosure, the microwell arrays are a consumable component of the assay system. In other embodiments, the microwell arrays may be reusable. In either case, they may be configured to be used as a stand-alone device for use in performing assays manually, or they may be configured to comprise a removable or fixed component of an instrument that provides for full or partial automation of the assay procedure.

[00178] The microwells of the array can be fabricated in a variety of shapes and sizes, which are chosen to optimize the efficiency of trapping a single cell and bead in each well. Appropriate well geometries include, but are not limited to, cylindrical, conical, hemispherical, rectangular, or polyhedral (*e.g.*, three dimensional geometries comprised of several planar faces, for example, hexagonal columns, octagonal columns, inverted triangular pyramids, inverted square pyramids, inverted pentagonal pyramids, inverted hexagonal pyramids, or inverted truncated pyramids). The microwells may comprise a shape that combines two or more of these geometries. For example, in one embodiment it may be partly

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cylindrical, with the remainder having the shape of an inverted cone. In another embodiment, it may include two side-by-side cylinders, one of larger diameter than the other, that are connected by a vertical channel (that is, parallel to the cylinder axes) that extends the full length (depth) of the cylinders. In general, the open end (or mouth) of each microwell will be located at an upper surface of the microwell array, but in some embodiments the openings may be located at a lower surface of the array. In general, the closed end (or bottom) of the microwell will be flat, but curved surfaces (*e.g.*, convex or concave) are also possible. In general, the shape (and size) of the microwells will be determined based on the types of cells and/or beads to be trapped in the microwells.

[00179] Microwell dimensions may be characterized in terms of the diameter and depth of the well. As used herein, the diameter of the microwell refers to the largest circle that can be inscribed within the planar cross-section of the microwell geometry. In one embodiment of the present disclosure, the diameter of the microwells may range from about 0.1 to about 5-fold the diameter of the cells and/or beads to be trapped within the microwells. In other embodiments, the microwell diameter is at least 0.1-fold, at least 0.5-fold, at least 1-fold, at least 2-fold, at least 3-fold, at least 4-fold, or at least 5-fold the diameter of the cells and/or beads to be trapped within the microwells. In yet other embodiments, the microwell diameter is at most 5-fold, at most 4-fold, at most 3-fold, at most 2-fold, at most 1-fold, at most 0.5-fold, or at most 0.1-fold the diameter of the cells and/or beads to be trapped within the microwells. In one embodiment, the microwell diameter is about 2.5-fold the diameter of the cells and/or beads to be trapped within the microwells. Those of skill in the art will appreciate that the microwell diameter may fall within any range bounded by any of these values (*e.g.,* from about 0.2-fold to about 3.5-fold the diameter of the cells and/or beads to be trapped within the microwells). Alternatively, the diameter of the microwells can be specified in terms of absolute dimensions. In one embodiment of the present disclosure, the diameter of the microwells may range from about 5 to about 50 microns. In other embodiments, the microwell diameter is at least 5 microns, at least 10 microns, at least 15 microns, at least 20 microns, at least 25 microns, at least 30 microns, at least 35 microns, at least 40 microns, at least 45 microns, or at least 50 microns. In yet other embodiments, the microwell diameter is at most 50 microns, at most 45 microns, at most 40 microns, at most 35 microns, at most 30 microns, at most 25 microns, at most 20 microns, at most 15 microns, at most 10 microns, or at most 5 microns. In one embodiment, the microwell diameter is about 30 microns. Those of skill in the art will appreciate that the microwell diameter may fall

within any range bounded by any of these values (*e.g.,* from about 28 microns to about 34 microns).

[00180] The microwell depth is chosen to optimize cell and bead trapping efficiency while also providing efficient exchange of assay buffers and other reagents contained within the wells. In one embodiment of the present disclosure, the depth of the microwells may range from about 0.1 to about 5-fold the diameter of the cells and/or beads to be trapped within the microwells. In other embodiments, the microwell depth is at least 0.1-fold, at least 0.5-fold, at least 1-fold, at least 2-fold, at least 3-fold, at least 4-fold, or at least 5-fold the diameter of the cells and/or beads to be trapped within the microwells. In yet other embodiments, the microwell depth is at most 5-fold, at most 4-fold, at most 3-fold, at most 2-fold, at most 1-fold, at most 0.5-fold, or at most 0.1-fold the diameter of the cells and/or beads to be trapped within the microwells. In one embodiment, the microwell depth is about 2.5-fold the diameter of the cells and/or beads to be trapped within the microwells. Those of skill in the art will appreciate that the microwell depth may fall within any range bounded by any of these values (*e.g.,* from about 0.2-fold to about 3.5-fold the diameter of the cells and/or beads to be trapped within the microwells). Alternatively, the diameter of the microwells can be specified in terms of absolute dimensions. In one embodiment of the present disclosure, the depth of the microwells may range from about 10 to about 60 microns. In other embodiments, the microwell depth is at least 10 microns, at least 20 microns, at least 25 microns, at least 30 microns, at least 35 microns, at least 40 microns, at least 50 microns, or at least 60 microns. In yet other embodiments, the microwell depth is at most 60 microns, at most 50 microns, at most 40 microns, at most 35 microns, at most 30 microns, at most 25 microns, at most 20 microns, or at most 10 microns. In one embodiment, the microwell depth is about 30 microns. Those of skill in the art will appreciate that the microwell depth may fall within any range bounded by any of these values (*e.g.,* from about 24 microns to about 36 microns).

[00181] The wells of the microwell array are arranged in a one dimensional, two dimensional, or three dimensional array, where three dimensional arrays may be achieved, for example, by stacking a series of two or more two dimensional arrays (that is, by stacking two or more substrates comprising microwell arrays). The pattern and spacing between wells is chosen to optimize the efficiency of trapping a single cell and bead in each well, as well as to maximize the number of wells per unit area of the array. The wells may be distributed according to a variety of random or non-random patterns, for example, they may be distributed entirely randomly across the surface of the array substrate, or they may be

arranged in a square grid, rectangular grid, or hexagonal grid. In one embodiment of the present disclosure, the center-to-center distance (or spacing) between wells may vary from about 15 microns to about 75 microns. In other embodiments, the spacing between wells is at least 15 microns, at least 20 microns, at least 25 microns, at least 30 microns, at least 35 microns, at least 40 microns, at least 45 microns, at least 50 microns, at least 55 microns, at least 60 microns, at least 65 microns, at least 70 microns, or at least 75 microns. In yet other embodiments, the microwell spacing is at most 75 microns, at most 70 microns, at most 65 microns, at most 60 microns, at most 55 microns, at most 50 microns, at most 45 microns, at most 40 microns, at most 35 microns, at most 30 microns, at most 25 microns, at most 20 microns, or at most 15 microns. In one embodiment, the microwell spacing is about 55 microns. Those of skill in the art will appreciate that the microwell depth may fall within any range bounded by any of these values (*e.g.,* from about 18 microns to about 72 microns).

[00182] The microwell array may comprise surface features between the microwells that are designed to help guide cells and beads into the wells and/or prevent them from settling on the surfaces between wells. Examples of suitable surface features include, but are not limited to, domed, ridged, or peaked surface features that encircle the wells and/or straddle the surface between wells.

[00183] The total number of wells in the microwell array is determined by the pattern and spacing of the wells and the overall dimensions of the array. In one embodiment of the present disclosure, the number of microwells in the array may range from about 96 to about 5,000,000 or more. In other embodiments, the number of microwells in the array is at least 96, at least 384, at least 1,536, at least 5,000, at least 10,000, at least 25,000, at least 50,000, at least 75,000, at least 100,000, at least 500,000, at least 1,000,000, or at least 5,000,000. In yet other embodiments, the number of microwells in the array is at most 5,000,000, at most 1,000,000, at most 75,000, at most 50,000, at most 25,000, at most 10,000, at most 5,000, at most 1,536, at most 384, or at most 96 wells. In one embodiment, the number of microwells in the array is about 96. In another embodiment, the number of microwells is about 150,000. Those of skill in the art will appreciate that the number of microwells in the array may fall within any range bounded by any of these values (*e.g.,* from about 100 to 325,000).

[00184] Microwell arrays may be fabricated using any of a number of fabrication techniques known to those of skill in the art. Examples of fabrication methods that may be used include, but are not limited to, bulk micromachining techniques such as photolithography and wet chemical etching, plasma etching, or deep reactive ion etching;

micro-molding and micro-embossing; laser micromachining; 3D printing or other direct write fabrication processes using curable materials; and similar techniques.

[00185] Microwell arrays may be fabricated from any of a number of substrate materials known to those of skill in the art, where the choice of material typically depends on the choice of fabrication technique, and vice versa. Examples of suitable materials include, but are not limited to, silicon, fused-silica, glass, polymers (*e.g.,* agarose, gelatin, hydrogels, polydimethylsiloxane (PDMS; elastomer), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), polyimide, cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET), and epoxy resins), metals or metal films (*e.g.,* aluminum, stainless steel, copper, nickel, chromium, and titanium), and the like. Typically, a hydrophilic material is desirable for fabrication of the microwell arrays (to enhance wettability and minimize non-specific binding of cells and other biological material), but hydrophobic materials that can be treated or coated (*e.g.,* by oxygen plasma treatment, or grafting of a polyethylene oxide surface layer) can also be used. The use of porous, hydrophilic materials for the fabrication of the microwell array may be desirable in order to facilitate capillary wicking/venting of entrapped air bubbles in the device. In some embodiments, the microwell array is fabricated with an optical adhesive. In some embodiments, the microwell array is fabricated with a plasma or corona treated material. The use of plasma or corona treated materials can make the material hydrophillic. In some embodients, plasma or corona treated materials, such as a hydrophillic material, can be more stable than non-treated materials. In some embodiments, the microwell array is fabricated from a single material. In other embodiments, the microwell array may comprise two or more different materials that have been bonded together or mechanically joined.

[00186] A variety of surface treatments and surface modification techniques may be used to alter the properties of microwell array surfaces. Examples include, but are not limited to, oxygen plasma treatments to render hydrophobic material surfaces more hydrophilic, the use of wet or dry etching techniques to smooth (or roughen) glass and silicon surfaces, adsorption and/or grafting of polyethylene oxide or other polymer layers to substrate surfaces to render them more hydrophilic and less prone to non-specific adsorption of biomolecules and cells, the use of silane reactions to graft chemically-reactive functional groups to otherwise inert silicon and glass surfaces, etc. Photodeprotection techniques can be used to selectively activate chemically-reactive functional groups at specific locations in the array structure, for example, the selective addition or activation of chemically-reactive functional groups such as

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primary amines or carboxyl groups on the inner walls of the microwells may be used to covalently couple oligonucleotide probes, peptides, proteins, or other biomolecules to the walls of the microwells. In general, the choice of surface treatment or surface modification utilized will depend both on the type of surface property that is desired and on the type of material from which the microwell array is made.

[00187] In some embodiments, it may be advantageous to seal the openings of microwells during, for example, cell lysis steps, to prevent cross hybridization of target nucleic acid between adjacent microwells. A microwell may be sealed using a cap such as a solid support or a bead, where the diameter of the bead is larger than the diameter of the microwell. For example, a bead used as a cap can be at least about 10, 20, 30, 40, 50, 60, 70, 80 or 90% larger than the diameter of the microwell. Alternatively, a cap may be at most about 10, 20, 30, 40, 50, 60, 70, 80 or 90% larger than the diameter of the microwell.

[00188] A bead used as a cap may comprise cross-linked dextran beads (*e.g.*, Sephadex). Cross-linked dextran can range from about 10 micrometers to about 80 micrometers. The cross-linked dextran of the bead cap can be from 20 micrometers to about 50 micrometers. A cap can comprise, for example, inorganic nanopore membranes (*e.g.*, aluminum oxides), dialysis membranes, glass slides, coverslips,and/or hydrophilic plastic film (*e.g.*, film coated with a thin film of agarose hydrated with lysis buffer).

[00189] In some embodiments, the cap may allow buffer to pass into and out of the microwell, while preventing macromolecules (*e.g.*, nucleic acids) from migrating out of the well. A macromolecule of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, or 20 or more nucleotides can be blocked from migrating into or out of the microwell by the cap. A macromolecule of at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, or 20 or more nucleotides can be blocked from migrating into or out of the microwell by the cap.

[00190] In some embodiments, a sealed microwell array can comprise a single layer of beads on top of the microwells. In some embodiments, a sealed microwell array can comprise multiple layers of beads on top of the microwells. A sealed microwell array can comprise about 1, 2, 3, 4, 5, or 6 or more layers of beads.

Mechanical fixtures

[00191] When performing multiplexed, single cell stochastic labeling/molecular indexing assays manually, it is convenient to mount the microwell array in a mechanical fixture to create a reaction chamber and facilitate the pipetting or dispensing of cell suspensions and assay reagents onto the array (**FIGs. 69** and **70**). In the example illustrated in **FIG. 69**, the

fixture accepts a microwell array fabricated on a 1 mm thick substrate, and provides mechanical support in the form of a silicone gasket to confine the assay reagents to a reaction chamber that is 16 mm wide x 35 mm long x approximately 4 mm deep, thereby enabling the use of 800 microliters to 1 milliliter of cell suspension and bead suspension (comprising bead-based oligonucleotide labels) to perform the assay.

[00192] The fixture consists of rigid, machined top and bottom plates (*e.g.,* aluminum) and a compressible (*e.g.,* silicone, polydimethylsiloxane) gasket for creating the walls of the chamber or well. Design features include: (i) Chamfered aperture edges and clearance for rotating microscope objectives in and out of position as needed (for viewing the microwell array at different magnifications). (ii) Controlled compression of the silicone gasket to ensure uniform, repeatable formation of a leak-proof seal with the microwell array substrate. (iii) Captive fasteners for convenient operation. (iv) A locating clamp mechanism for secure and repeatable positioning of the array. (v) Convenient disassembly for removal of the array during rinse steps.

[00193] The top and bottom plates may be fabricated using any of a variety of techniques (*e.g.,* conventional machining, CNC machining, injection molding, 3D printing, etc.) using a variety of materials (*e.g.,* aluminum, anodized aluminum, stainless steel, teflon, polymethylmethacrylate (PMMA), polycarbonate (PC), or similar rigid polymer materials).

[00194] The silicone (polydimethylsiloxane; PDMS) gasket may be configured to create multiple chambers (see **FIG. 71**) in order to run controls and experiments (or replicate experiments, or multiple independent experiments) in parallel. The gasket is molded from PDMS or similar elastomeric material using a Teflon mold that includes draft angles for the vertical gasket walls to provide for good release characteristics. Alternatively, molds can be machined from aluminum or other materials (*e.g.*, black delrin, polyetherimide (ultem), etc.), and coated with Teflon if necessary to provide for good release characteristics. The gasket mold designs are inverted, i.e. so that the top surface of the molded part (i.e. the surface at the interface with a glass slide or silicon wafer used to cover the mold during casting) becomes the surface for creating a seal with the microwell array substrate during use, thereby avoiding potential problems with mold surface roughness and surface contamination in creating a smooth gasket surface (to ensure a leak-proof seal with the array substrate), and also providing for a flexible choice of substrate materials and the option of pre-assembly by using the microwell array substrate as a base during casting. The gasket mold designs may also include force focusing ridges at the boundaries of the well areas, i.e. the central mesa(s) in the mold (which form the well(s)) have raised ridges at the locations which become the perimeter

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of the well(s), so that a cover placed on top of the mold after filling rests on a small contact area at the precise location where good edge profile is critical for forming a leak-proof seal between the gasket and substrate during use.

Instrument Systems

[00195] The present disclosure also includes instrument systems and consumables to support the automation of multiplexed, single cell stochastic labeling/molecular indexing assays. Such systems may include consumable cartridges that incorporate microwell arrays integrated with flow cells, as well as the instrumentation necessary to provide control and analysis functionality such as (i) fluidics control, (ii) temperature control, (iii) cell and/or bead distribution and collection mechanisms, (iv) cell lysis mechanisms, (v) imaging capability, and (vi) image processing. In some embodiments, the input for the system comprises a cell sample and the output comprises a bead suspension comprising beads having attached oligonucleotides that incorporate sample tags, cell tags, and molecular indexing tags. In other embodiments, the system may include additional functionality, such as thermal cycling capability for performing PCR amplification, in which case the input for the system comprises a cell sample and the output comprises an oligonucleotide library resulting from amplification of the oligonucleotides incorporating sample tags, cell tags, and molecular indexing tags that were originally attached to beads. In yet other embodiments, the system may also include sequencing capability, with or without the need for oligonucleotide amplification, in which case the input for the system is a cell sample and the output comprises a dataset further comprising the sequences of all sample tag, cell tag, and molecular indexing tags associated with the target sequences of interest.

Microwell array flow cells

[00196] In many embodiments of the automated assay system, the microwell array substrate will be packaged within a flow cell that provides for convenient interfacing with the rest of the fluid handling system and facilitates the exchange of fluids, *e.g.,* cell and bead suspensions, lysis buffers, rinse buffers, etc., that are delivered to the microwell array. Design features may include: (i) one or more inlet ports for introducing cell samples, bead suspensions, and/or other assay reagents, (ii) one or more microwell array chambers designed to provide for uniform filling and efficient fluid-exchange while minimizing back eddies or dead zones, and (iii) one or more outlet ports for delivery of fluids to a sample collection point and/or a waste reservoir. In some embodiments, the design of the flow cell may include a plurality of microarray chambers that interface with a plurality of microwell arrays such that one or more cell samples may be processed in parallel. In some embodiments, the design

of the flow cell may further include features for creating uniform flow velocity profiles, i.e. "plug flow", across the width of the array chamber to provide for more uniform delivery of cells and beads to the microwells, for example, by using a porous barrier located near the chamber inlet and upstream of the microwell array as a "flow diffuser", or by dividing each array chamber into several subsections that collectively cover the same total array area, but through which the divided inlet fluid stream flows in parallel. In some embodiments, the flow cell may enclose or incorporate more than one microwell array substrate. In some embodiments, the integrated microwell array / flow cell assembly may constitute a fixed component of the system. In some embodiments, the microwell array / flow cell assembly may be removable from the instrument.

[00197] In general, the dimensions of fluid channels and the array chamber(s) in flow cell designs will be optimized to (i) provide uniform delivery of cells and beads to the microwell array, and (ii) to minimize sample and reagent consumption. In some embodiments, the width of fluid channels will be between 50 microns and 20 mm. In other embodiments, the width of fluid channels may be at least 50 microns, at least 100 microns, at least 200 microns, at least 300 microns, at least 400 microns, at least 500 microns, at least 750 microns, at least 1 mm, at least 2.5 mm, at least 5 mm, at least 10 mm, or at least 20 mm. In yet other embodiments, the width of fluid channels may at most 20 mm, at most 10 mm, at most 5 mm, at most 2.5 mm, at most 1 mm, at most 750 microns, at most 500 microns, at most 400 microns, at most 300 microns, at most 200 microns, at most 100 microns, or at most 50 microns. In one embodiment, the width of fluid channels is about 2 mm. Those of skill in the art will appreciate that the width of the fluid channels may fall within any range bounded by any of these values (*e.g.,* from about 250 microns to about 3 mm).

[00198] In some embodiments, the depth of the fluid channels will be between 50 microns and 10 mm. In other embodiments, the depth of fluid channels may be at least 50 microns, at least 100 microns, at least 200 microns, at least 300 microns, at least 400 microns, at least 500 microns, at least 750 microns, at least 1 mm, at least 1.25 mm, at least 1.5 mm, at least 1.75 mm, at least 2 mm, at least 2.5 mm, at least 3 mm, at least 3.5 mm, at least 4 mm, at least 4.5 mm, at least 5 mm, at least 5.5 mm, at least 6 mm, at least 6.5 mm, at least 7 mm, at least 7.5 mm, at least 8 mm, at least 8.5 mm, at least 9 mm, or at least 9.5 mm. In other embodiments, the depth of fluid channels may be at most 10 mm, at most 9.5 mm, at most 9 mm, at most 8.5 mm, at most 8 mm, at most 7.5 mm, at most 7 mm, at most 6.5 mm, at most 6 mm, at most 5.5 mm, at most 5 mm, at most 4.5 mm, at most 4 mm, at most 3.5 mm, at most 3 mm, at most 2 mm, at most 1.75 mm, at most 1.5 mm, at most 1.25 mm, at most 1

mm, at most 750 microns, at most 500 microns, at most 400 microns, at most 300 microns, at most 200 microns, at most 100 microns, or at most 50 microns. In one embodiment, the depth of the fluid channels is about 1 mm. Those of skill in the art will appreciate that the depth of the fluid channels may fall within any range bounded by any of these values (*e.g.,* from about 800 microns to about 1 mm).

[00199] Flow cells may be fabricated using a variety of techniques and materials known to those of skill in the art. In general, the flow cell will be fabricated as a separate part and subsequently either mechanically clamped or permanently bonded to the microwell array substrate. Examples of suitable fabrication techniques include conventional machining, CNC machining, injection molding, 3D printing, alignment and lamination of one or more layers of laser or die-cut polymer films, or any of a number of microfabrication techniques such as photolithography and wet chemical etching, dry etching, deep reactive ion etching, or laser micromachining. Once the flow cell part has been fabricated it may be attached to the microwell array substrate mechanically, *e.g.,* by clamping it against the microwell array substrate (with or without the use of a gasket), or it may be bonded directly to the microwell array substrate using any of a variety of techniques (depending on the choice of materials used) known to those of skill in the art, for example, through the use of anodic bonding, thermal bonding, ultrasonic welding, or any of a variety of adhesives or adhesive films, including epoxy-based, acrylic-based, silicone-based, UV curable, polyurethane-based, or cyanoacrylate-based adhesives.

[00200] Flow cells may be fabricated using a variety of materials known to those of skill in the art. Examples of suitable materials include, but are not limited to, silicon, fused-silica, glass, any of a variety of polymers, *e.g.,* polydimethylsiloxane (PDMS; elastomer), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), polyimide, cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET), epoxy resins, metals (*e.g.,* aluminum, stainless steel, copper, nickel, chromium, and titanium), or a combination of these materials.

Cartridges

[00201] In many embodiments of the automated assay system, the microwell array, with or without an attached flow cell, will be packaged within a consumable cartridge that interfaces with the instrument system and which may incorporate additional functionality. Design features of cartridges may include (i) one or more inlet ports for creating fluid connections with the instrument and/or manually introducing cell samples, bead suspensions, and/or other

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assay reagents into the cartridge, (ii) one or more bypass channels, i.e. for self-metering of cell samples and bead suspensions, to avoid overfilling and/or back flow, (iii) one or more integrated microwell array / flow cell assemblies, or one or more chambers within which the microarray substrate(s) are positioned, (iv) integrated miniature pumps or other fluid actuation mechanisms for controlling fluid flow through the device, (v) integrated miniature valves for compartmentalizing pre-loaded reagents and/or controlling fluid flow through the device, (vi) vents for providing an escape path for trapped air, (vii) one or more sample and reagent waste reservoirs, (viii) one or more outlet ports for creating fluid connections with the instrument and/or providing a processed sample collection point, (ix) mechanical interface features for reproducibly positioning the removable, consumable cartridge with respect to the instrument system, and for providing access so that external magnets can be brought into close proximity with the microwell array, (x) integrated temperature control components and/or a thermal interface for providing good thermal contact with the instrument system, and (xi) optical interface features, *e.g.,* a transparent window, for use in optical interrogation of the microwell array. In some embodiments, the cartridge is designed to process more than one sample in parallel. In some embodiments of the device, the cartridge may further comprise one or more removable sample collection chamber(s) that are suitable for interfacing with stand-alone PCR thermal cyclers and/or sequencing instruments. In some embodiments of the device, the cartridge itself is suitable for interfacing with stand-alone PCR thermal cyclers and/or sequencing instruments.

[00202] In some embodiments of the device, the cartridge may further comprise components that are designed to create physical and/or chemical barriers that prevent diffusion of (or increase path lengths and diffusion times for) large molecules in order to minimize cross-contamination between microwells. Examples of such barriers include, but are not limited to, a pattern of serpentine channels used for delivery of cells and beads to the microwell array, a retractable platen or deformable membrane that is pressed into contact with the surface of the microwell array substrate during lysis or incubation steps, the use of larger beads, *e.g.,* Sephadex beads as described previously, to block the openings of the microwells, or the release of an immiscible, hydrophobic fluid from a reservoir within the cartridge during lysis or incubation steps, to effectively separate and compartmentalize each microwell in the array. Any or all of these barriers, or an embodiment without such barriers, may be combined with raising the viscosity of the solution in and adjacent to the microwells, *e.g.,* through the addition of solution components such as glycerol or polyethylene glycol.

[00203] In general, the dimensions of fluid channels and the array chamber(s) in cartridge designs will be optimized to (i) provide uniform delivery of cells and beads to the microwell array, and (ii) to minimize sample and reagent consumption. In some embodiments, the width of fluid channels will be between 50 microns and 20 mm. In other embodiments, the width of fluid channels may be at least 50 microns, at least 100 microns, at least 200 microns, at least 300 microns, at least 400 microns, at least 500 microns, at least 750 microns, at least 1 mm, at least 2.5 mm, at least 5 mm, at least 10 mm, or at least 20 mm. In yet other embodiments, the width of fluid channels may at most 20 mm, at most 10 mm, at most 5 mm, at most 2.5 mm, at most 1 mm, at most 750 microns, at most 500 microns, at most 400 microns, at most 300 microns, at most 200 microns, at most 100 microns, or at most 50 microns. In one embodiment, the width of fluid channels is about 2 mm. Those of skill in the art will appreciate that the width of the fluid channels may fall within any range bounded by any of these values (*e.g.,* from about 250 microns to about 3 mm).

[00204] In some embodiments, the depth of the fluid channels in cartridge designs will be between 50 microns and 10 mm. In other embodiments, the depth of fluid channels may be at least 50 microns, at least 100 microns, at least 200 microns, at least 300 microns, at least 400 microns, at least 500 microns, at least 750 microns, at least 1 mm, at least 1.25 mm, at least 1.5 mm, at least 1.75 mm, at least 2 mm, at least 2.5 mm, at least 3 mm, at least 3.5 mm, at least 4 mm, at least 4.5 mm, at least 5 mm, at least 5.5 mm, at least 6 mm, at least 6.5 mm, at least 7 mm, at least 7.5 mm, at least 8 mm, at least 8.5 mm, at least 9 mm, or at least 9.5 mm. In yet other embodiments, the depth of fluid channels may be at most 10 mm, at most 9.5 mm, at most 9 mm, at most 8.5 mm, at most 8 mm, at most 7.5 mm, at most 7 mm, at most 6.5 mm, at most 6 mm, at most 5.5 mm, at most 5 mm, at most 4.5 mm, at most 4 mm, at most 3.5 mm, at most 3 mm, at most 2 mm, at most 1.75 mm, at most 1.5 mm, at most 1.25 mm, at most 1 mm, at most 750 microns, at most 500 microns, at most 400 microns, at most 300 microns, at most 200 microns, at most 100 microns, or at most 50 microns. In one embodiment, the depth of the fluid channels is about 1 mm. Those of skill in the art will appreciate that the depth of the fluid channels may fall within any range bounded by any of these values (*e.g.,* from about 800 microns to about 1 mm).

[00205] Cartridges may be fabricated using a variety of techniques and materials known to those of skill in the art. In general, the cartridges will be fabricated as a series of separate component parts (**FIG. 72**) and subsequently assembled (**FIGs. 72** and **73**) using any of a number of mechanical assembly or bonding techniques. Examples of suitable fabrication techniques include, but are not limited to, conventional machining, CNC machining, injection

molding, thermoforming, and 3D printing. Once the cartridge components have been fabricated they may be mechanically assembled using screws, clips, and the like, or permanently bonded using any of a variety of techniques (depending on the choice of materials used), for example, through the use of thermal or ultrasonic bonding/welding or any of a variety of adhesives or adhesive films, including epoxy-based, acrylic-based, silicone-based, UV curable, polyurethane-based, or cyanoacrylate-based adhesives.

[00206] Cartridge components may be fabricated using any of a number of suitable materials, including but not limited to silicon, fused-silica, glass, any of a variety of polymers, *e.g.,* polydimethylsiloxane (PDMS; elastomer), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), polyimide, cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET), epoxy resins, or metals (*e.g.,* aluminum, stainless steel, copper, nickel, chromium, and titanium).

[00207] As described above, the inlet and outlet features of the cartridge may be designed to provide convenient and leak-proof fluid connections with the instrument, or may serve as open reservoirs for manual pipetting of samples and reagents into or out of the cartridge. Examples of convenient mechanical designs for the inlet and outlet port connectors include, but are not limited to, threaded connectors, swaged connectors, Luer lock connectors, Luer slip or "slip tip" connectors, press fit connectors, and the like. In some embodiments, the inlet and outlet ports of the cartridge may further comprise caps, spring-loaded covers or closures, phase change materials, or polymer membranes that may be opened or punctured when the cartridge is positioned in the instrument, and which serve to prevent contamination of internal cartridge surfaces during storage and/or which prevent fluids from spilling when the cartridge is removed from the instrument. As indicated above, in some embodiments the one or more outlet ports of the cartridge may further comprise a removable sample collection chamber that is suitable for interfacing with stand-alone PCR thermal cyclers and/or sequencing instruments.

[00208] As indicated above, in some embodiments the cartridge may include integrated miniature pumps or other fluid actuation mechanisms for control of fluid flow through the device. Examples of suitable miniature pumps or fluid actuation mechanisms include, but are not limited to, electromechanically- or pneumatically-actuated miniature syringe or plunger mechanisms, chemical propellants, membrane diaphragm pumps actuated pneumatically or by an external piston, pneumatically-actuated reagent pouches or bladders, or electro-osmotic pumps.

[00209] As described above, in some embodiments the cartridge may include miniature valves for compartmentalizing pre-loaded reagents and/or controlling fluid flow through the device. Examples of suitable miniature valves include, but are not limited to, one-shot "valves" fabricated using wax or polymer plugs that can be melted or dissolved, or polymer membranes that can be punctured; pinch valves constructed using a deformable membrane and pneumatic, hydraulic, magnetic, electromagnetic, or electromechanical (solenoid) acutation, one-way valves constructed using deformable membrane flaps, and miniature gate valves.

[00210] As indicated above, in some embodiments the cartridge may include vents for providing an escape path for trapped air. Vents may be constructed according to a variety of techniques known to those of skill in the art, for example, using a porous plug of polydimethylsiloxane (PDMS) or other hydrophobic material that allows for capillary wicking of air but blocks penetration by water. Vents may also be constructed as apertures through hydrophobic barrier materials, such that wetting to the aperture walls does not occur at the pressures used during operation.

[00211] In general, the mechanical interface features of the cartridge provide for easily removable but highly precise and repeatable positioning of the cartridge relative to the instrument system. Suitable mechanical interface features include, but are not limited to, alignment pins, alignment guides, mechanical stops, and the like. In some embodiments, the mechanical design features will include relief features for bringing external apparatus, *e.g.,* magnets or optical components, into close proximity with the microwell array chamber (**FIG. 72**).

[00212] In some embodiments, the cartridge will also include temperature control components or thermal interface features for mating to external temperature control modules. Examples of suitable temperature control elements include, but are not limited to, resistive heating elements, miniature infrared-emitting light sources, Peltier heating or cooling devices, heat sinks, thermistors, thermocouples, and the like. Thermal interface features will typically be fabricated from materials that are good thermal conductors (*e.g.,* copper, gold, silver, aluminium, etc.) and will typically comprise one or more flat surfaces capable of making good thermal contact with external heating blocks or cooling blocks.

[00213] In many embodiments, the cartridge will include optical interface features for use in optical imaging or spectroscopic interrogation of the microwell array. Typically, the cartridge will include an optically transparent window, *e.g.,* the microwell substrate itself or the side of the flow cell or microarray chamber that is opposite the microwell array,

fabricated from a material that meets the spectral requirements for the imaging or spectroscopic technique used to probe the microwell array. Examples of suitable optical window materials include, but are not limited to, glass, fused-silica, polymethylmethacrylate (PMMA), polycarbonate (PC), cyclic olefin polymers (COP), or cyclic olefin copolymers (COC). Typically, the cartridge will include a second optically transparent or translucent window or region which can be used to illuminate the microwell array in transverse, reflected, or oblique illumination orientations.

Instruments

[00214] The present disclosure also includes instruments for use in the automation of multiplexed, single cell stochastic labeling/molecular indexing assays. As indicated above, these instruments may provide control and analysis functionality such as (i) fluidics control, (ii) temperature control, (iii) cell and/or bead distribution and collection mechanisms, (iv) cell lysis mechanisms, (v) magnetic field control, (vi) imaging capability, and (vii) image processing. In some embodiments, the instrument system may comprise one or more modules (one possible embodiment of which is illustrated schematically in **FIG. 74**), where each module provides one or more specific functional feature sets to the system. In other embodiments, the instrument system may be packaged such that all system functionality resides within the same package. **FIG. 75** provides a schematic illustration of the process steps included in one embodiment of the automated system. As indicated above, in some embodiments, the system may comprise additional functional units, either as integrated components or as modular components of the system, that expand the functional capabilities of the system to include PCR amplification (or other types of oligonucleotide amplification techniques) and oligonucleotide sequencing.

[00215] In general, the instrument system will provide fluidics capability for delivering samples and/or reagents to the one or more microarray chamber(s) or flow cell(s) within one or more assay cartridge(s) connected to the system. Assay reagents and buffers may be stored in bottles, reagent and buffer cartridges, or other suitable containers that are connected to the cartridge inlets. The system may also include waste reservoirs in the form of bottles, waste cartridges, or other suitable waste containers for collecting fluids downstream of the assay cartridge(s). Control of fluid flow through the system will typically be performed through the use of pumps (or other fluid actuation mechanisms) and valves. Examples of suitable pumps include, but are not limited to, syringe pumps, programmable syringe pumps, peristaltic pumps, diaphragm pumps, and the like. In some embodiments, fluid flow through the system may be controlled by means of applying positive pneumatic pressure at the one or

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more inlets of the reagent and buffer containers, or at the inlets of the assay cartridge(s). In some embodiments, fluid flow through the system may be controlled by means of drawing a vacuum at the one or more outlets of the waste reservoirs, or at the outlets of the assay cartridge(s). Examples of suitable valves include, but are not limited to, check valves, electromechanical two-way or three-way valves, pneumatic two-way and three-way valves, and the like. In some embodiments, pulsatile flow may be applied during assay wash/rinse steps to facilitate complete and efficient exchange of fluids within the one or more microwell array flow cell(s) or chamber(s).

[00216] As indicated above, in some embodiments the instrument system may include mechanisms for further facilitating the uniform distribution of cells and beads over the microwell array. Examples of such mechanisms include, but are not limited to, rocking, shaking, swirling, recirculating flow, low frequency agitation (for example, using a rocker plate or through pulsing of a flexible (*e.g.,* silicone) membrane that forms a wall of the chamber or nearby fluid channel), or high frequency agitation (for example, through the use of piezoelectric transducers). In some embodiments, one or more of these mechanisms is utilized in combination with physical structures or features on the interior walls of the flow cell or array chamber, *e.g.,* mezzanine/top hat structures, chevrons, or ridge arrays, to facilitate mixing and/or to help prevent pooling of cells or beads within the array chamber. Flow-enhancing ribs on upper or lower surfaces of the flow cell or array chamber may be used to control flow velocity profiles and reduce shear across the microwell openings (i.e. to prevent cells or beads from being pulled out of the microwells during reagent exchange and rinse steps).

[00217] In some embodiments, the instrument system may include mechanical cell lysis capability as an alternative to the use of detergents or other reagents. Sonication using a high frequency piezoelectric transducer is one example of a suitable technique.

[00218] In some embodiments, the instrument system will include temperature control functionality for the purpose of facilitating the accuracy and reproducibility of assay results, for example, cooling of the microwell array flow cell or chamber may be advantageous for minimizing molecular diffusion between microwells. Examples of temperature control components that may be incorporated into the instrument system design include, but are not limited to, resistive heating elements, infrared light sources, Peltier heating or cooling devices, heat sinks, thermistors, thermocouples, and the like. In some embodiments of the system, the temperature controller may provide for programmable changes in temperature over specified time intervals.

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[00219] As indicated elsewhere in this disclosure, many embodiments of the disclosed methods utilize magnetic fields for removing beads from the microwells upon completion of the assay. In some embodiments, the instrument system may further comprise use of magnetic fields for transporting beads into or out of the microwell array flow cell or chamber. Examples of suitable means for providing control of magnetic fields include, but are not limited to, use of electromagnets in fixed position(s) relative to the cartridge, or the use of permanent magnets that are mechanically repositioned as necessary. In some embodiments of the instrument system, the strength of the applied magnetic field(s) will be varied by varying the amount of current applied to one or more electromagnets. In some embodiments of the instrument system, the strength of the applied magnetic fields will be varied by changing the position of one or more permanent magnets relative to the position of the microarray chamber(s) using, for example, stepper motor-driven linear actuators, servo motor-driven linear actuators, or cam shaft mechanisms. In some embodiments of the instrument system, the use of pulsed magnetic fields may be advantageous, for example, to prevent clustering of magnetic beads. In some embodiments, a magnet in close proximity to the array or chamber may be moved, once or multiple times, between at least two positions relative to the microwell array. Motion of the magnets can serve to agitate beads within microwells, to facilitate removal of beads from microwells, or to collect magnetic beads at a desired location.

[00220] As indicated above, in many embodiments the instrument system will include optical imaging and/or other spectroscopic capabilities. Such functionality may be useful, for example, for inspection of the microwell array(s) to determine whether or not the array has been uniformly and optimally populated with cells and/or beads. Any of a variety of imaging modes may be utilized, including but not limited to, bright-field, dark-field, and fluorescence/luminescence imaging. The choice of imaging mode will impact the design of microwell arrays, flow cells, and cartridge chambers in that the array substrate and/or opposing wall of the flow cell or array chamber will necessarily need to be transparent or translucent over the spectral range of interest. In some embodiments, each microwell array may be imaged in its entirety within a single image. In some embodiments, a series of images may be "tiled" to create a high resolution image of the entire array. In some embodiment, a single image that represents a subsection of the array may be used to evaluate properties, *e.g.,* cell or bead distributions, for the array as a whole. In some embodiments, dual wavelength excitation and emission (or multi-wavelength excitation and/or emission) imaging may be performed. Any of a variety of light sources may be used to provide the

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imaging and/or excitation light, including but not limited to, tungsten lamps, tungsten-halogen lamps, arc lamps, lasers, light emitting diodes (LEDs), or laser diodes. Any of a variety of image sensors may be used for imaging purposes, including but not limited to, photodiode arrays, charge-coupled device (CCD) cameras, or CMOS image sensors. The optical system will typically include a variety of optical components for steering, shaping, filtering, and/or focusing light beams through the system. Examples of suitable optical components include, but are not limited to, lenses, mirrors, prisms, diffraction gratings, colored glass filters, narrowband interference filters, broadband interference filters, dichroic reflectors, optical fibers, optical waveguides, and the like. In some embodiments, the instrument system may use an optically transparent microarray substrate as a waveguide for delivering excitation light to the microwell array. The choice of imaging mode may also enable the use of other types of assays to be run in parallel with stochastic labeling / molecular indexing assays, for example, the use of trypan blue live cell / dead cell assays with bright field imaging, the use of fluorescence-based live cell / dead cell assays with fluorescence imaging, etc. Correlation of viability data for individual cells with the cell tag associated with each bead in the associated microwell may provide an additional level of discrimination in analyzing the data from multiplexed, single cell assays. Alternatively, viability data in the form of statistics for multiple cells may be employed for enhancing the analytical capabilities and quality assurance of the assay.

[00221] In some embodiments, the system may comprise non-imaging and/or non-optical capabilities for probing the microwell array. Examples of non-imaging and/or non-optical techniques for detecting trapped air bubbles, determining the cell and/or bead distribution over the array, etc., include but are not limited to measurements of light scattering, ultraviolet/visible/infrared absorption measurements (*e.g.*, using stained cells and/or beads that incorporate dyes), coherent raman scattering, and conductance measurements (*e.g.*, using microfabricated arrays of electrodes in register with the microwell arrays).

System processor and software

[00222] In general, instrument systems designed to support the automation of multiplexed, single cell stochastic labeling/molecular indexing assays will include a processor or computer, along with software to provide (i) instrument control functionality, (ii) image processing and analysis capability, and (iii) data storage, analysis, and display functionality.

[00223] In many embodiments, the instrument system will comprise a computer (or processor) and computer-readable media that includes code for providing a user interface as well as manual, semi-automated, or fully-automated control of all system functions, i.e.

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control of the fluidics system, the temperature control system, cell and/or bead distribution functions, magnetic bead manipulation functions, and the imaging system. Examples of fluid control functions provided by the instrument control software include, but are not limited to, volumetric fluid flow rates, fluid flow velocities, the timing and duration for sample and bead addition, reagent addition, and rinse steps. Examples of temperature control functions provided by the instrument control software include, but are not limited to, specifying temperature set point(s) and control of the timing, duration, and ramp rates for temperature changes. Examples of cell and/or bead distribution functions provided by the instrument control software include, but are not limited to, control of agitation parameters such as amplitude, frequency, and duration. Examples of magnetic field functions provided by the instrument control software include, but are not limited to, the timing and duration of the applied magnetic field(s), and in the case of electromagnets, the strength of the magnetic field as well. Examples of imaging system control functions provided by the instrument control software include, but are not limited to, autofocus capability, control of illumination and/or excitation light exposure times and intensities, control of image acquisition rate, exposure time, and data storage options.

[00224] In some embodiments of the instrument system, the system will further comprise computer-readable media that includes code for providing image processing and analysis capability. Examples of image processing and analysis capability provided by the software include, but are not limited to, manual, semi-automated, or fully-automated image exposure adjustment (*e.g.,* white balance, contrast adjustment, signal-averaging and other noise reduction capability, etc.), automated object identification (i.e. for identifying cells and beads in the image), automated statistical analysis (i.e. for determining the number of cells and/or beads identified per unit area of the microwell array, or for identifying wells that contain more than one cell or more than one bead), and manual measurement capabilities (*e.g.,* for measuring distances between objects, etc.). In some embodiments, the instrument control and image processing/analysis software will be written as separate software modules. In some embodiments, the instrument control and image processing/analysis software will be incorporated into an integrated package. In some embodiments, the system software may provide integrated real-time image analysis and instrument control, so that cell and bead sample loading steps can be prolonged or repeated until optimal cell/bead distributions are achieved.

[00225] In some embodiments of the instrument system, the system will comprise computer-readable media that includes code for providing sequence data analysis. Examples

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of sequence data analysis functionality that may be provided by the data analysis software includes, but is not limited to, (i) algorithms for determining the number of reads per gene per cell, and the number of unique transcript molecules per gene per cell, based on the data provided by sequencing the oligonucleotide library created by running the assay, (ii) statistical analysis of the sequencing data, *e.g.,* principal component analysis, for predicting confidence intervals for determinations of the number of transcript molecules per gene per cell, etc., (iii) sequence alignment capabilities for alignment of gene sequence data with known reference sequences, (iv) decoding/demultiplexing of sample barcodes, cell barcodes, and molecular barcodes, and (v) automated clustering of molecular labels to compensate for amplification or sequencing errors.

[00226] In general, the computer or processor included in the presently disclosed instrument systems, as illustrated in **FIG. 76**, may be further understood as a logical apparatus that can read instructions from media 511 and/or a network port 505, which can optionally be connected to server 509 having fixed media 512. The system 500, such as shown in **FIG. 76** can include a CPU 501, disk drives 503, optional input devices such as keyboard 515 and/or mouse 516 and optional monitor 507. Data communication can be achieved through the indicated communication medium to a server at a local or a remote location. The communication medium can include any means of transmitting and/or receiving data. For example, the communication medium can be a network connection, a wireless connection or an internet connection. Such a connection can provide for communication over the World Wide Web. It is envisioned that data relating to the present disclosure can be transmitted over such networks or connections for reception and/or review by a party 522 as illustrated in **FIG. 76**.

[00227] **FIG. 77** is a block diagram illustrating a first example architecture of a computer system 100 that can be used in connection with example embodiments of the present disclosure. As depicted in **FIG. 77**, the example computer system can include a processor 102 for processing instructions. Non-limiting examples of processors include: Intel XeonTM processor, AMD OpteronTM processor, Samsung 32-bit RISC ARM 1176JZ(F)-S v1.0TM processor, ARM Cortex-A8 Samsung S5PC100TM processor, ARM Cortex-A8 Apple A4TM processor, Marvell PXA 930TM processor, or a functionally-equivalent processor. Multiple threads of execution can be used for parallel processing. In some embodiments, multiple processors or processors with multiple cores can also be used, whether in a single computer system, in a cluster, or distributed across systems over a network comprising a plurality of computers, cell phones, and/or personal data assistant devices.

[00228] As illustrated in **FIG. 77**, a high speed cache 104 can be connected to, or incorporated in, the processor 102 to provide a high speed memory for instructions or data that have been recently, or are frequently, used by processor 102. The processor 102 is connected to a north bridge 106 by a processor bus 108. The north bridge 106 is connected to random access memory (RAM) 110 by a memory bus 112 and manages access to the RAM 110 by the processor 102. The north bridge 106 is also connected to a south bridge 114 by a chipset bus 116. The south bridge 114 is, in turn, connected to a peripheral bus 118. The peripheral bus can be, for example, PCI, PCI-X, PCI Express, or other peripheral bus. The north bridge and south bridge are often referred to as a processor chipset and manage data transfer between the processor, RAM, and peripheral components on the peripheral bus 118. In some alternative architectures, the functionality of the north bridge can be incorporated into the processor instead of using a separate north bridge chip.

[00229] In some embodiments, system 100 can include an accelerator card 122 attached to the peripheral bus 118. The accelerator can include field programmable gate arrays (FPGAs) or other hardware for accelerating certain processing. For example, an accelerator can be used for adaptive data restructuring or to evaluate algebraic expressions used in extended set processing.

[00230] Software and data are stored in external storage 124 and can be loaded into RAM 110 and/or cache 104 for use by the processor. The system 100 includes an operating system for managing system resources; non-limiting examples of operating systems include: Linux, Windows™, MACOS™, BlackBerry OS™, iOS™, and other functionally-equivalent operating systems, as well as application software running on top of the operating system for managing data storage and optimization in accordance with example embodiments of the present invention.

[00231] In this example, system 100 also includes network interface cards (NICs) 120 and 121 connected to the peripheral bus for providing network interfaces to external storage, such as Network Attached Storage (NAS) and other computer systems that can be used for distributed parallel processing.

[00232] **FIG. 78** is a diagram showing a network 200 with a plurality of computer systems 202a, and 202b, a plurality of cell phones and personal data assistants 202c, and Network Attached Storage (NAS) 204a, and 204b. In example embodiments, systems 212a, 212b, and 212c can manage data storage and optimize data access for data stored in Network Attached Storage (NAS) 214a and 214b. A mathematical model can be used for the data and be evaluated using distributed parallel processing across computer systems 212a, and 212b, and

cell phone and personal data assistant systems 212c. Computer systems 212a, and 212b, and cell phone and personal data assistant systems 212c can also provide parallel processing for adaptive data restructuring of the data stored in Network Attached Storage (NAS) 214a and 214b. **FIG. 78** illustrates an example only, and a wide variety of other computer architectures and systems can be used in conjunction with the various embodiments of the present invention. For example, a blade server can be used to provide parallel processing. Processor blades can be connected through a back plane to provide parallel processing. Storage can also be connected to the back plane or as Network Attached Storage (NAS) through a separate network interface.

[00233] In some example embodiments, processors can maintain separate memory spaces and transmit data through network interfaces, back plane or other connectors for parallel processing by other processors. In other embodiments, some or all of the processors can use a shared virtual address memory space.

[00234] **FIG. 79** is a block diagram of a multiprocessor computer system 300 using a shared virtual address memory space in accordance with an example embodiment. The system includes a plurality of processors 302a-f that can access a shared memory subsystem 304. The system incorporates a plurality of programmable hardware memory algorithm processors (MAPs) 306a-f in the memory subsystem 304. Each MAP 306a-f can comprise a memory 308a-f and one or more field programmable gate arrays (FPGAs) 310a-f. The MAP provides a configurable functional unit and particular algorithms or portions of algorithms can be provided to the FPGAs 310a-f for processing in close coordination with a respective processor. For example, the MAPs can be used to evaluate algebraic expressions regarding the data model and to perform adaptive data restructuring in example embodiments. In this example, each MAP is globally accessible by all of the processors for these purposes. In one configuration, each MAP can use Direct Memory Access (DMA) to access an associated memory 308a-f, allowing it to execute tasks independently of, and asynchronously from, the respective microprocessor 302a-f. In this configuration, a MAP can feed results directly to another MAP for pipelining and parallel execution of algorithms.

[00235] The above computer architectures and systems are examples only, and a wide variety of other computer, cell phone, and personal data assistant architectures and systems can be used in connection with example embodiments, including systems using any combination of general processors, co-processors, FPGAs and other programmable logic devices, system on chips (SOCs), application specific integrated circuits (ASICs), and other processing and logic elements. In some embodiments, all or part of the computer system can

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be implemented in software or hardware. Any variety of data storage media can be used in connection with example embodiments, including random access memory, hard drives, flash memory, tape drives, disk arrays, Network Attached Storage (NAS) and other local or distributed data storage devices and systems.

[00236] In example embodiments, the computer subsystem of the present disclosure can be implemented using software modules executing on any of the above or other computer architectures and systems. In other embodiments, the functions of the system can be implemented partially or completely in firmware, programmable logic devices such as field programmable gate arrays (FPGAs) as referenced in **FIG. 79**, system on chips (SOCs), application specific integrated circuits (ASICs), or other processing and logic elements. For example, the Set Processor and Optimizer can be implemented with hardware acceleration through the use of a hardware accelerator card, such as accelerator card 122 illustrated in **FIG. 77**.

Oligonucleotides (*e.g.*, Molecular Barcodes)

[00237] The methods and kits disclosed herein may comprise one or more oligonucleotides or uses thereof. The oligonucleotides may be attached to a solid support disclosed herein. Attachment of the oligonucleotide to the solid support may occur through functional group pairs on the solid support and the oligonucleotide. The oligonucleotide may be referred to as a molecular bar code. The oligonucleotide may be referred to as a label (*e.g.*, molecular label, cellular label) or tag (*e.g.*, sample tag).

[00238] Oligonucleotides may comprise a universal label. A universal label may be the same for all oligonucleotides in a sample. A universal label may be the same for oligonucleotides in a set of oligonucleotides. A universal label may be the same for two or more sets of oligonucleotides. A universal label may comprise a sequence of nucleic acids that may hybridize to a sequencing primer. Sequencing primers may be used for sequencing oligonucleotides comprising a universal label. Sequencing primers (*e.g.*, universal sequencing primers) may comprise sequencing primers associated with high-throughput sequencing platforms. A universal label may comprise a sequence of nucleic acids that may hybridize to a PCR primer. A universal label may comprise a sequence of nucleic acids that may hybridize to a sequencing primer and a PCR primer. The sequence of nucleic acids of the universal label that may hybridize to a sequencing and/or PCR primer may be referred to as a primer binding site. A universal label may comprise a sequence that may be used to initiate transcription of the oligonucleotide. A universal label may comprise a sequence that may be used for extension of the oligonucleotide or a region within the oligonucleotide. A universal

label may be at least about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length. A universal label may comprise at least about 10 nucleotides. A universal label may be at most about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length.

[00239] Oligonucleotides may comprise a cellular label. A cellular label may comprise a nucleic acid sequence that may provide information for which cell the oligonucleotide is contacted to (*e.g.*, determining which nucleic acid originated from which cell). At least 60%, 70%, 80%, 85%, 90%, 95%, 97%, 99% or 100% of oligonucleotides on the same solid support may comprise the same cellular label. At least 60% of oligonucleotides on the same solid support may comprise the same cellular label. At least 95% of oligonucleotides on the same solid support may comprise the same cellular label. All the oligonucleotides on a same solid support may comprise the same cellular label. The cellular label of the oligonucleotides on a first solid support may be different than the cellular labels of the oligonucleotides on the second solid support.

[00240] A cellular label may be at least about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length. A cellular label may be at most about 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 12, 10, 9, 8, 7, 6, 5, 4 or fewer or more nucleotides in length. A cellular label may comprise between about 5 to about 200 nucleotides. A cellular label may comprise between about 10 to about 150 nucleotides. A cellular label may comprise between about 20 to about 125 nucleotides in length.

[00241] Oligonucleotides may comprise a molecular label. A molecular label may comprise a nucleic acid sequence that may provide identifying information for the specific nucleic acid species hybridized to the oligonucleotide. Oligonucleotides conjugated to a same solid support may comprise different molecular labels. In this way, the molecular label may distinguish the types of target nucleic acids (*e.g.*, genes), that hybridize to the different oligonucleotides. A molecular label may be at least about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length. A molecular label may be at most about 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 12, 10, 9, 8, 7, 6, 5, 4 or fewer nucleotides in length.

[00242] Oligonucleotides may comprise a sample label (*e.g.*, sample index). A sample label may comprise a nucleic acid sequence that may provide information about from where a target nucleic acid originated. For example, a sample label may be different on different solid supports used in different experiments. A sample label may be at least about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length. A sample label may be at most

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about 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 12, 10, 9, 8, 7, 6, 5, 4 or fewer nucleotides in length.

[00243] An oligonucleotide may comprise a universal label, a cellular label, a molecular label and a sample label, or any combination thereof. In combination, the sample label may be used to distinguish target nucleic acids between samples, the cellular label may be used to distinguish target nucleic acids from different cells in the sample, the molecular label may be used to distinguish the different target nucleic acids in the cell (*e.g.*, different copies of the same target nucleic acid), and the universal label may be used to amplify and sequence the target nucleic acids.

[00244] A universal label, a molecular label, a cellular label, linker label and/or a sample label may comprise a random sequence of nucleotides. A random sequence of nucleotides may be computer generated. A random sequence of nucleotides may have no pattern associated with it. A universal label, a molecular label, a cellular label, linker label and/or a sample label may comprise a non-random (*e.g.*, the nucleotides comprise a pattern) sequence of nucleotides. Sequences of the universal label, a molecular label, a cellular label, linker label and/or a sample label may be commercially available sequences. Sequences of the universal label, a molecular label, a cellular label, linker label and/or a sample label may be comprise randomer sequences. Randomer sequences may refer to oligonucleotide sequences composed of all possible sequences for a given length of the randomer. Alternatively, or additionally, a universal label, a molecular label, a cellular label, linker label and/or a sample label may comprise a predetermined sequence of nucleotides.

[00245] **FIG. 1** shows an exemplary oligonucleotide of the disclosure comprising a universal label, a cellular label and a molecular label.

[00246] **FIG. 3** shows an exemplary oligonucleotide coupled solid support comprising a solid support (301) coupled to an oligonucleotide (312). The oligonucleotide (312) comprises a chemical group (5' amine, 302), a universal label (303), a cellular label (311), a molecular label (Molecular BC, 311), and a target binding region (oligodT, 310). In this schematic, the cellular label (311) comprises a first cell label (CL Part 1, 304), a first linker (Linker1, 305), a second cell label (CL Part 2, 306), a second linker (Linker2, 307), a third cell label (CL Part 3, 308). The cellular label (311) is common for each oligonucleotide on the solid support. The cellular labels (311) for two or more beads may be different. The cellular labels (311) for two or more beads may differ by the cell labels (*e.g.*, CL Part 1 (304), CL Part 2 (306), CL Part 3 (308)). The cellular labels (311) for two or more beads may differ by the first cell label (304), second cell label (306), third cell label (308), or a combination thereof. The first and second

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linkers (303, 305) of the cellular labels (311) may be identical for two or more oligonucleotide coupled solid supports. The universal label (303) may be identical for two or more oligonucleotide coupled solid supports. The universal label (303) may be identical for two or more oligonucleotides on the same solid support. The molecular label (311) may be different for at least two or more oligonucleotides on the solid support. The solid support may comprise 100 or more oligonucleotides. The solid support may comprise 1000 or more oligonucleotides. The solid support may comprise 10000 or more oligonucleotides. The solid support may comprise 100000 or more oligonucleotides.

[00247] In addition to a universal label, a cellular label, and a molecular label, an oligonucleotide may comprise a target binding region. A target binding region may comprise a nucleic acid sequence that may bind to a target nucleic acid (*e.g.*, a cellular nucleic acid to be analyzed). A target binding region may be a gene specific sequence. For example, a target binding region may comprise a nucleic acid sequence that may attach (*e.g.*, hybridize) to a specific location of a specific target nucleic acid. A target binding region may comprise a non-specific target nucleic acid sequence. A non-specific target nucleic acid sequence may refer to a sequence that may bind to multiple target nucleic acids, independent of the specific sequence of the target nucleic acid. For example, target binding region may comprise a random multimer sequence or an oligo dT sequence (*e.g.*, a stretch of thymidine nucleotides that may hybridize to a poly-adenylation tail on mRNAs). A random multimer sequence can be, for example, a random dimer, trimer, quatramer, pentamer, hexamer, septamer, octamer, nonamer, decamer, or higher multimer sequence of any length. A target binding region may be at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length. A target binding region may be at most about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length.

[00248] An oligonucleotide may comprise a plurality of labels. For example an oligonucleotide may comprise at least about 1, 2, 3, 4, 5, 6, 7, or 8 or more universal labels. An oligonucleotide may comprise at most about 1, 2, 3, 4, 5, 6, 7, or 8 or more universal labels. An oligonucleotide may comprise at least about 1, 2, 3, 4, 5, 6, 7, or 8 or more cellular labels. An oligonucleotide may comprise at most about 1, 2, 3, 4, 5, 6, 7, or 8 or more cellular labels. An oligonucleotide may comprise at least about 1, 2, 3, 4, 5, 6, 7, or 8 or more molecular labels. An oligonucleotide may comprise at most about 1, 2, 3, 4, 5, 6, 7, or 8 or more molecular labels. An oligonucleotide may comprise at least about 1, 2, 3, 4, 5, 6, 7, or 8 or more sample labels. An oligonucleotide may comprise at most about 1, 2, 3, 4, 5, 6, 7, or 8 or more sample labels. An oligonucleotide may comprise at least about 1, 2, 3, 4, 5, 6, 7, or 8

or more target binding regions. An oligonucleotide may comprise at most about 1, 2, 3, 4, 5, 6, 7, or 8 or more target binding regions.

[00249] When an oligonucleotide comprises more than one of a type of label (*e.g.*, more than one cellular label or more than one molecular label), the labels may be interspersed with a linker label sequence. A linker label sequence may be at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length. A linker label sequence may be at most about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more nucleotides in length. In some instances, a linker label sequence is 12 nucleotides in length. A linker label sequence may be used to facilitate the synthesis of the oligonucleotide, such as diagrammed in **FIG. 2A**.

[00250] The number of oligonucleotides conjugated to a solid support may be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10-fold more than the number of target nucleic acids in a cell. In some instances, at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% of the oligonucleotides are bound by a target nucleic acid. In some instances, at most 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% of the oligonucleotides are bound by a target nucleic acid. In some instances, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 or more different target nucleic acids are captured by the oligonucleotides on a solid support. In some instances, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 or more different target nucleic acids are captured by the oligonucleotides on a solid support.

[00251] A polymer may comprise additional solid supports. For example, a polymer may be dotted with beads. The beads may be spatially located at different regions of the polymer. The beads or supports comprising oligonucleotides of the disclosure may be spatially addressed. The beads or supports may comprise a barcode corresponding to a spatial address on the polymer. For example, each bead or support of a plurality of beads or supports may comprise barcode that corresponds to a position on a polymer, such as a position on an array or a particular microwall of a plurality of microwells. The spatial address can be decoded to determine the location from which a bead or support was positioned. For example, a spatial address, such as a barcode, can be decoded by hybridization of an oligonucleotide to the barcode or by sequencing the barcode. Alternatively, beads or supports can bear other types of barcodes, such as graphical features, chemical groups, colors, fluorescence, or combinations any combination thereof, for spatial address decoding purposes.

[00252] The methods and kits disclosed herein may comprise one or more sets of molecular barcodes. One or more molecular barcodes may comprise a sample index region and a label region. Two or more molecular barcodes of a set of molecular barcodes may comprise the same sample index region and two or more different label regions. Two or more molecular

barcodes of two or more sets of molecular barcodes may comprise two or more different sample index regions. Two or more molecular barcodes from a set of molecular barcodes may comprise different label regions. Two or more molecular barcodes of two or more sets of molecular barcodes may comprise the same label region. Molecular barcodes from two or more sets of molecular barcodes may differ by their sample index regions. Molecular barcodes from two or more sets of molecular barcodes may be similar based on their label regions.

[00253] The molecular barcodes may further comprise a target specific region, an adapter region, a universal PCR region, a target specific region or any combination thereof. The molecular barcode may comprise a universal PCR region and a target specific region. The molecular barcode may comprise one or more secondary structures. The molecular barcode may comprise a hairpin structure. The molecular barcode may comprise a target specific region and a cleavable stem.

[00254] The methods and kits disclosed herein may comprise one or more sets of sample tags. One or more sample tags may comprise a sample index region. One or more sample tags may comprise a sample index region. Two or more sample tags of a set of sample tags may comprise the same sample index region. Two or more sample tags of two or more sets of sample tags may comprise two or more different sample index regions.

[00255] The sample tags may further comprise a target specific region, an adapter region, a universal PCR region, a target specific region or any combination thereof. The sample tag may comprise a universal PCR region and a target specific region. The sample tag may comprise one or more secondary structures. The sample tag may comprise a hairpin structure. The sample tag may comprise a target specific region and a cleavable stem.

[00256] The methods and kits disclosed herein may comprise one or more sets of, molecular identifier labels. One or more molecular identifier labels may comprise a label region. One or more molecular identifier labels may comprise a label region. Two or more molecular identifier labels of a set of molecular identifier labels may comprise two or more different label regions. Two or more molecular identifier labels of two or more sets of molecular identifier labels may comprise two or more identical label regions. The molecular identifier labels may further comprise a target specific region, an adapter region, a universal PCR region, a target specific region or any combination thereof. The molecular identifier label may comprise a universal PCR region and a target specific region. The molecular identifier label may comprise one or more secondary structures. The molecular identifier label may

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comprise a hairpin structure. The molecular identifier label may comprise a target specific region and a cleavable stem.

[00257] The molecular barcode, sample tag or molecular identifier label may comprise at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or base pairs. In another example, the sample tag or molecular identifier label comprises at least about 1500, 2,000; 2500, 3,000; 3500, 4,000; 4500, 5,000; 5500, 6,000; 6500, 7,000; 7500, 8,000; 8500, 9,000; 9500, or 10,000 nucleotides or base pairs.

[00258] The molecular barcodes, sample tags or molecular identifier labels may be multimers, *e.g.,* random multimers. A multimer sequence can be, for example, a non-random or random dimer, trimer, quatramer, pentamer, hexamer, septamer, octamer, nonamer, decamer, or higher multimer sequence of any length. The tags may be randomly generated from a set of mononucleotides. The tags may be assembled by randomly incorporating mononucleotides.

[00259] The molecular barcodes, sample tags or molecular identifier labels may also be assembled without randomness, to generate a library of different tags which are not randomly generated but which includes sufficient numbers of different tags to practice the methods.

[00260] In some embodiments a molecular barcode, sample tag or molecular identifier label may comprise a cutback in a target nucleic acid. The cutback may be, for example, an enzymatic digestion of one or both ends of a target nucleic acid. The cutback may be used in conjunction with the addition of added molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label). The combination of the cutback and the added tags may contain information related to the particular starting molecule. By adding a random cutback to the molecular barcode, sample tag or molecular identifier label, a smaller diversity of the added tags may be necessary for counting the number of target nucleic acids when detection allows a determination of both the random cutback and the added oligonucleotides.

[00261] The molecular barcode, sample tag or molecular identifier label may comprise a target specific region. The target specific region may comprise a sequence that is complementary to the molecule. In some instances, the molecule is an mRNA molecule and the target specific region comprises an oligodT sequence that is complementary to the polyA tail of the mRNA molecule. The target specific region may also act as a primer for DNA and/or RNA synthesis. For example, the oligodT sequence of the target specific region may act as a primer for first strand synthesis of a cDNA copy of the mRNA molecule.

Alternatively, the target specific region comprises a sequence that is complementary to any portion of the molecule. In other instances, the target specific region comprises a random sequence that may be hybridized or ligated to the molecule. The target specific region may enable attachment of the sample tag or molecular identifier label to the molecule. Attachment of the sample tag or molecular identifier label may occur by any of the methods disclosed herein (*e.g.*, hybridization, ligation). In some instances, the target specific region comprises a sequence that is recognized by one or more restriction enzymes. The target specific region may comprise at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or base pairs. In another example, the target specific region comprises at least about 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, or 10000 nucleotides or base pairs. Preferably, the target specific region comprises at least about 5-10, 10-15, 10-20, 10-30, 15-30, or 20-30 nucleotides or base pairs.

[00262] In some instances, the target specific region is specific for a particular gene or gene product. For example, the target specific region comprises a sequence complementary to a region of a p53 gene or gene product. Therefore, the sample tags and molecular identifier labels may only attach to molecules comprising the p53-specific sequence. Alternatively, the target specific region is specific for a plurality of different genes or gene products. For example, the target specific region comprises an oligodT sequence. Therefore, the sample tags and molecular identifier labels may attach to any molecule comprising a polyA sequence. In another example, the target specific region comprises a random sequence that is complementary to a plurality of different genes or gene products. Thus, the sample tag or molecular identifier label may attach to any molecule with a sequence that is complementary to the target specific region. In other instances, the target specific region comprises a restriction site overhang (*e.g.*, EcoRI sticky-end overhang). The sample tag or molecular identifier label may ligate to any molecule comprising a sequence complementary to the restriction site overhang.

[00263] In some instances, the target specific region is specific for a particular microRNA or microRNA product. For example, the target specific region comprises a sequence complementary to a region of a specific microRNA or microRNA product. For example, the target specific regions comprise sequences complementary to regions of a specific panel of microRNAs or panel of microRNA products. Therefore, the sample tags and molecular identifier labels may only attach to molecules comprising the micoRNA-specific sequence. Alternatively, the target specific region is specific for a plurality of different micoRNAs or

micoRNA products. For example, the target specific region comprises a sequence complimentary to a region comprised in two or more microRNAs, such as a panel of microRNAs containing a common sequence. Therefore, the sample tags and molecular identifier labels may attach to any molecule comprising the common microRNA sequence. In another example, the target specific region comprises a random sequence that is complementary to a plurality of different microRNAs or microRNA products. Thus, the sample tag or molecular identifier label may attach to any microRNA molecule with a sequence that is complementary to the target specific region. In other instances, the target specific region comprises a restriction site overhang (*e.g.*, EcoRI sticky-end overhang). The sample tag or molecular identifier label may ligate to any microRNA molecule comprising a sequence complementary to the restriction site overhang.

[00264] The molecular barcode or molecular identifier label disclosed herein often comprises a label region. The label region may be used to uniquely identify occurrences of target species thereby marking each species with an identifier that may be used to distinguish between two otherwise identical or nearly identical targets. The label region of the plurality of sample tags and molecular identifier labels may comprise a collection of different semiconductor nanocrystals, metal compounds, peptides, oligonucleotides, 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, different isotopes, or any combination thereof. The label region may comprise a degenerative sequence. The label region may comprise at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or base pairs. In another example, the label region comprises at least about 1500; 2,000; 2500, 3,000; 3500, 4,000; 4500, 5,000; 5500, 6,000; 6500, 7,000; 7500, 8,000; 8500, 9,000; 9500, or 10,000 nucleotides or base pairs. Preferably, the label region comprises at least about 10-30, 15-40, or 20-50 nucleotides or base pairs.

[00265] In some instances, the molecular barcode, sample tag or molecular identifier label comprises a universal primer binding site. The universal primer binding site allows the attachment of a universal primer to the labeled-molecule and/or labeled-amplicon. Universal primers are well known in the art and include, but are not limited to, -47F (M13F), alfaMF, AOX3', AOX5', BGH_r, CMV_-30, CMV_-50, CVM_f, LACrmt, lamgda gt10F, lambda gt 10R, lambda gt11F, lambda gt11R, M13 rev, M13Forward(-20), M13Reverse, male, p10SEQP_pQE, pA_-120, pet_4, pGAP Forward, pGL_RVpr3, pGLpr2_R, pKLAC1_4,

pQE_FS, pQE_RS, puc_U1, puc_U2, revers_A, seq_IRES_tam, seq_IRES_zpet, seq_ori, seq_PCR, seq_pIRES-, seq_pIRES+, seq_pSecTag, seq_pSecTag+, seq_retro+PSI, SP6, T3-prom, T7-prom, and T7-term_Inv. Attachment of the universal primer to the universal primer binding site may be used for amplification, detection, and/or sequencing of the labeled-molecule and/or labeled-amplicon. The universal primer binding site may comprise at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or base pairs. In another example, the universal primer binding site comprises at least about 1500; 2,000; 2500, 3,000; 3500, 4,000; 4500, 5,000; 5500, 6,000; 6500, 7,000; 7500, 8,000; 8500, 9,000; 9500, or 10,000 nucleotides or base pairs. Preferably, the universal primer binding site comprises 10-30 nucleotides or base pairs.

[00266] The molecular barcode, sample tag or molecular identifier label may comprise an adapter region. The adapter region may enable hybridization of one or more probes. The adapter region may enable hybridization of one or more HCR probes.

[00267] The molecular barcode, sample tag or molecular identifier label may comprise one or more detectable labels.

[00268] The molecular barcode, sample tag or molecular identifier label may act as an initiator for a hybridization chain reaction (HCR). The adapter region of the sample tag or molecular identifier label may act as an initiation for HCR. The universal primer binding site may act as an initiator for HCR.

[00269] In some instances, the molecular barcode, sample tag or molecular identifier label is single-stranded. In other instances, the molecular barcode, sample tag or molecular identifier label is double-stranded. The molecular barcode, sample tag or molecular identifier label may be linear. Alternatively, the molecular barcode, sample tag or molecular identifier label comprises a secondary structure. As used herein, "secondary structure" includes tertiary, quaternary, etc…structures. In some instances, the secondary structure is a hairpin, a stem-loop structure, an internal loop, a bulge loop, a branched structure or a pseudoknot, multiple stem loop structures, cloverleaf type structures or any three dimensional structure. In some instances, the secondary structure is a hairpin. The hairpin may comprise an overhang sequence. The overhang sequence of the hairpin may act as a primer for a polymerase chain reaction and/or reverse transcription reaction. The overhang sequence comprises a sequence that is complementary to the molecule to which the sample tag or molecular identifier label is attached and the overhang sequence hybridizes to the molecule. The overhang sequence may be ligated to the molecule and acts as a template for a polymerase chain reaction and/or

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reverse transcription reaction. In some embodiments, molecular barcode, the sample tag, or molecular identifier label comprises nucleic acids and/or synthetic nucleic acids and/or modified nucleic acids.

[00270] In some instances, the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) comprises at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label). In other instances, the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) comprises at least about 200; 300; 400; 500; 600; 700; 800; 900; 1,000; 2,000; 3,000; 4,000; 5,000; 6,000; 7,000; 8,000; 9,000; or 10000 different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label). Alternatively; the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) comprises at least about 20,000; 30,000; 40,000; 50,000; 60,000; 70,000; 80,000; 90,000; or 100,000 different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label).

[00271] The number of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is often in excess of the number of molecules to be labeled. In some instances, the number of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 times greater than the number of molecules to be labeled.

[00272] The number of different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is often in excess of the number of different molecules to be labeled. In some instances, the number of different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and

molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is at least about 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 times greater than the number of different molecules to be labeled.

[00273] In some instances, stochastic labeling of a molecule comprises a plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label), wherein the concentration of the different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is the same. In such instances, the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) comprises equal numbers of each different molecular barcode, sample tag or molecular identifier label.

[00274] In some instances, stochastic labeling of a molecule comprises a plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label), wherein the concentration of the different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is different. In such instances, the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) comprises different numbers of each different molecular barcode, sample tag or molecular identifier label.

[00275] In some instances, some molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) are present at higher concentrations than other molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label). In some instances, stochastic labeling with different concentrations of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) extends the sample measurement dynamic range without increasing the

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number of different labels used. For example, consider stochastically labeling 3 nucleic acid sample molecules with 10 different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) all at equal concentration. We expect to observe 3 different labels. Now instead of 3 nucleic acid molecules, consider 30 nucleic acid molecules, and we expect to observe all 10 labels. In contrast, if we still used 10 different stochastic labels and alter the relative ratios of the labels to 1:2:3:4…10, then with 3 nucleic acid molecules, we would expect to observe between 1-3 labels, but with 30 molecules we would expect to observe only approximately 5 labels thus extending the range of measurement with the same number of stochastic labels.

[00276] The relative ratios of the different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) may be 1:X, where X is at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100. Alternatively, the relative ratios of "n" different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is 1:A:B:C:…Zn, where A, B, C…Zn is at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100.

[00277] In some instances, the concentration of two or more different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is the same. For "n" different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label), the concentration of at least 2, 3, 4, …n different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is the same. Alternatively, the concentration of two or more different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is different. For "n" different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular

label), the concentration of at least 2, 3, 4,…n different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is different. In some instances, for "n" different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label), the difference in concentration for at least 2, 3, 4,…n different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) is at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000-fold.

[00278] In some instances, at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 100% of the different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) have the same concentration. Alternatively, at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 100% of the different molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) in the plurality of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) have a different concentration.

[00279] As shown in FIG. 65, molecular barcodes (1004) may be synthesized separately. The molecular barcodes (1004) may comprise a universal PCR region (1001), one or more identifier regions (1002), and a target specific region. The one or more identifier regions may comprise a sample index region, label region, or a combination thereof. The one or more identifier regions may be adjacent. The one or more identifier regions may be non-adjacent. The individual molecular barcodes may be pooled to produce a plurality of molecular barcodes (1005) comprising a plurality of different identifier regions. Sample tags may be synthesized in a similar manner as depicted in FIG. 65, wherein the one or more identifier regions comprise a sample index region. Molecular identifier labels may be synthesized in a similar manner as depicted in FIG. 65, wherein the one or more identifier regions comprises a label region.

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[00280] The target specific region may be ligated to the identifier region to produce a molecular barcode comprising a target specific region. 5' and 3' exonucleases may be added to the reaction to remove non-ligated products. The molecular barcode may comprise the universal primer binding site, label region and target specific region and may be resistant to 5' and 3' exonucleases. As used herein, the terms "universal primer binding site" and "universal PCR region" may be used interchangeably and refer to a sequence that can be used to prime an amplification reaction. The 3' phosphate group from the ligated identifier region may be removed to produce a molecular barcode without a 3' phosphate group. The 3' phosphate group may be removed enzymatically. For example, a T4 polynucleotide kinase may be used to remove the 3' phosphate group.

[00281] Another method of synthesizing molecular barcodes is depicted in FIG. 66A. As shown in FIG. 66A, a molecular barcode (1128) may be synthesized by ligating two or more oligonucleotide fragments (1121 and 1127). One oligonucleotide fragment (1121) may comprise a universal primer binding site (1122), identifier region (1123) and a first splint (1123). The other oligonucleotide fragment (1128) may comprise a second splint (1125) and a target specific region (1126). A ligase (*e.g.*, T4 DNA ligase) may be used to join the two oligonucleotide fragments (1121 and 1127) to produce a molecular barcode (1128). Double stranded ligation of the first splint (1124) and second splint (1125) may produce a molecular barcode (1128) with a bridge splint (1129).

[00282] An alternative method of synthesizing a molecular barcode by ligating two oligonucleotide fragments is depicted in FIG. 66B. As shown in FIG. 66B, a molecular barcode (1158) is synthesized by ligating two oligonucleotide fragments (1150 and 1158). One oligonucleotide fragment (1150) may comprise a universal primer binding site (1151), one or more identifier region (1152), and a ligation sequence (1153). The other oligonucleotide fragment (1158) may comprise a ligation sequence (1154) that is complementary to the ligation sequence (1153) of the first oligonucleotide fragment (1150), a complement of a target specific region (1155), and a label (1156). The oligonucleotide fragment (1159) may also comprise a 3' phosphate which prevents extension of the oligonucleotide fragment. As shown in Step 1 of FIG. 66B, the ligation sequences (1153 and 1154) of the two oligonucleotide fragments may anneal and a polymerase may be used to extend the 3' end of the first oligonucleotide fragment (1150) to produce molecular barcode (1158). The molecular barcode (1158) may comprise a universal primer binding site (1151), one or more identifier regions (1152), ligation sequence (1153), and a target specific sequence (1157). The target specific sequence (1157) of the molecular barcode (1158) may

be the complement of the complement of the target specific region (1155) of the second oligonucleotide fragment (1159). The oligonucleotide fragment comprising the label (1156) may be removed from the molecular barcode (1158). For example, the label (1156) may comprise biotin and oligonucleotide fragments (1159) comprising the biotin label (1156) may be removed via streptavidin capture. In another example, the label (1156) may comprise a 5' phosphate and oligonucleotide fragments (1159) comprising the 5' phosphate (1156) may be removed via an exonuclease (*e.g.*, Lambda exonuclease).

[00283] As depicted in FIG. 66C, a first oligonucleotide fragment (1170) comprising a universal primer binding site (1171), one or more identifier regions (1172), a first ligation sequence (1173) is annealed to a second oligonucleotide fragment (1176) comprising a second ligation sequence (1174) and an RNA complement of the target sequence (1175). Step 1 may comprise annealing the first and second ligation sequences (1173 and 1174) followed by reverse transcription of the RNA complement of the target sequence (1175) to produce molecular barcode (1177) comprising a universal primer binding site (1171), one or more identifier regions (1172), a first ligation sequence (1173), and a target specific region (1178). The oligonucleotide fragments comprising the RNA complement of the target sequence may be selectively degraded by RNAse treatment.

[00284] The sequences of the molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) may be optimized to minimize dimerization of molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label). The molecular barcode, sample tag or molecular identifier label dimer may be amplified and result in the formation of an amplicon comprising two universal primer binding sites on each end of the amplicon and a target specific region and a unique identifier region. Because the concentration of the molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) are far greater that the number of DNA templates, these molecular barcode, sample tag or molecular identifier label dimers may outcompete the labeled DNA molecules in an amplification reaction. Unamplified DNAs lead to false negatives, and amplified molecular barcode, sample tag or molecular identifier label dimers lead to high false positives. Thus, the molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) may be optimized to minimize molecular barcode, sample tag or molecular identifier label dimer formation. Alternatively, molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*,

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molecular label) that dimerize are discarded, thereby eliminating molecular barcode, sample tag or molecular identifier label dimer formation.

[00285] Alternatively, molecular barcode, sample tag or molecular identifier label dimer formation may be eliminated or reduced by incorporating one or more modifications into the molecular barcode, sample tag or molecular identifier label sequence. A molecular barcode, sample tag or molecular identifier label comprising a universal primer binding site, unique identifier region, and target specific region comprising uracils and a 3' phosphate group is annealed to a target nucleic acid. The target nucleic acid may be a restriction endonuclease digested fragment. The restriction endonuclease may recognize the recognition site. PCR amplification may comprise one or more forward primers and one or more reverse primers. PCR amplification may comprise nested PCR with a forward primer specific for the universal primer binding site of the molecular barcode, sample tag or molecular identifier label and a forward primer specific for the target specific region of the molecular barcode, sample tag or molecular identifier label and reverse primers that are specific for the target nucleic acid. The target nucleic acid may be amplified using a Pfu DNA polymerase, which cannot amplify template comprising one or more uracils. Thus, any dimerized molecular barcodes, sample tags (*e.g.*, sample index region, sample label), cellular label, and molecular identifier labels (*e.g.*, molecular label) cannot be amplified by Pfu DNA polymerase.

Methods to synthesize oligonucleotides (*e.g.*, molecular barcodes)

[00286] An oligonucleotide may be synthesized. An oligonucleotide may be synthesized, for example, by coupling (*e.g.*, by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) of a 5' amino group on the oligonucleotide to the carboxyl group of the functionalized solid support.

[00287] Uncoupled oligonucleotides may be removed from the reaction mixture by multiple washes. The solid supports may be split into wells (*e.g.*, 96 wells). Each solid support may be split into a different well. Oligonucleotide synthesis may be performed using the split/pool method of synthesis. The split/pool method may utilize a pool of solid supports comprising reactive moieties (*e.g.*, oligonucleotides to be synthesized). This pool may be split into a number of individual pools of solid supports. Each pool may be subjected to a first reaction that may result in a different modification to the solid supports in each of the pools (*e.g.*, a different nucleic acid sequence added to the oligonucleotide). After the reaction, the pools of solid supports may be combined, mixed, and split again. Each split pool may be subjected to a second reaction or randomization that again is different for each of the pools. The process may be continued until a library of target compounds is formed.

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[00288] Using split/pool synthesis, the nucleic acid sequence to be added to the oligonucleotide may be incorporated by primer extension (*e.g.*, Klenow extension). The nucleic acid sequence to be added to the oligonucleotide may be referred to as a primer fragment. Each primer fragment for each individual pool may comprise a different sequence (*e.g.*, either in the cellular label, the molecular label, the sample label, or any combination thereof). The primer fragment may comprise a sequence that may hybridize to the linker label sequence of the oligonucleotide (*e.g.*, the oligonucleotide coupled to the solid support). The primer fragment may further comprise a second cell label and a second linker label sequence. Primer extension may be used to introduce the second cell label sequence and the second linker label sequence onto the oligonucleotide coupled to the solid support (See **FIG. 2B**). After primer extension incorporates the new sequences, the solid supports may be combined. The combined solid supports may be heated to denature the enzyme. The combined solid supports may be heated to disrupt hybridization. The combined solid supports may be split into wells again. The process may be repeated to add additional sequences to the solid support-conjugated oligonucleotide.

[00289] The split/pool process may lead to the creation of at least about 1000, 10000, 100000, 500000, or 1000000 or more different oligonucleotides. The process may lead to the creation of at most about 1000, 10000, 100000, 500000, or 1000000 or more different oligonucleotides.

[00290] Split pool synthesis may comprise chemical synthesis. Different oligonucleotides may be synthesized using DMT chemistry on solid supports in individual reactions, then pooled into reactions for synthesis. The split/pool process may be repeated 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more times. The split/pool process may be repeated 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more times. The split/pool process may be repeated 2 or more times. The split/pool process may be repeated 3 or more times. The split/pool process may be repeated 5 or more times. The split/pool process may be repeated 10 or more times.

[00291] Further disclosed herein are methods of producing one or more sets of labeled beads (*e.g.*, oligonucleotide conjugated beads). The method of producing the one or more sets of labeled beads may comprise attaching one or more nucleic acids to one or more beads, thereby producing one or more sets of labeled beads. The one or more nucleic acids may comprise one or more molecular barcodes. The one or more nucleic acids may comprise one or more sample tags (*e.g.*, sample labels, sample index regions). The one or more nucleic acids may comprise one or more cellular labels. The one or more nucleic acids may comprise

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one or more molecular identifier labels (*e.g.*, molecular labels). The one or more nucleic acids may comprise a) a primer region; b) a sample index region; and c) a linker or adaptor region. The one or more nucleic acids may comprise a) a primer region; b) a label region (*e.g.*, molecular label); and c) a linker or adaptor region. The one or more nucleic acids may comprise a) a sample index region (*e.g.*, sample tag); and b) a label region (*e.g.*, molecular label). The one or more nucleic acids may comprise a) a sample index region; and b) a cellular label. The one or more nucleic acids may comprise a) a cellular label; and b) a molecular label. The one or more nucleic acids may comprise a) a sample index region; b) cellular label; and c) a molecular label. The one or more nucleic acids may further comprise a primer region. The one or more nucleic acids may further comprise a target specific region. The one or more nucleic acids may further comprise a linker region. The one or more nucleic acids may further comprise an adaptor region. The one or more nucleic acids may further comprise a sample index region. The one or more nucleic acids may further comprise a label region.

[00292] Alternatively, the method comprises: a) depositing a plurality of first nucleic acids into a plurality of wells, wherein two or more different wells of the plurality of wells may comprise two or more different nucleic acids of the plurality of nucleic acids; b) contacting one or more wells of the plurality of wells with one or fewer beads to produce a plurality of single label beads, wherein a single label bead of the plurality of first labeled beads comprises a bead attached to a nucleic acid of the plurality of first nucleic acids; c) pooling the plurality of first labeled beads from the plurality of wells to produce a pool of first labeled beads; d) distributing the pool of first labeled beads to a subsequent plurality of wells, wherein two or more wells of the subsequent plurality of wells comprise two or more different nucleic acids of a plurality of subsequent nucleic acids; and e) attaching one or more nucleic acids of the plurality of subsequent nucleic acids to one or more first labeled beads to produce a plurality of uniquely labeled beads.

Libraries

[00293] Disclosed herein are methods of producing molecular libraries. The method may comprise: (a) stochastically labeling two or more molecules from two or more samples to produce labeled molecules, wherein the labeled molecules comprise (i) a molecule region based on or derived from the two or more molecules, (ii) a sample index region for use in differentiating two or more molecules from two or more samples; and (iii) a label region for use in differentiating two or more molecules from a single sample. Stochastic labeling may comprise the use of one or more sets of molecular barcodes. Stochastic labeling may

comprise the use of one or more sets of sample tags. Stochastic labeling may comprise the use of one or more sets of molecular identifier labels.

[00294] Stochastically labeling the two or more molecules may comprise contacting the two or more samples with a plurality of sample tags and the plurality of molecule specific labels to produce the plurality of labeled nucleic acids. The contacting can be random. The method may further comprise amplifying one or more of the labeled molecules, thereby producing an enriched population of labeled molecules of the library. The method may further comprise conducting one or more assays on the two or more molecules from the two or more samples. The method may further comprise conducting one or more pull-down assays.

[00295] The method of producing a labeled nucleic acid library may further comprise adding one or more controls to the two or more of samples. The one or more controls may be stochastically labeled to produce labeled controls. The one or more controls may be used to measure an efficiency of producing the labeled molecules.

[00296] The libraries disclosed herein may be used in a variety of applications. For example, the library could be used for sequencing applications. The library may 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.

Sample Preparation and Applications

[00297] The oligonucleotides (*e.g.*, molecular bar code, sample tag, molecular label, cellular label) disclosed herein may be used in a variety of methods. The oligonucleotides may be in methods for nucleic acid analysis. Nucleic acid analysis may include, but is not limited to, genotyping, gene expression, copy number variation, and molecular counting.

[00298] The disclosure provides for methods of multiplex nucleic acid analysis. The method may comprise (a) contacting one or more oligonucleotides from a cell with one or more oligonucleotides attached to a support, wherein the one or more oligonucleotides attached to the support comprise (i) a cell label region comprising two or more randomer sequences connected by a non-random sequence; and (ii) a molecular label region; and (b) conducting one or more assays on the one or more oligonucleotides from the cell.

[00299] Further disclosed herein are methods of producing single cell nucleic acid libraries. The method may comprise (a) contacting one or more oligonucleotides from a cell with one or more oligonucleotides attached to a support, wherein the one or more oligonucleotides attached to the support comprise (i) a cell label region comprising two or more randomer

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sequences connected by a non-random sequence; and (ii) a molecular label region; and (b) conducting one or more assays on the one or more oligonucleotides from the cell.

[00300] In some instances, the method comprises adding a one or more cells onto a microwell array. The number of cells to be added may be determined from counting. Excess or unbound cells may be washed away using a buffer (*e.g.*, phosphobuffered saline buffer, HEPES, Tris). The number of cells that may be captured by the wells of the microwell array may be related to the size of the cell. For example, depending on the design of the microwell, larger cells may be more easily captured than smaller cells, as depicted in **FIG. 6**. Different microwells (*e.g.*, different dimensions) may be used for capturing different cell types.

[00301] The methods described here allow for the addition of sequences that can nucleic acids for sequencing or other molecular analyses. These methods can allow detection of nucleic acid variants, mutants, polymorphisms, inversions, deletions, reversions and other qualitative events found in a population of RNA or DNA molecules. For example, the methods can allow for identification of target frequencies (*e.g.*, gene expression or allelic distribution). For example, the methods also allow for identification of mutations or SNPs in a genome or transcriptome, such as from a diseased or non-diseased subject. The methods also allow for determining the presence or absence of contamination or infections in a biological sample from a subject, such as foreign organisms or viruses, such as a bacteria or a fungus.

[00302] Cells can be added into microwells by any method. In some embodiments, cells are added to microwells as a diluted cell sample. In some embodiments, cells are added to microwells and allowed to settle in the microwells by gravity. In some embodiments, cells are added to microwells and centrifugatiion is used to settle the cells in the microwells. In some embodiments, cells are added to microwells by injecting one or more cells into one or more microwells. For example, a single cell can be added to a microwell by injecting the single cell in to a microwell. The injecting of a cell can be through the use of any device or method, such as through the use of a micro manipulator. In some embodiments, cell can be added to microwells using a magnet. For example, cells can coated on their surface with magnetic particles, such as magnetic microparticles or magnetic nanoparticles and added to microwells using a magnet or a magnetic field.

[00303] The microwell array comprising cells may be contacted with an oligonucleotide conjugated solid support (*e.g.*, bead). Uncaptured oligonucleotide conjugated solid supports may be removed (*e.g.*, washed away with buffer). **FIG. 5** depicts a microwell array with captured solid supports. A microwell may comprise at least one solid support. A microwell

may comprise at least two solid supports. A microwell may comprise at most one solid support. A microwell may comprise at most two solid supports. A microwell may comprise at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more solid supports. A microwell may comprise at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more solid supports. Some of the microwells of the microwell array may comprise one solid support and some of the microwells of the microwell array may comprise two or more solid supports, as shown in **FIG. 5.** The microwell may not need to be covered for any of the methods of the disclosure. In other words, microwells may not need to be sealed during the method. When the microwells are not covered (*e.g.*, sealed), the wells may be spaced apart such that the contents of one microwell may not diffuse into another microwell.

[00304] Alternatively, or additionally, cells may be captured and/or purified prior to being contacted with an oligonucleotide conjugated support. Methods to capture and/or purify cells may comprise use of antibodies, molecular scaffolds, and/or beads. Cells may be purified by flow cytometry. Commercially available kits may be used to capture or purify cells. For example, Dynabeads(R) may be used to isolate cells. Magnetic isolation may be used to purify cells. Cells may be purified by centrifugation.

[00305] Cells may be contacted with oligonucleotide conjugated supports by creating a suspension comprising cells and the supports. The suspension may comprise a gel. Cells may be immobilized on a support or in a solution prior to contact with the oligonucleotide conjugated supports. Alternatively, cells may be added to a suspension comprising the oligonucleotide conjugated support. For example, cells may be added to a hydrogel that is embedded with oligonucleotide conjugated supports.

[00306] A single cell may be contacted with a single oligonucleotide coupled solid support. A single cell may be contacted with multiple oligonucleotide conjugated solid supports. Multiple cells may interact with a single oligonucleotide conjugated solid support. Multiple cells may interact with multiple oligonucleotide conjugated solid supports. The oligonucleotide conjugated solid supports may be cell-type specific. Alternatively, the oligonucleotide conjugated support may interact with two or more different cell types.

Lysis

[00307] Cells in the microwells may be lysed. Lysis may be performed by mechanical lysis, heat lysis, optical lysis, and/or chemical lysis. Chemical lysis may include the use of digestive enzymes such as proteinase K, pepsin, and trypsin. Lysis may be performed by the addition of a lysis buffer to the microwells. A lysis buffer may comprise Tris HCl. A lysis buffer may comprise at least about 0.01, 0.05, 0.1, 0.5, or 1M or more Tris HCl. A lysis

buffer may comprise at most about 0.01, 0.05, 0.1, 0.5, or 1M or more Tris HCL. A lysis buffer may comprise about 0.1 M Tris HCl. The pH of the lysis buffer may be at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more. The pH of the lysis buffer may be at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more. In some instances, the pH of the lysis buffer is about 7.5. The lysis buffer may comprise a salt (*e.g.*, LiCl). The concentration of salt in the lysis buffer may be at least about 0.1, 0.5, or 1M or more. The concentration of salt in the lysis buffer may be at most about 0.1, 0.5, or 1M or more. In some instances, the concentration of salt in the lysis buffer is about 0.5M. The lysis buffer may comprise a detergent (*e.g.*, SDS, Li dodecyl sufate, triton X, tween, NP-40). The concentration of the detergent in the lysis buffer may be at least about 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 6, or 7% or more. The concentration of the detergent in the lysis buffer may be at most about 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 6, or 7% or more. In some instances, the concentration of the detergent in the lysis buffer is about 1% Li dodecyl sulfate. The time used in the method for lysis may be dependent on the amount of detergent used. In some instances, the more detergent used, the less time needed for lysis. The lysis buffer may comprise a chelating agent (*e.g.*, EDTA, EGTA). The concentration of a chelating agent in the lysis buffer may be at least about 1, 5, 10, 15, 20, 25, or 30mM or more. The concentration of a chelating agent in the lysis buffer may be at most about 1, 5, 10, 15, 20, 25, or 30mM or more. In some instances, the concentration of chelating agent in the lysis buffer is about 10 mM. The lysis buffer may comprise a reducing reagent (*e.g.*, beta-mercaptoethanol, DTT). The concentration of the reducing reagent in the lysis buffer may be at least about 1, 5, 10, 15, or 20 mM or more. The concentration of the reducing reagent in the lysis buffer may be at most about 1, 5, 10, 15, or 20 mM or more. In some instances, the concentration of reducing reagent in the lysis buffer is about 5 mM. In some instances, a lysis buffer may comprise about 0.1M TrisHCl, about pH 7.5, about 0.5M LiCl, about 1% lithium dodecyl sulfate, about 10mM EDTA, and about 5mM DTT.

[00308] Lysis may be performed at a temperature of about 4, 10, 15, 20, 25, or 30 C. Lysis may be performed for about 1, 5, 10, 15, or 20 or more minutes. A lysed cell may comprise at least about 100000, 200000, 300000, 400000, 500000, 600000, or 700000 or more target nucleic acid molecules. A lysed cell may comprise at most about 100000, 200000, 300000, 400000, 500000, 600000, or 700000 or more target nucleic acid molecules. **FIG. 7** illustrates exemplary statistics about the concentration of target nucleic acid (i.e., mRNA) that may be obtained from lysis.

Sealing

[00309] The microwells of the microwell array may be sealed during lysis. Sealing may be useful for preventing cross hybridization of target nucleic acid between adjacent microwells. A microwell may be sealed using a cap as shown in **FIG. 8A and B**. A cap may be a solid support. A cap may comprise a bead. The diameter of the bead may be larger than the diameter of the microwell. For example, a cap may be at least about 10, 20, 30, 40, 50, 60, 70, 80 or 90% larger than the diameter of the microwell. For example, a cap may be at most about 10, 20, 30, 40, 50, 60, 70, 80 or 90% larger than the diameter of the microwell.

[00310] A cap may comprise cross-linked dextran beads (*e.g.*, Sephadex). Cross-linked dextran may range from about 10 micrometers to about 80 micrometers. The cross-linked dextran of the cap may be from 20 micrometers to about 50 micrometers. A cap may comprise, for example, anopore inorganic membranes (*e.g.*, aluminum oxides), dialysis membranes, glass slides, coverslips, and/or hydrophilic plastic film (*e.g.*, film coated with a thin film of agarose hydrated with lysis buffer).

[00311] The cap may allow buffer to pass through into and out of the microwell, but may prevent macromolecules (*e.g.*, nucleic acid) from migrating out of the well. A macromolecule of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, or 20 or more nucleotides may be blocked from migrating into or out of the microwell by the cap. A macromolecule of at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, or 20 or more nucleotides may be blocked from migrating into or out of the microwell by the cap.

[00312] A sealed microwell array may comprise a single layer of beads on top of the microwells. A sealed microwell array may comprise multiple layers of beads on top of the microwells. A sealed microwell array may comprise about 1, 2, 3, 4, 5, or 6 or more layers of beads.

[00313] Depositing a bead, or plurality of beads, onto a solid support (*e.g.*, a microwell array) can be random or non-random. For example, contacting a bead with a microwell array can be a random or non-random contacting. In some embodiments, the bead is contacted with a microwell array randomly. In some embodiments, the bead is contacted with a microwell array non-randomly. Depositing of a plurality of beads to a microwell array can be random or non-random. For example, the contacting of a plurality of beads to a microwell array can be a random or non-random contacting. In some embodiments, the plurality of beads is contacted to a microwell array randomly. In some embodiments, the plurality of beads is contacted to a microwell array non-randomly.

Stochastic labeling of molecules

[00314] Wherein the sample tag or molecular identifier label is an oligonucleotide, attachment of the oligonucleotide to a nucleic acid may occur by a variety of methods, including, but not limited to, hybridization of the oligonucleotide to the nucleic acid. In some instances, the oligonucleotide comprises a target specific region. The target specific region may comprise a sequence that is complementary to at least a portion of the molecule to be labeled. The target specific region may hybridize to the molecule, thereby producing a labeled nucleic acid. Hybridization of the oligonucleotide to the nucleic acid may be followed by a nucleic acid extension reaction. The nucleic acid extension reaction may be reverse transcription.

[00315] Attaching, alternatively referred to as contacting, the plurality of nucleic acids with the sample tag may comprise hybridizing the sample tag to one or more of the plurality of nucleic acids. Contacting the plurality of nucleic acids with the sample tag may comprise performing a nucleic acid extension reaction. The nucleic acid extension reaction may be a reverse transcription reaction.

[00316] Contacting the plurality of nucleic acids with the molecular identifier label may comprise hybridizing the molecular identifier label to one or more of the plurality of nucleic acids. Contacting the plurality of nucleic acids with the molecular identifier label may comprise performing a nucleic acid extension reaction. The nucleic acid extension reaction may comprise reverse transcription.

[00317] Contacting the plurality of nucleic acids with the molecular identifier label may comprise hybridizing the sample tag to one or more of the plurality of nucleic acids. Contacting the plurality of nucleic acids with the molecular identifier label may comprise hybridizing the molecular identifier label to the sample tag.

[00318] Contacting the plurality of nucleic acids with the sample tag may comprise hybridizing the molecular identifier label to one or more of the plurality of nucleic acids. Contacting the plurality of nucleic acids with the sample tag may comprise hybridizing the sample tag to the molecular identifier label.

[00319] Attachment of the sample tag and/or the molecular identifier label to a nucleic acid may occur by ligation. Contacting the plurality of nucleic acids with the sample tag may comprise ligating the sample tag to any one of the plurality of nucleic acids. Contacting the plurality of nucleic acids with the molecular identifier label may comprise ligating the molecular identifier label to one or more of the plurality of nucleic acids. Contacting the plurality of nucleic acids with the sample tag may comprise ligating the molecular identifier label one or more the nucleic acids. Contacting the plurality of nucleic acids with the

molecular identifier label may comprise ligating the sample tag to one or more of the nucleic acids. Ligation techniques comprise blunt-end ligation and sticky-end ligation. Ligation reactions may include DNA ligases such as DNA ligase I, DNA ligase III, DNA ligase IV, and T4 DNA ligase. Ligation reactions may include RNA ligases such as T4 RNA ligase I and T4 RNA ligase II.

[00320] Methods of ligation are described, for example in Sambrook et al. (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.

[00321] In some instances, both ends of the oligonucleotide are attached to the molecule. For example, both ends of the oligonucleotide may be hybridized and/or ligated to one or more ends of the molecule. In some instances, attachment of both ends of the oligonucleotide to both ends of the molecule results in the formation of a circularized labeled nucleic acid. Both ends of the oligonucleotide may also be attached to the same end of the molecule. For example, the 5' end of the oligonucleotide is ligated to the 3' end of the molecule and the 3' end of the oligonucleotide is hybridized to the 3'end of the molecule, resulting in a labeled nucleic acid with a hairpin structure at one end. In some instances the oligonucleotide is attached to the middle of the molecule.

[00322] In some instances, attachment of the oligonucleotide to the nucleic acid comprises attaching one or more oligonucleotide linkers to the plurality of nucleic acids. The method may further comprise attaching one or more oligonucleotide linkers to the sample-tagged nucleic acids. The method may further comprise attaching one or more oligonucleotide linkers to the labeled nucleic acids. Attaching one or more oligonucleotide linkers to a

nucleic acid, sample tag or molecular identifier label may comprise ligating one or more oligonucleotide linkers to a nucleic acid, sample tag or molecular identifier label. The one or more linkers may comprise at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100 nucleotides. In some instances, the linker may comprise at least about 1000 nucleotides.

[00323] In some instances, attachment of the molecular barcode to the molecule comprises the use of one or more adaptors. As used herein, the terms "adaptors" and "adaptor regions" may be used interchangeably. Adaptors may comprise a target specific region, which allows the attachment of the adaptor to the molecule, and an oligonucleotide specific region, which allows attachment of the molecular barcode to the adaptor. Adaptors may further comprise a universal primer. Adaptors may further comprise a universal PCR region. Adaptors may be attached to the molecule and/or molecular barcodes by methods including, but not limited to, hybridization and/or ligation.

[00324] Methods for ligating adaptors to fragments of nucleic acid are well known. Adaptors may be double-stranded, single-stranded or partially single-stranded. In some 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 be about 1 to about 8 bases, or 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 EcoP151 (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).

[00325] In some instances, stochastically counting the number of copies of a nucleic acid in a plurality of samples comprises detecting the adaptor, a complement of the adaptor, a reverse complement of the adaptor or a portion thereof to determine the number of different labeled nucleic acids. Detecting the adaptor, a complement of the adaptor, a reverse complement of the adaptor or a portion thereof may comprise sequencing the adaptor, a complement of the adaptor, a reverse complement of the adaptor or a portion thereof.

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[00326] The molecular barcode may be attached to any region of a molecule. For example, the molecular barcode may be attached to the 5' or 3' end of a polynucleotide (*e.g.*, DNA, RNA). For example, the target-specific region of the molecular barcode comprises a sequence that is complementary to a sequence in the 5' region of the molecule. The target-specific region of the molecular barcode may also comprise a sequence that is complementary to a sequence in the 3' region of the molecule. In some instances, the molecular barcode is attached a region within a gene or gene product. For example, genomic DNA is fragmented and a sample tag or molecular identifier label is attached to the fragmented DNA. In other instances, an RNA molecule is alternatively spliced and the molecular barcode is attached to the alternatively spliced variants. In another example, the polynucleotide is digested and the molecular barcode is attached to the digested polynucleotide. In another example, the target-specific region of the molecular barcode comprises a sequence that is complementary to a sequence within the molecule.

[00327] A molecular barcode, sample tag (*e.g.*, sample index), cellular label, or molecular identifier label (*e.g.*, molecular label) comprising a hairpin may act as a probe for a hybridization chain reaction (HCR), and, thus, may be referred to as an HCR probe. The HCR probe may comprise a molecular barcode comprising a hairpin structure. The HCR probe may comprise a sample tag comprising a hairpin structure. The HCR probe may comprise a molecular identifier label comprising a hairpin structure. Further disclosed herein is a stochastic label-based hybridization chain reaction (HCR) method comprising stochastically labeling one or more nucleic acid molecules with an HCR probe, wherein the HCR probe comprises a molecular barcode comprising a hairpin and the one or more nucleic acid molecules act as initiators for a hybridization chain reaction. Further disclosed herein is a stochastic label-based hybridization chain reaction (HCR) method comprising stochastically labeling one or more nucleic acid molecules with an HCR probe, wherein the HCR probe comprises a sample tag comprising a hairpin and the one or more nucleic acid molecules act as initiators for a hybridization chain reaction. Further disclosed herein is a stochastic label-based hybridization chain reaction (HCR) method comprising stochastically labeling one or more nucleic acid molecules with an HCR probe, wherein the HCR probe comprises a molecular identifier label comprising a hairpin and the one or more nucleic acid molecules act as initiators for a hybridization chain reaction.

[00328] The HCR probe may comprise a hairpin with an overhang region. The overhang region of the hairpin may comprise a target specific region. The overhang region may comprise an oligodT sequence. The sample comprising the one or more nucleic acid

molecules may be treated with one or more restriction nucleases prior to stochastic labeling. The overhang region may comprise a restriction enzyme recognition sequence. The sample comprising the one or more nucleic acid molecules may be contacted with one or more adapters prior to stochastic labeling to produce an adapter-nucleic acid molecule hybrid. The overhang region and the stem may be complementary to the one or more adapters. The HCR probe may comprise a hairpin with a loop. The loop of the HCR probe may comprise a label region and/or sample index region.

[00329] Hybridization of a first HCR probe to the nucleic acid molecules may result in the formation of a labeled nucleic acid, wherein the first HCR probe is linearized to produce a first linearized HCR probe. The first linearized HCR probe of the labeled nucleic acid may act as an initiator for hybridization of a second HCR probe to the labeled nucleic acid to produce a labeled nucleic acid with two linearized HCR probes. The second linearized HCR probe may act as an initiator for another hybridization reaction. This process may be repeated multiple times to produce a labeled nucleic acid with multiple linearized HCR probes. The detectable labels on the HCR probe may enable detection of the labeled nucleic acid. The detectable labels may be any type of label (*e.g.*, fluorphore, chromophore, small molecule, nanoparticle, hapten, enzyme, antibody, magnet). The detectable labels may comprise fragments of a single label. The detectable labels may generate a detectable signal when they are in close proximity. When the HCR probe is a hairpin, the detectable labels may be too far away to produce a detectable signal. When the HCR probe is linearized and multiple linearized HCR probes are hybridized together, the detectable labels may be in close enough proximity to generate a detectable signal. For example, a HCR probe may comprise two pyrene moieties as detectable labels. Alternatively, the detectable labels may be nanoparticles. The stochastic label-based HCR method may enable attachment of multiple hairpin HCR probes to a labeled nucleic acid, which may result in signal amplification. Stochastic label-based HCR may increase the sensitivity of detection, analysis and/or quantification of the nucleic acid molecules. Stochastic label-based HCR may increase the accuracy of detection, analysis, and/or quantification of one or more nucleic acid molecules.

[00330] After lysis the target nucleic acid of the cells may hybridize to the oligonucleotide conjugated to the solid support. The target nucleic acid may hybridize to the target binding region of the oligonucleotide. The nucleic acid may hybridize to any region of the olignucleotide.

[00331] In some instances, not all oligonucleotides may bind a target nucleic acid. This is because in some instances, the number of oligonucleotides is larger than the number of target

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nucleic acids. The number of oligonucleotides conjugated to a solid support may be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10-fold more than the number of target nucleic acids in a cell. At least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% of the oligonucleotides may be bound by a target nucleic acid. At most 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% of the oligonucleotides may be bound by a target nucleic acid. In some instances, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 or more different target nucleic acids may be captured by the oligonucleotides on a solid support. In some instances, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 or more different target nucleic acids may be captured by the oligonucleotides on a solid support.

[00332] In some instances, at least about 40, 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 100% of the number of copies of a target nucleic acid are bound to oligonucleotides on a solid support. In some instances, at most about 40, 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 100% of the number of copies of a target nucleic acid are bound to oligonucleotides on a solid support.

Retrieval

[00333] After lysis, the solid supports may be retrieved. Retrieval of the solid supports may be performed by using a magnet. Retrieval of the solid supports may be performed by melting the microwell array and/or sonication. Retrieval of the solid supports may comprise centrifugation. Retrieval of the solid supports may comprise size exclusion. In some instances, at least about 50, 60, 70, 80, 90, 95, or 100% of the solid supports are recovered from the microwells. In some instances, at most about 50, 60, 70, 80, 90, 95, or 100% of the solid supports are recovered from the microwells.

Reverse Transcription

[00334] The methods disclosed herein may further comprise reverse transcription of a labeled-RNA molecule to produce a labeled-cDNA molecule. In some instances, at least a portion of the oligonucleotide acts as a primer for the reverse transcription reaction. The oligodT portion of the oligonucleotide may act as a primer for first strand synthesis of the cDNA molecule.

[00335] In some instances the labeled cDNA molecule may be used as a molecule for a new stochastic labeling reaction. The labeled cDNA may have a first tag or set of tags from attachment to the RNA prior to reverse transcription and a second tag or set of tags attached to the cDNA molecule. These multiple labeling reactions can, for example, be used to determine the efficiency of events that occur between the attachment of the first and second tags, *e.g.,* an optional amplification reaction or the reverse transcription reaction.

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[00336] In another example, an oligonucleotide is attached to the 5' end of an RNA molecule to produce a labeled-RNA molecule. Reverse transcription of the labeled-RNA molecule may occur by the addition of a reverse transcription primer. In some instances, the reverse transcription primer is an oligodT primer, random hexanucleotide primer, or a target-specific oligonucleotide primer. Generally, oligodT primers are 12–18 nucleotides in length and bind to the endogenous poly(A)+ tail at the 3' end of mammalian mRNA. Random hexanucleotide primers may bind to mRNA at a variety of complementary sites. Target-specific oligonucleotide primers typically selectively prime the mRNA of interest.

[00337] In some instances, the method comprises repeatedly reverse transcribing the labeled-RNA molecule to produce multiple labeled-cDNA molecules. The methods disclosed herein may comprise conducting at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 reverse transcription reactions. The method may comprise conducting at least about 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 reverse transcription reactions.

[00338] Nucleic acid synthesis (*e.g.*, cDNA synthesis) may be performed on the retrieved solid supports. Nucleic acid synthesis may be performed in a tube and/or on a rotor to keep the solid supports suspended. The resulting synthesized nucleic acid may be used in subsequent nucleic acid amplification and/or sequencing technologies. Nucleic acid synthesis may comprise generating cDNA copies on a RNA attached to the oligonucleotide on the solid support. Generating cDNA copies may comprise using a reverse transcriptase (RT) or DNA polymerases having RT activity. This may result in the production of single-stranded cDNA molecules. After nucleic acid synthesis, unused oligonucleotides may be removed from the solid support. Removal of the oligonucleotides may occur by exonuclease treatment (*e.g.*, by ExoI).

[00339] In some embodiments, nucleic acids can be removed from the solid support using chemical cleavage. For example, a chemical group or a modified base present in a nucleic acid can be used to facilitate its removal from a solid support. For example, an ezyme can be used to remove a nucleic acid from a solid support. For example, a nucleic acid can be removed from a solid support through a restriction endonucelase digestion. For example, treatment of a nucleic acid containing a dUTP or ddUTP with uracil-d-glycosylase (UDG) can be used to remove a nucleic acid from a solid support. For example, a nucleic acid can be removed from a solid support using an enyme that performs nucleotide excision, such as a base excision repair enzyme, such as an apurinic/apyrimidinic (AP) endonuclease. In some embodiments, a nucleic acid can be removed from a solid support using a photocleavable

group and light. In some embodiments, a cleavable linker can be used to remove a nucleic acid from the solid support. For example, the cleavable linker can comprise at least one of biotin/avidin, biotin/streptavidin, biotin/neutravidin, Ig-protein A, a photo-labile linker, acid or base labile linker group, or an aptamer.

[00340] In some embodiments, nucleic acids are not amplified. In some embodiments, nucleic acids are not amplified prior to sequencing the nucelic acids. In some embodiments, nucleic acids not attached to a solid support can be directly sequenced without prior amplification. In some embodiments, nucleic acids can be directly sequenced without performing amplification when attached to a solid support, for example, nucleic acids attached to a solid support can be directly sequenced while attached to the solid support. In some embodiments, a nucleic acid that has been removed from a solid support can be directly sequenced. For example, a nucleic acid that has been removed from a solid support can be directly sequenced without performing amplification. Any sequencing platform conducive to sequencing without amplification can be used to perform the sequencing.

Amplification

[00341] After the nucleic acid has been synthesized (*e.g.*, reverse transcribed), it may be amplified. Amplification may be performed in a multiplex manner, wherein multiple target nucleic acid sequences are amplified simultaneously. Amplification may add sequencing adaptors to the nucleic acid. Amplification may be performed by polymerase chain reaction (PCR). PCR may refer to a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. PCR may encompass derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, digital PCR, and assembly PCR.

[00342] The method may further comprise conducting one or more amplification reactions to produce labeled nucleic acid amplicons. The labeled nucleic acids may be amplified prior to detecting the labeled nucleic acids. The method may further comprise combining the first and second samples prior to conducting the one or more amplification reactions.

[00343] The amplification reactions may comprise amplifying at least a portion of the sample tag. The amplification reactions may comprise amplifying at least a portion of the label. The amplification reactions may comprise amplifying at least a portion of the sample tag, label, nucleic acid, or a combination thereof. The amplification reactions may comprise amplifying at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 100% of the plurality of nucleic acids. The method may further comprise conducting one or more

cDNA synthesis reactions to produce one or more cDNA copies of the sample-tagged nucleic acids or molecular identifier labeled nucleic acids.

[00344] Amplification of the labeled nucleic acids may comprise PCR-based methods or non-PCR based methods. Amplification of the labeled nucleic acids may comprise exponential amplification of the labeled nucleic acids. Amplification of the labeled nucleic acids may comprise linear amplification of the labeled nucleic acids.

[00345] In some instances, amplification of the labeled nucleic acids comprises non-PCR based methods. Examples of non-PCR based methods include, but are not limited to, multiple displacement amplification (MDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), real-time SDA, rolling circle amplification, or circle-to-circle amplification. Other non-PCR-based amplification methods include multiple cycles of DNA-dependent RNA polymerase-driven RNA transcription amplification or RNA-directed DNA synthesis and transcription to amplify DNA or RNA targets (WO 89/01050; WO 88/10315; and U.S. Pat. Nos. 5,130,238; 5,409,818; 5,466,586; 5,514,545; 5,554,517; 5,888,779; 6,063,603; and 6,197,554), a ligase chain reaction (LCR), a Qβ replicase (Qβ) method as described in U.S. Pat. No. 4,786,600, use of palindromic probes, strand displacement amplification, oligonucleotide-driven amplification using a restriction endonuclease, an amplification method in which a primer is hybridized to a nucleic acid sequence and the resulting duplex is cleaved prior to the extension reaction and amplification, strand displacement amplification using a nucleic acid polymerase lacking 5' exonuclease activity (U.S. Pat. No. 6,214,587), rolling circle amplification, and ramification extension amplification (RAM) (U.S. Pat. No. 5,942,391).

[00346] Amplification of the labeled nucleic acids may comprise hybridization chain reaction (HCR) based methods (Dirks and Pierce, PNAS, 2004; Zhang et al., Anal Chem, 2012). HCR based methods may comprise DNA-based HCR. HCR based methods may comprise one or more labeled probes. The one or more labeled probes may comprise one or more sample tags or molecular identifier labels, or the complement thereof, disclosed herein.

[00347] In some instances, the methods disclosed herein further comprise conducting a polymerase chain reaction on the labeled nucleic acid (*e.g.*, labeled-RNA, labeled-DNA, labeled-cDNA) to produce a labeled-amplicon. The labeled-amplicon may be double-stranded molecule. The double-stranded molecule may comprise a double-stranded RNA molecule, a double-stranded DNA molecule, or a RNA molecule hybridized to a DNA molecule. One or both of the strands of the double-stranded molecule may comprise the sample tag or molecular identifier label. Alternatively, the labeled-amplicon is a single-

stranded molecule. The single-stranded molecule may comprise DNA, RNA, or a combination thereof. The nucleic acids of the present invention may comprise synthetic or altered nucleic acids.

[00348] The polymerase chain reaction may be performed by methods such as PCR, HD-PCR, Next Gen PCR, digital RTA, or any combination thereof. Additional PCR methods include, but are not limited to, allele-specific PCR, Alu PCR, assembly PCR, asymmetric PCR, droplet PCR, emulsion PCR, helicase dependent amplification HDA, hot start PCR, inverse PCR, linear-after-the-exponential (LATE)-PCR, long PCR, multiplex PCR, nested PCR, hemi-nested PCR, quantitative PCR, RT-PCR, real time PCR, single cell PCR, touchdown PCR or combinations thereof.

[00349] Multiplex PCR reactions may comprise nested PCR reactions. The method may comprise a pair of primers wherein a first primer that anneals to any one of the plurality of nucleic acids at least 300 to 400 nucleotides from the 3' end of any one of the plurality of nucleic acids and a second primer that anneals to any one of the plurality of nucleic acids at least 200 to 300 nucleotides from the 3' end of any one of the plurality of nucleic acids, wherein the first primer and second primer generate complementary DNA synthesis towards the 3' end of any one of the plurality of nucleic acids.

[00350] In some instances, conducting a polymerase chain reaction comprises annealing a first target specific primer to the labeled nucleic acid. Alternatively or additionally, conducting a polymerase chain reaction further comprises annealing a universal primer to a universal primer binding site region of the sample tag or molecular identifier label, wherein the sample tag or molecular identifier label is on a labeled nucleic acid or labeled-amplicon. The methods disclosed herein may further comprise annealing a second target specific primer to the labeled nucleic acid and/or labeled-amplicon.

[00351] In some instances, the method comprises repeatedly amplifying the labeled nucleic acid to produce multiple labeled-amplicons. The methods disclosed herein may comprise conducting at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amplification reactions. Alternatively, the method comprises conducting at least about 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amplification reactions.

[00352] 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 89:117 (1990)), transcription amplification (Kwoh et al., Proc. Natl. Acad. Sci. USA 86, 1173 (1989) and W088/10315), self-sustained sequence replication (Guatelli et al., Proc. Nat. Acad. Sci. USA, 87, 1874 (1990) and W090/06995),

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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-PCR) (U.S. Patent Nos. 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 21(12):531-5; Barker et al. Genome Res. 2004 May;14(5):901-7; Dean et al. Proc Natl Acad Sci U S A. 2002; 99(8):5261-6; Walker et al. 1992, Nucleic Acids Res. 20(7):1691-6, 1992 and Paez, et al. Nucleic Acids Res. 2004; 32(9):e71), Qbeta Replicase, described in PCT Patent Application No. PCT/US87/00880 and nucleic acid based sequence amplification (NABSA). (See, U.S. Patent Nos. 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.

[00353] 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 may 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 may 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.

[00354] Amplification may further comprise adding one or more control nucleic acids to one or more samples comprising a plurality of nucleic acids. Amplification may further comprise adding one or more control nucleic acids to a plurality of nucleic acids. The control nucleic acids may comprise a control label.

[00355] Amplification may comprise use of one or more non-natural nucleotides. Non-natural nucleotides may comprise photolabile and/or triggerable nucleotides. Examples of non-natural nucleotides include, but are not limited to, peptide nucleic acid (PNA), morpholino and locked nucleic acid (LNA), as well as glycol nucleic acid (GNA) and threose nucleic acid (TNA). Non-natural nucleotides may be added to one or more cycles of an

amplification reaction. The addition of the non-natural nucleotides may be used to identify products as specific cycles or time points in the amplification reaction.

[00356] Conducting the one or more amplification reactions may comprise the use of one or more primers. The one or more primers may comprise one or more oligonucleotides. The one or more oligonucleotides may comprise at least about 7-9 nucleotides. The one or more oligonucleotides may comprise less than 12-15 nucleotides. The one or more primers may anneal to at least a portion of the plurality of labeled nucleic acids. The one or more primers may anneal to the 3' end and/or 5' end of the plurality of labeled nucleic acids. The one or more primers may anneal to an internal region of the plurality of labeled nucleic acids. The internal region may be at least about 50, 100, 150, 200, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 650, 700, 750, 800, 850, 900 or 1000 nucleotides from the 3' ends the plurality of labeled nucleic acids. The one or more primers may comprise a fixed panel of primers. The one or more primers may comprise at least one or more custom primers. The one or more primers may comprise at least one or more control primers. The one or more primers may comprise at least one or more housekeeping gene primers. The one or more oligonucleotides may comprise a sequence selected from a group consisting of sequences in Table 23. The one or more primers may comprise a universal primer. The universal primer may anneal to a universal primer binding site. The one or more custom primers may anneal to the first sample tag, the second sample tag, the molecular identifier label, the nucleic acid or a product thereof. The one or more primers may comprise a universal primer and a custom primer. The custom primer may be designed to amplify one or more target nucleic acids. The target nucleic acids may comprise a subset of the total nucleic acids in one or more samples. The target nucleic acids may comprise a subset of the total labeled nucleic acids in one or more samples The one or more primers may comprise at least 96 or more custom primers. The one or more primers may comprise at least 960 or more custom primers. The one or more primers may comprise at least 9600 or more custom primers. The one or more custom primers may anneal to two or more different labeled nucleic acids. The two or more different labeled nucleic acids may correspond to one or more genes.

[00357] Disclosed herein is a method of selecting a custom primer comprising: a) a first pass, wherein primers chosen may comprise: i) no more than three sequential guanines, no more than three sequential cytosines, no more than four sequential adenines, and no more than four sequential thymines; ii) at least 3, 4, 5, or 6 nucleotides that are guanines or

cytosines; and iii) a sequence that does not easily form a hairpin structure; b) a second pass, comprising: i) a first round of choosing a plurality of sequences that have high coverage of all transcripts; and ii) one or more subsequent rounds, selecting a sequence that has the highest coverage of remaining transcripts and a complementary score with other chosen sequences no more than 4; and c) adding sequences to a picked set until coverage saturates or total number of customer primers is less than or equal to about 96.

[00358] The method of selecting the custom primer may further comprise selecting the at least one common primer based on one or more mRNA transcripts, non-coding transcripts including structural RNAs, transcribed pseudogenes, model mRNA provided by a genome annotation process, sequences corresponding to the genomic contig, or any combination thereof.

[00359] The method of selecting the custom primer may further comprise a primer selection method that enriches for one or more subsets of nucleic acids. The one or more subsets may comprise low abundance mRNAs.

[00360] The method of selecting the custom primer may further comprise a computational algorithm. Primers used in the method may be designed with the use of the Primer 3, a computer program which suggests primer sequences based on a user defined input sequence. Other primer designs may also be used, or primers may be selected by eye without the aid of computer programs. There are many options available with the program to tailor the primer design to most applications. Primer3 may consider many factors, including, but not limited to, oligo melting temperature, length, GC content, 3′ stability, estimated secondary structure, the likelihood of annealing to or amplifying undesirable sequences (for example interspersed repeats) and the likelihood of primer–dimer formation between two copies of the same primer. In the design of primer pairs, Primer3 may consider product size and melting temperature, the likelihood of primer– dimer formation between the two primers in the pair, the difference between primer melting temperatures, and primer location relative to particular regions of interest to be avoided.

[00361] The methods, compositions and kits disclosed herein may comprise one or more primers disclosed in Tables 23-24.

Sequencing

[00362] In some aspects, determining the number of different labeled nucleic acids may comprise determining the sequence of the labeled nucleic acid or any product thereof (*e.g.*, labeled-amplicons, labeled-cDNA molecules). In some instances, an amplified target nucleic acid may be subjected to sequencing. Determining the sequence of the labeled nucleic acid or

any product thereof may comprise conducting a sequencing reaction to determine the sequence of at least a portion of the sample tag, molecular identifier label, at least a portion of the labeled nucleic acid, a complement thereof, a reverse complement thereof, or any combination thereof. In some instances only the sample tag or a portion of the sample tag is sequenced. In some instances only the molecular identifier label or a portion of the molecular identifier label is sequenced.

[00363] Determining the sequence of the labeled nucleic acid or any product thereof may be performed by sequencing methods such as Helioscope™ single molecule sequencing, Nanopore DNA sequencing, Lynx Therapeutics' Massively Parallel Signature Sequencing (MPSS), 454 pyrosequencing, Single Molecule real time (RNAP) sequencing, Illumina (Solexa) sequencing, SOLiD sequencing, Ion Torrent™, Ion semiconductor sequencing, Single Molecule SMRT(™) sequencing, Polony sequencing, DNA nanoball sequencing, and VisiGen Biotechnologies approach. Alternatively, determining the sequence of the labeled nucleic acid or any product thereof may use sequencing platforms, including, but not limited to, Genome Analyzer IIx, HiSeq, and MiSeq offered by Illumina, Single Molecule Real Time (SMRT™) technology, such as the PacBio RS system offered by Pacific Biosciences (California) and the Solexa Sequencer, True Single Molecule Sequencing (tSMS™) technology such as the HeliScope™ Sequencer offered by Helicos Inc. (Cambridge, MA).

[00364] In some embodiments, the labeled nucleic acids comprise nucleic acids representing from about 0.01% of the genes of an organism's genome to about 100% of the genes of an organism's genome. For example, about 0.01% of the genes of an organism's genome to about 100% of the genes of an organism's genome can be sequenced using a target complimentary region comprising a plurality of multimers by capturing the genes containing a complimentary sequence from the sample. In some embodiments, the labeled nucleic acids comprise nucleic acids representing from about 0.01% of the transcripts of an organism's transcriptome to about 100% of the transcripts of an organism's transcriptome. For example, about 0.501% of the transcripts of an organism's transcriptome to about 100% of the transcripts of an organism's transcriptome can be sequenced using a target complimentary region comprising a poly-T tail by capturing the mRNAs from the sample.

[00365] In some instances, determining the sequence of the labeled nucleic acid or any product thereof comprises paired-end sequencing, nanopore sequencing, high-throughput sequencing, shotgun sequencing, dye-terminator sequencing, multiple-primer DNA sequencing, primer walking, Sanger dideoxy sequencing, Maxim-Gilbert sequencing, pyrosequencing, true single molecule sequencing, or any combination thereof. Alternatively,

the sequence of the labeled nucleic acid or any product thereof may be determined by electron microscopy or a chemical-sensitive field effect transistor (chemFET) array.

[00366] Determination of the sequence of a nucleic acid (*e.g.*, amplified nucleic acid, labeled nucleic acid, cDNA copy of a labeled nucleic acid, etc.) may be performed using variety of sequencing methods including, but not limited to, sequencing by hybridization (SBH), sequencing by ligation (SBL), quantitative incremental fluorescent nucleotide addition sequencing (QIFNAS), stepwise ligation and cleavage, fluorescence resonance energy transfer (FRET), molecular beacons, TaqMan reporter probe digestion, pyrosequencing, fluorescent in situ sequencing (FISSEQ), FISSEQ beads, wobble sequencing, multiplex sequencing, polymerized colony (POLONY) sequencing; nanogrid rolling circle sequencing (ROLONY), allele-specific oligo ligation assays (*e.g.*, oligo ligation assay (OLA), single template molecule OLA using a ligated linear probe and a rolling circle amplification (RCA) readout, ligated padlock probes, and/or single template molecule OLA using a ligated circular padlock probe and a rolling circle amplification (RCA) readout) and the like. High-throughput sequencing methods, such as cyclic array sequencing using platforms such as Roche 454, Illumina Solexa, ABI-SOLiD, ION Torrents, Complete Genomics, Pacific Bioscience, Helicos, Polonator platforms, may also be utilized. Sequencing may comprise MiSeq sequencing. Sequencing may comprise HiSeq sequencing. Sequencing may read the cell label, the molecular label and/or the gene that was on the original oligonucleotide.

[00367] In another example, determining the sequence of labeled nucleic acids or any product thereof comprises RNA-Seq or microRNA sequencing. Alternatively, determining the sequence of labeled nucleic acids or any products thereof comprises protein sequencing techniques such as Edman degradation, peptide mass fingerprinting, mass spectrometry, or protease digestion.

[00368] The sequencing reaction can, in certain embodiments, occur on a solid or semi-solid support, in a gel, in an emulsion, on a surface, on a bead, in a drop, in a continuous follow, in a dilution, or in one or more physically separate volumes.

[00369] Sequencing may comprise sequencing at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more nucleotides or base pairs of the labeled nucleic acid. In some instances, sequencing comprises sequencing at least about 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more nucleotides or base pairs of the labeled nucleic acid. In other instances, sequencing comprises sequencing at least about 1500; 2,000; 3,000; 4,000; 5,000; 6,000; 7,000; 8,000; 9,000; or 10,000 or more nucleotides or base pairs of the labeled nucleic acid.

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[00370] Sequencing may comprise at least about 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more sequencing reads per run. In some instances, sequencing comprises sequencing at least about 1500; 2,000; 3,000; 4,000; 5,000; 6,000; 7,000; 8,000; 9,000; or 10,000 or more sequencing reads per run. Sequencing may comprise less than or equal to about 1,600,000,000 sequencing reads per run. Sequencing may comprise less than or equal to about 200,000,000 reads per run.

[00371] Determining the number of different labeled nucleic acids may comprise one or more arrays.

[00372] Determining the number of different labeled nucleic acids may comprise contacting the labeled nucleic acids with the one or more probes.

[00373] Probes, as described herein, may comprise a sequence that is complementary to at least a portion of the labeled nucleic acid or labeled-amplicon. The plurality of probes may be arranged on the solid support in discrete regions, wherein a discrete region on the solid support comprises probes of identical or near-identical sequences. In some instances, two or more discrete regions on the solid support comprise two different probes comprising sequences complementary to the sequence of two different unique identifier regions of the oligonucleotide tag.

[00374] In some instances, the plurality of probes is hybridized to the array. The plurality of probes may allow hybridization of the labeled-molecule to the array. The plurality of probes may comprise a sequence that is complementary to the stochastic label oligo dT. Alternatively, or additionally, the plurality of probes comprises a sequence that is complementary to the molecule.

[00375] Determining the number of different labeled nucleic acids may comprise contacting the labeled nucleic acids with an array of a plurality of probes. Determining the number of different labeled nucleic acids may comprise contacting the labeled nucleic acids with a glass slide of a plurality of probes.

[00376] Determining the number of different labeled nucleic acids may comprise labeled probe hybridization, target-specific amplification, target-specific sequencing, sequencing with labeled nucleotides specific for target small nucleotide polymorphism, sequencing with labeled nucleotides specific for restriction enzyme digest patterns, sequencing with labeled nucleotides specific for mutations, or a combination thereof.

[00377] Determining the number of different labeled nucleic acids may comprise flow cytometry sorting of a sequence-specific label. Determining the number of different labeled nucleic acids may comprise detection of the labeled nucleic acids attached to the beads.

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Detection of the labeled nucleic acids attached to the beads may comprise fluorescence detection.

[00378] Determining the number of different labeled nucleic acids may comprise counting the plurality of labeled nucleic acids by fluorescence resonance energy transfer (FRET), between a target-specific probe and a labeled nucleic acid or a target-specific labeled probe.

Detection of labeled nucleic acids

[00379] The methods disclosed herein may further comprise detection of the labeled nucleic acids and/or labeled-amplicons. Detection of the labeled nucleic acids and/or labeled-amplicons may comprise hybridization of the labeled nucleic acids to surface, *e.g.,* a solid support. The method may further comprise immunoprecipitation of a target sequence with a nucleic-acid binding protein. Detection of the labeled nucleic acids and/or labeled amplicons may enable or assist in determining the number of different labeled nucleic acids.

[00380] In some instances, the method further comprises contacting the labeled nucleic acids and/or labeled-amplicons with a detectable label to produce a detectable-label conjugated labeled nucleic acid. The methods disclosed herein may further comprise detecting the detectable-label conjugated labeled nucleic acid. Detection of the labeled nucleic acids or any products thereof (*e.g.*, labeled-amplicons, detectable-label conjugated labeled nucleic acid) may comprise detection of at least a portion of the sample tag or molecular identifier label, molecule, detectable label, a complement of the sample tag or molecular identifier label, a complement of the molecule, or any combination thereof.

[00381] Detection of the labeled nucleic acids or any products thereof may comprise an emulsion or a droplet. For example, the labeled nucleic acids or any products thereof may be in an emulsion or droplet. A droplet can be a small volume of a first liquid that is encapsulated by an immiscible second liquid, such as a continuous phase of an emulsion (and/or by a larger droplet). The volume of a droplet, and/or the average volume of droplets in an emulsion, can, for example, be less than about one microliter (or between about one microliter and one nanoliter or between about one microliter and one picoliter), less than about one nanoliter (or between about one nanoliter and one picoliter), or less than about one picoliter (or between about one picoliter and one femtoliter), among others. A droplet (or droplets of an emulsion) can have a diameter (or an average diameter) of less than about 1000, 100, or 10 micrometers, or about 1000 to 10 micrometers, among others. A droplet can be spherical or nonspherical. Droplets can be generated having an average diameter of about, less than about, or more than about 0.001, 0.01, 0.05, 0.1, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 100, 120, 130, 140, 150, 160, 180, 200, 300, 400, or 500 microns. Droplets can have an

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average diameter of about 0.001 to about 500, about 0.01 to about 500, about 0.1 to about 500, about 0.1 to about 100, about 0.01 to about 100, or about 1 to about 100 microns. A droplet can be a simple droplet or a compound droplet. The term emulsion, as used herein, can refer to a mixture of immiscible liquids (such as oil and water). Oil-phase and/or water-in-oil emulsions allow for the compartmentalization of reaction mixtures within aqueous droplets. The emulsions can comprise aqueous droplets within a continuous oil phase. The emulsions provided herein can be oil-in-water emulsions, wherein the droplets are oil droplets within a continuous aqueous phase. When an emulsion or droplet is used to isolate, for example, spatially isolate, single cells, a solid support may not be used. Thus the nucleic acids to be tagged and analyzed may not be bound to a solid support and in such instances; a cellular label can correspond to the single cell or population of cells present in the emulsion or droplet when tagged. The emulsion or droplet can thus effectively isolate the tagging or labeling steps with a single cell or plurality of cells and the cellular label can be used to identify the nucleic acids that came from the single cell or plurality of cells. In some embodiments, droplets can be applied to microwells, for example, similarly to application of beads to microwell arrays.

[00382] Alternatively, detection of the labeled nucleic acids or any products thereof comprises one or more solutions. In other instances, detection of the labeled nucleic acids comprises one or more containers.

[00383] Detection of the labeled nucleic acids or any products thereof (*e.g.*, labeled-amplicons, detectable-label conjugated labeled nucleic acid) may comprise detecting each labeled nucleic acid or products thereof. For example, the methods disclosed herein comprise sequencing at least a portion of each labeled nucleic acid, thereby detecting each labeled nucleic acid.

[00384] In some instances, detection of the labeled nucleic acids and/or labeled-amplicons comprises electrophoresis, spectroscopy, microscopy, chemiluminescence, luminescence, fluorescence, immunofluorescence, colorimetry, or electrochemiluminescence methods. For example, the method comprises detection of a fluorescent dye. Detection of the labeled nucleic acid or any products thereof may comprise colorimetric methods. For example, the colorimetric method comprises the use of a colorimeter or a colorimetric reader. A non-limiting list of colorimeters and colorimetric readers include Sensovation's Colorimetric Array Imaging Reader (CLAIR), ESEQuant Lateral Flow Immunoassay Reader, SpectraMax 340PC 38, SpectraMax Plus 384, SpectraMax 190, VersaMax, VMax, and EMax.

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[00385] Additional methods used alone or in combination with other methods to detect the labeled nucleic acids and/or amplicons may comprise the use of an array detector, fluorescence reader, non-fluorescent detector, CR reader, luminometer, or scanner. In some instances, detecting the labeled nucleic acids and/or labeled-amplicons comprises the use of an array detector. Examples of array detectors include, but are not limited to, diode-array detectors, photodiode array detectors, HLPC photodiode array detectors, array detectors, Germanium array detectors, CMOS and CCD array detectors, Gated linear CCD array detectors, InGaAs photodiode array systems, and TE cooled CCD systems. The array detector may be a microarray detector. Non-limiting examples of microarray detectors include microelectrode array detectors, optical DNA microarray detection platforms, DNA microarray detectors, RNA microarray detectors, and protein microarray detectors.

[00386] In some instances, a fluorescence reader is used to detect the labeled nucleic acid and/or labeled-amplicons. The fluorescence reader may read 1, 2, 3, 4, 5, or more color fluorescence microarrays or other structures on biochips, on slides, or in microplates. In some instances, the fluorescence reader is a Sensovation Fluorescence Array imaging Reader (FLAIR). Alternatively, the fluorescence reader is a fluorescence microplate reader such as the Gemini XPS Fluorescence microplate reader, Gemini EM Fluorescence microplate reader, Finstruments® Fluoroskan filter based fluorescence microplate reader, PHERAstar microplate reader, FlUOstar microplate reader, POLARstar Omega microplate reader, FLUOstar OPTIMA multi-mode microplate reader and POLARstar OPTIMA multi-mode microplate reader. Additional examples of fluorescence readers include PharosFXTM and PharosFX Plus systems.

[00387] In some instances, detection of the labeled nucleic acid and/or labeled-amplicon comprises the use of a microplate reader. In some instances, the microplate reader is an xMarkTM microplate absorbance spectrophotometer, iMark microplate absorbance reader, EnSpire® Multimode plate reader, EnVision Multilabel plate reader, VICTOR X Multilabel plate reader, FlexStation, SpectraMax Paradigm, SpectraMax M5e, SpectraMax M5, SpectraMax M4, SpectraMax M3, SpectraMax M2-M2e, FilterMax F series, Fluoroskan Ascent FL Microplate Fluoremeter and Luminometer, Fluoroskan Ascent Microplate Fluoremeter, Luminoskan Ascent Microplate Luminometer, Multiskan EX Microplate Photometer, Muliskan FC Microplate Photometer, and Muliskan GO Microplate Photometer. In some instances, the microplate reader detects absorbance, fluorescence, luminescence, time-resolved fluorescence, light scattering, or any combination thereof. In some embodiments, the microplate reader detects dynamic light scattering. The microplate reader,

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may in some instances, detect static light scattering. In some instances, detection of the labeled nucleic acids and/or labeled-amplicons comprises the use of a microplate imager. In some instances, the microplate imager comprises ViewLux uHTS microplate imager and BioRad microplate imaging system.

[00388] Detection of labeled nucleic acids and/or products thereof may comprise the use of a luminometer. Examples of luminometers include, but are not limited to, SpectraMax L, GloMax®-96 microplate luminometer, GloMax®-20/20 single-tube luminometer, GloMax®-Multi+ with InstinctTM software, GloMax®-Multi Jr single tube multimode reader, LUMIstar OPTIMA, LEADER HC+ luminometer, LEADER 450i luminometer, and LEADER 50i luminometer.

[00389] In some instances, detection of the labeled nucleic acids and/or labeled-amplicons comprises the use of a scanner. Scanners include flatbed scanners such as those provided by Cannon, Epson, HP, Fujitsu, and Xerox. Additional examples of flatbed scanners include the FMBIO® fluorescence imaging scanners (*e.g.*, FMBIO® II, III, and III Plus systems). Scanners may include microplate scanners such as the Arrayit ArrayPixTM microarray microplate scanner. In some instances, the scanner is a Personal Molecular ImagerTM (PMI) system provided by Bio-rad.

[00390] Detection of the labeled nucleic acid may comprise the use of an analytical technique that measures the mass-to-charge ratio of charged particles, *e.g.,* mass spectrometry. In some embodiments the mass-to-charge ratio of charged particles is measured in combination with chromatographic separation techniques. In some embodiments sequencing reactions are used in combination with mass-to-charge ratio of charged particle measurements. In some embodiments the tags comprise isotopes. In some embodiments the isotope type or ratio is controlled or manipulated in the tag library.

[00391] Detection of the labeled nucleic acids or any products thereof comprises the use of small particles and/or light scattering. For example, the amplified molecules (*e.g.*, labeled-amplicons) are attached to haptens or directly to small particles and hybridized to the array. The small particles may be in the nanometer to micrometer range in size. The particles may be detected when light is scattered off of its surface.

[00392] A colorimetric assay may be used where the small particles are colored, or haptens may be stained with colorimetric detection systems. In some instances, a flatbed scanner may be used to detect the light scattered from particles, or the development of colored materials. The methods disclosed herein may further comprise the use of a light absorbing material. The light absorbing material may be used to block undesirable light scatter or reflection. The light

absorbing material may be a food coloring or other material. In some instances, detection of the labeled nucleic acid or any products thereof comprises contacting the labeled nucleic acids with an off-axis white light.

[00393] In some mebodiments, two or more different types of biological materials from a sample can be detected simultaneously. For example, two or more different types of biological materials selected from the group consisiting of DNA, RNA (*e.g.,* microRNA, mRNA, etc.), nucleotide, protein, and carbohydrate, from a sample can be detected simultaneously. For example, DNA and RNA from a sample can be detected simultaneously using the methods described herein.

Data Analysis

[00394] The sequencing data may be used to count the number of target nucleic acid molecules in a cell. For example, a plurality of copies of a target nucleic acid in a cell may bind to a different oligonucleotide on the solid support. When the plurality of target nucleic acids are amplified and sequenced, they may comprise different molecular labels. The number of molecular labels for a same target nucleic acid may be indicative of the number of copies of the target nucleic acid in the cell. Determining the copy number of a target nucleic acid may be useful for removing amplification bias when determining the concentration of a target nucleic acid in a cell.

[00395] The sequencing data may be used to genotype a subject. By comparing target nucleic acids with different cellular labels, the copy number variation and/or concentration of the target nucleic acid may be determined. By comparing concentrations of target nucleic acids with different cellular labels, the sequencing data may be used to determine cellular genotype heterogeneity. For example, a first cell of a sample may comprise a target nucleic acid at high concentrations, whereas a second cell of the sample may not comprise the target nucleic acid, or may comprise the target nucleic acid at low concentrations, thereby indicating the heterogeneity of the cellular sample.

[00396] Determining cellular genotype heterogeneity may be useful for diagnosing, prognosing, and determining a course of treatment of a disease. For example, if a first cell of a sample comprises the target nucleic acid, but a second cell of the sample does not comprise the target nucleic acid, but comprises a second target nucleic acid, then a course of a treatment may include an agent (*e.g.*, drug) to target the first genotype and an agent (*e.g.*, drug) to target the second genotype.

[00397] In some embodiments, certain sequence types can be linked to a DNA or RNA profile. For example, T-cell receptor and/or B-cell receptor sequences can be linked to a

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transcription profile, microRNA profile, or genomic mutation profile of a sample, such as a single cell. In some embodiments, certain sequence types can be linked to an antigenicity or protein expression profile. For example, T-cell receptor and/or B-cell receptor sequences can be linked to to an antigenicity or protein expression profile via binding antibodies to a surface, such as a surface comprising proteins, such as protein targets of antibodies comprising the T-cell receptor and/or B-cell receptor sequences.

[00398] In some embodiments, the presence or absence of a sequence, such as a viral sequence, can be linked to a DNA or RNA profile. For example, the presence or absence of a sequence, such as a viral sequence, can be linked to a transcription profile, microRNA profile, or genomic mutation profile of a sample, such as a single cell.

Kits

[00399] The present disclosure provides kits for carrying out the methods of the disclosure. A kit may comprise one or more of: a microwell array, an oligonucleotide, and a solid support. A kit may comprise a reagent for reconstituting and/or diluting the oligonucleotides and/or solid support. A kit may comprise reagents for conjugating the oligonucleotides to the solid support. A kit may further comprise one or more additional reagents, where such additional reagents may be selected from: a wash buffer; a control reagent, an amplification agent for amplifying (*e.g.*, performing cDNA synthesis and PCR) a target nucleic acid, and a conjugation agent for conjugating an oligonucleotide to the solid support. Components of a subject kit may be in separate containers, or may be combined in a single container.

[00400] A kit may comprise instructions for using the components of the kit to practice the subject methods. The instructions for practicing the subject methods may be recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or subpackaging) etc. In some embodiments, the instructions may be present as an electronic storage data file present on a suitable computer readable storage medium, *e.g.,* CD-ROM, diskette, flash drive, etc. In some embodiments, the actual instructions may not be present in the kit, but means for obtaining the instructions from a remote source, *e.g.,* via the internet, are provided. For example a kit may comprise a web address where the instructions may be viewed and/or from which the instructions may be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

[00401] Further disclosed herein are kits for use in analyzing two or more molecules from two or more samples. The kits disclosed herein may comprise a plurality of beads, a primer

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